Analysis of the JA-Ile-independent function of COI1 in *Arabidopsis thaliana* upon infection with *Verticillium longisporum*

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Summary

CORONATINE INSENSITIVE 1 (COI1) perceives the plant hormone jasmonoyl-isoleucine (JA-Ile) together with proteins of the JASMONATE ZIM-domain (JAZ) family. JA-Ile induces signalling cascades in defence and developmental processes. It has been shown that in *Arabidopsis thaliana*, COI1 without its ligand conveys susceptibility to the soil-born vascular pathogen *Verticillium longisporum*. Grafting experiments have shown that presence of *COI1* in roots mediates susceptibility to the pathogen. Root transcriptome analysis has revealed that a number of salicylic acid defence-associated genes are constitutively expressed in *coi1.* The observation that COI1 acts as a JA-Ile-independent repressor of root gene expression led us to postulate that this novel COI1 function operates independently of the canonical JA signalling machinery.

In this thesis, we show that *coi1* plants complemented with a COI1 protein, that was severely impaired in its interaction with JAZ proteins ($COI1_{AA}$), were compromised in wound-induced induction of the JA-signalling marker gene *VEGETATIVE STORAGE PROTEIN 2 (VSP2)*. Moreover, $COI1_{AA}$ could not restore fertility in sterile *coi1* plants. In contrast, $COI1_{AA}$ was able to repress gene expression in roots. Hence, in roots, COI1 has a second function other than its role in JA-Ile perception, in which it acts as a suppressor of defence gene expression independently of JA-Ile and most likely independently of JAZ proteins. We furthermore show that after infection with *V. longisporum*, approximately half of the COI1-repressed genes in roots are induced to similar levels as in *coi1*. We hence postulate that COI1-mediated repression is inactivated upon infection with *V. longisporum* leading to induction of these genes. Gene induction requires the transcription factor SYSTEMIC ACQUIRED RESISTANCE 1 (SARD1) which is itself repressed by COI1. Equally, constitutive expression of genes in *coi1* was abolished by mutations in *SARD1* and its close homologue *CALMODULIN BINDING PROTEIN 60-LIKE G*. In contrast, overexpression of *SARD1* in wild-type roots did not lead to activation of gene expression, likely because the repressive effect of COI1 on gene expression could not be overcome. The repressor function of COI1 was only observed in roots and not in shoots. As roots need to balance perception of microbe-associated molecular patterns with maintaining an intact rhizosphere, we speculate that COI1 acts as a regulator of the onset of defence responses in roots.

List of Abbreviations

AIM1 - ABNORMAL INFLORESCENCE MERISTEM 1

AOC - ALLENE OXIDE CYCLASE

AOS - ALLENE OXIDE SYNHASE

ASK1 - ARABIDOPSIS SKP1-LIKE 1

A. thaliana - *Arabidopsis thaliana*

bHLH - basic helix-loop-helix

B. napus - *Brassica napus*

CAMTA - Calmodulin-binding transcription factor

CBP60g - CALMODULIN-BINDING PROTEIN 60G

COI1 - CORONATINE INSENSITIVE 1

$COI1_{AA}$ - COI1 protein with amino acid residues Glu203 and Tyr302 changed to Alanine

CUL1 - CULLIN 1

CYP - CYTOCHROME P450 family protein

Cys - cysteine

DAMP - damage-associated molecular pattern

DLO1 - DMR6-LIKE OXYGENASE 1

DNA - deoxyribonucleic acid

dpi - days past inoculation

ECS1 - Pathogen-inducible protein CXC75

EDS5 - ENHANCED DISEASE SUSCEPTIBILITY 5

EIL1 - EIN3-LIKE 1

EIN3 - ETHYLENE INSENSITIVE 3

ET - ethylene

ETI - effector-triggered immunity

flg22 - conserved 22 amino acid sequence of flagellin

FLS2 - FLAGELLIN SENSITIVE 2

F. oxysporum - *Fusarium oxysporum*

GLP - Germin-like protein

Glu - Glutamic acid

HA-tag - Human influenza hemagglutinin tag

HR - hypersensitive response

ICS1 - ISOCHORISMATE SYNTHASE 1

IGs - indole glucosinolates

JA - jasmonic acid

JA-Ile – jasmonoyl isoleucine

JAZ - JASMONATE ZIM-domain

jazD - *jaz* decuple mutant

LRR - leucine-rich repeat

LTP4.4 - LIPID TRANSFER PROTEIN 4.4

MAMP - microbe-associated molecular-pattern

MAPK - mitogen-activated protein kinase

MED25 - MEDIATOR 25

MeJA - methyl jasmonate

NAC - No apical meristem (NAM), Arabidopsis transcription activation factor (ATAF), Cup- shaped cotyledon (CUC)

NHP - N-hydroxypipecolic acid

NINJA - NOVEL INTERACTOR OF JAZ

NLP - Necrosis and ethylene-inducing peptide 1 (Nep1)-like protein

NLR - nucleotide-binding (NB) leucine-rich repeat (LRR) protein

NPR1 - NON-EXPRESSER OF PATHOGENESIS-RELATED GENES 1

NPR3 - NPR1-LIKE PROTEIN 3

OPR3 - OPDA REDUCTASE 3

ORA59 - OCTADECANOID-RESPONSIVE ARABIDOPSIS 59

PAL - PHENYLALANINE AMMONIA-LYASE

PAMP - pathogen-associated molecular pattern

PBS3 - avrPphB SUSCEPTIBLE 3

PDF1.2 - PLANT DEFENSIN 1.2

Pep1 - plant elicitor peptide 1

PGM - PHOSPHOGLYCERATE MUTASE

PRLIP2 - PATHOGENESISRELATED LIPASE 2

PRR - pathogen recognition receptors

PTI - PAMP-triggered immunity

RBX1 - RING-BOX 1

RLK - receptor-like kinase

RLP - receptor-like protein

RNA - ribonucleic acid

RNA-seq - RNA sequencing

ROS - reactive oxygen species

SA - salicylic acid

SAR - Systemic acquired resistance

SARD1 - SAR-DEFICIENT 1

SCF^{COI1} - SKP1/CULLIN/F-BOX E3 ligase complex formed with COI1

sGFP - superfolder GREEN FLUORESCENT PROTEIN

sid2 - *salicylic acid induction-deficient 2*

TPL - TOPLESS

Tyr - Tyrosine

V. dahliae - *Verticillium dahliae*

V. longisporum - *Verticillium longisporum*

VND7 - VASCULAR-RELATED NAC DOMAIN 7

VSP2 - VEGETATTIVE STORAGE PROTEIN 2

WT_{aos} - wild-type plant originating from the segregating offspring of heterozygous *aos* population

$WT_{coi1\text{-}t}$ - wild-type plant originating from the segregating offspring of heterozygous *coi1-t* population

ZIM - ZINC-FINGER EXPRESSED IN INFLORESCENCE MERISTEM

I. General Introduction

1. The plant immune system

1.1 PAMP-triggered immunity and effector-triggered immunity

Plants have a sessile lifestyle and face a myriad of pathogenic microorganisms. Nevertheless, due to carefully regulated and broadly effective defence responses, plant disease is the exception and not the norm. Instead of having mobile defence cells, plants rely on the reaction of each cell and the propagation of signals from infection sites (Jones and Dangl, 2006). Plants have several layers of defence that constitute potent protection against infection by potential pathogens.

At the forefront of plant defences are structural barriers such as waxy cuticles and cell walls, limiting access of prospective pathogens to inner cells (Malinovsky *et al.*, 2014; Serrano *et al.*, 2014). At sites of penetration, plants can reinforce or create new barriers by deposition of structural elements such as callose or lignin to restrict pathogen entry or spread (Lee *et al.*, 2019; Wang *et al.*, 2021).

In opposition, pathogens attack these structural barriers with enzymes degrading their individual components (Kubicek *et al.*, 2014). If the plant's barriers are breached, a second layer of defence senses the imminent danger. On the surface of plant cell membranes pathogen recognition receptors (PRRs) detect conserved molecules associated with microorganisms, so called pathogen-associated molecular patterns (PAMPs) or more broadly and accurately microbe-associated molecular-patterns (MAMPs). PRRs transduce this information to the inside of the cell (Jones and Dangl, 2006). PRRs can be divided into receptor-like kinases (RLK) and receptor-like proteins (RLP). RLKs possess a cytosolic kinase domain for intracellular signal transduction upon ligand binding outside the cell. RLPs lack an intracellular kinase domain and rely on interaction partners for internal signal transduction (Macho and Zipfel, 2014).

An example of a MAMP is flagellin, the main constituent of bacterial flagella (Gómez-Gómez and Boller, 2002). In fact, a conserved 22 amino acid sequence of flagellin, flg22, is sufficient to elicit an immune response after binding to the PRR FLAGELLIN SENSITIVE 2 (FLS2) (Felix *et al.*, 1999; Chinchilla *et al.*, 2006). Another example of MAMPs are Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs), small proteins found in diverse microbes across kingdoms (Pemberton and Salmond, 2004; Oome *et al.*, 2014). Again, a conserved small fragment of NLPs is sufficient for recognition by the PRR RLP23 (Albert *et al.*, 2015). Special about these NLPs is that they trigger plant defence responses but at the same time contribute to a pathogen's virulence and can be cytotoxic (Qutob *et al.*, 2006).

In addition to non-self-recognition, plants can sense pathogen attack by self-recognition. Receptors on the cell surface can sense damage-associated molecular patterns (DAMPs). These are molecules like cell wall fragments, peptides or nucleotides that are secreted by attacked cells or released from the cytosol of damaged cells into the intercellular space (Hou *et al.*, 2019). A well-studied example of a DAMP is plant elicitor peptide 1 (Pep1), a 23 amino-acid long peptide derived from PRECURSOR OF PEPTIDE 1 (PROPEP1) (Huffaker *et al.*, 2006). Pep1 is perceived by PEP RECEPTOR 1 (PEPR1) and PEPR2 on the plasma membrane, leading to downstream immune responses in the cell. Cleavage of PROPEP1 is achieved by the Ca^{2+}-dependent caspase METACASPASE4 (MC4), the mode of transport to the apoplast is still elusive though (Hander *et al.*, 2019).

Downstream of MAMP and DAMP recognition, the information is passed on via signalling cascades. Cumulatively, the following broad responses aimed to fight off pathogen attack are termed PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). Early responses upon MAMP/DAMP sensing are cytosolic calcium influxes, reactive oxygen species (ROS) bursts and the activation of mitogen-activated protein kinases (MAPKs) (Zhang and Zhou, 2010; Bigeard *et al.*, 2015; Zipfel and Oldroyd, 2017). Calcium is an important second messenger that activates further signal transducers such as calcium-dependent protein kinases (CDPKs) and transcription factors (Gao *et al.*, 2014; Bigeard *et al.*, 2015). ROS production is mediated by respiratory burst oxidase homologs (RBOHs) and ROS act as another type of second

messengers that are also thought to possess antimicrobial properties themselves (Kadota *et al.*, 2015). The MAPK signal transduction cascade leads to transcriptional reprogramming by targeting transcription factors of the WRKY family resulting, e.g. in the production of the phytoalexin camalexin (Kim and Zhang, 2004; Mao *et al.*, 2011). Pathogen detection also triggers the production of phytohormones that regulate distinct branches of defence responses (Bari and Jones, 2009). Hormone-mediated defence signalling pathways will be discussed in more detail in the next chapter (2. Hormone-mediated signalling pathways).

Pathogens can secrete effectors to supress PTI, leading to so called effector triggered susceptibility (ETS) (Jones and Dangl, 2006). In turn, plants have another third layer of defence. Effector-triggered immunity (ETI) aims at disarming pathogens by recognition of such effectors (Dangl and Jones, 2001). The receptors for such effectors, nucleotide-binding (NB) leucine-rich repeat (LRR) proteins (NLRs), are found intracellularly (Dangl and Jones, 2001). Triggering of ETI leads to similar but stronger and faster responses than PTI, along with a form of programmed cell death, termed the hypersensitive response (HR) (Thordal-Christensen, 2020). HR is initiated by the plant at the point of penetration to restrict pathogen spread (Balint-Kurti, 2019).

Despite the overlapping defence outputs of PTI and ETI, the two responses were long seen as two separate tiers of immunity. Recent studies have changed the understanding of the interplay between PTI and ETI using systems that allow induction of ETI without PTI (Ngou *et al.*, 2021; Yuan *et al.*, 2021). When only ETI is triggered, components of PTI accumulated in the cell (Ngou *et al.*, 2021; Yuan *et al.*, 2021). ETI is hence replenishing PTI constituents to strengthen PTI weakened by effectors. Conversely, HR in ETI is strongly enhanced by activation of PRRs (Ngou *et al.*, 2021). The model of PTI and ETI as separate responses thus has to be revised in favour of a mutually potentiated interplay of PTI and ETI in plant defence.

1.2 Immune responses in roots

Roots are constantly submerged in an environment full of MAMPs. However, our current knowledge of immunity is mostly based on the study of aerial plant parts. Great progress has been made recently in the investigation of root specific immune response elicitation.
Roots have been shown to perceive and react to MAMPs via PRRs. flg22 elicitation of roots leads to induction of defence genes like *MYB DOMAIN PROTEIN 51* (*MYB51)* and *WRKY11* and induces production of camalexin and callose deposition (Millet *et al.*, 2010). Other MAMPS and DAMPs, like chitin, Pep1 and NLPs are also recognised by roots (Poncini *et al.*, 2017; Zhou *et al.*, 2020).

In contrast to shoots, root immune responses are highly dependent on cell types and developmental stages. flg22 treatment of seedling roots only elicits defence responses in the elongation zone, whereas chitin induces defences in cells of the mature zones of seedling roots (Millet *et al.*, 2010). In line with this, it has been shown that receptor expression varies in different tissues, for example, FLS2 is higher expressed in lateral roots, as they are an easy entry point for pathogens (Beck *et al.*, 2014).

Moreover, not all cells have the same ability to respond to elicitors, even if they had the required PRR. Emonet *et al.* (2021) showed that ectopically expressing FLS2 in the vascular meristem of Arabidopsis seedlings did not lead to induction of defences upon treatment with flg22. Rich-Griffin *et al.* (2020) showed that differential responses in different cells are underpinned by specific signalling networks in different tissues. Responses to flg22 and Pep1 are differentially regulated by specific pairs of transcription factor families in different cell types in roots. Treatment of epidermal cells with flg22 induced immune responses specifically regulated by WRKY12, 18, 36, 45 together with AT-hook motif nuclear localized protein (AHL) transcription factors AHL12, 20, 25. In contrast, Pep1 treatment induced cascades coordinated by WRKY12, 18, 36, 45 and No apical meristem (NAM), Arabidopsis transcription activation factor (ATAF), Cup-shaped cotyledon (CUC) (NAC) family transcription factors ANAC46, 55, 55_2, 58 in epidermal cells. Cortex cells had different signalling networks upon the same flg22

treatment with response curated by WRKY12, 38, 45 and Arabidopsis thaliana homeobox (ATHB) transcription factors ATHB15 and 51 (Rich-Griffin *et al.*, 2020).

Elicitation with MAMPs induces calcium signalling with the Ca^{2+} wave emerging from the elongation zone into other root zones, spreading the signal even to cells without PRRs themselves (Keinath *et al.*, 2015; Emonet *et al.*, 2021). This might aid roots in balancing crucial danger perception with overreactions in the omnipresent MAMP environment (Keinath *et al.*, 2015; Emonet *et al.*, 2021). Additionally, it has been shown that roots have another way of balancing responses instead of spatial distribution of PRRs and cell type specific responses. In accordance with earlier observations, Zhou *et al.* (2020) showed that responses to most MAMPs in seedlings were restricted to undifferentiated root zones. However, they observed highly localised defence responses in the differentiated zone where damage occurred, e.g. at sites of lateral root emergence. Upon localised laser ablation, cells in the differentiated zones became responsive to MAMPs. This coincided with the upregulation of PRR receptor transcription in damaged cells (Zhou *et al.*, 2020). However, as triggering of differentiated root zones with a mix of DAMPs and MAMPs together did not lead to defence response induction, other so far unknown signals accompanying damage play a role in defence initiation. Challenging roots with the commensal bacterium *Pseudomonas protegens* CHA0 did not cause defence reactions in the differentiated root zone, but challenge with the root damage causing pathogen *Ralstonia solanacearum* GMI1000 did lead to induction of defence responses in the differentiated root (Zhou *et al.*, 2020). Hence, differentiated roots possess a regulation mechanism to balance tolerating non-harmful or beneficial microbes and defending against harmful pathogens. In line with this, the DAMP Pep1 elicits much stronger responses in roots compared to MAMPS, suggesting that DAMPs might be the more reliable danger signal for roots in an environment crowded with MAMPs (Poncini *et al.*, 2017).

For many abiotic stresses and for nodulation regulation extensive root to shoot signalling has been demonstrated (Shabala *et al.*, 2015; Ko and Helariutta, 2017). Signal propagation is achieved via Ca^{2+} and ROS waves, electrical signalling, and the transport of hormones, secondary metabolites, proteins, peptides and RNAs in the vasculature (Lucas *et al.*, 2013;

Shabala *et al.*, 2015). Reports on defence-related root to shoot signalling are less plentiful. Wang *et al.* (2019) describe a synergistic system of ROS and electrical signalling between roots and shoots in *Solanum lycopersicum* in the jasmonic acid (JA)-mediated defence against the root knot nematode *Meloidogyne incognita* (for JA defences see 2.2 The JA/ET-mediated defence) (Wang *et al.*, 2019). Infection of roots with *M. incognita* leads to accumulation of JA in roots and shoots. Grafting experiments showed that resistance to *M. incognita* was principally depending on JA synthesis from the shoot and not the root (Wang *et al.*, 2019). After infection the root sends interdependent H_2O_2 and electrical signals up the stem which lead to the activation of MPK1 and MPK2 in leaves. Activation of MAPKs initiates the biosynthesis of JA, which is then transported down to roots to mediate defences against *M. incognita*.

2. Hormone-mediated signalling pathways

Hormone-mediated defence responses are complex networks of signalling cascades. Each hormone induces a different signalling pathway leading to downstream transcriptional reprogramming. Different hormone pathways can synergistically or antagonistically modify the plant defence output (Tsuda *et al.*, 2009; Mine *et al.*, 2018; Aerts *et al.*, 2021). Two major hormone-mediated defence pathways are the salicylic acid (SA)-mediated defence and the jasmonic acid (JA)-mediated defence response. In general, the SA defence pathway is effective against biotrophs; pathogens that derive nutrients from living hosts (Glazebrook, 2005). The JA defence pathway is generally launched in response to wounding and herbivory and also converges with the ethylene (ET) pathway to generate defence output against necrotrophic pathogens that kill host plants and feed off the dead tissue (Glazebrook, 2005).

2.1 The SA-mediated defence

2.1.1 SA biosynthesis

Biosynthesis of SA can be achieved through two pathways; the Isochorismate Synthase (ICS) pathway and the Phenylalanine Ammonia-Lyase (PAL) pathway. Figure 1 presents an overview of both biosynthesis pathways. Differences in the importance of both pathways for SA production exist between different plant species. In Arabidopsis, the ICS pathway is crucial for biosynthesis of pathogen-triggered SA accumulation (Wildermuth *et al.*, 2001). Chorismate is the starting point of both pathways which is a product of the shikimate pathway in plastids (Eberhard *et al.*, 1993; Wildermuth *et al.*, 2001).

The ICS pathways starts in plastids by conversion of chorismate to isochorismate (Strawn *et al.*, 2007). Two enzymes can do this conversion, ISOCHORISMATE SYNTHASE 1 (ICS1) and ICS2, with ICS1 being of far greater importance for SA production in Arabidopsis (Wildermuth

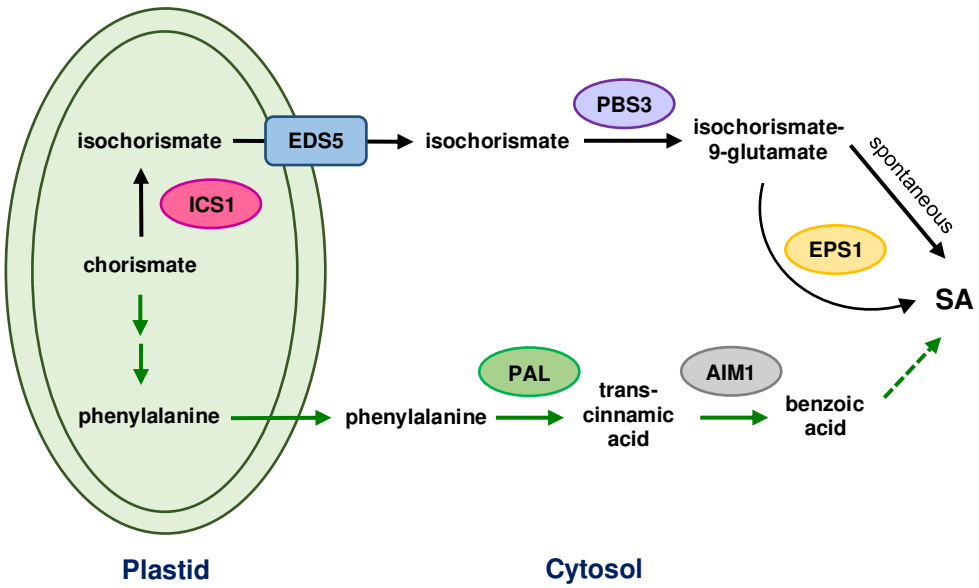

Figure 1. Overview of SA biosynthesis through the ICS (top pathway) and the PAL pathway (bottom pathway).
Both pathways start from chorismate in the plastid. The ICS pathway (black arrows) involves conversion of chorismate via ICS1 to isochorismate, which is exported from the plastid, most likely through EDS5. In the cytosol, isochorismate is further modified by PBS3. Spontaneous decomposition leads to the end product SA. EPS1 can additionally boost this final reaction. In the PAL pathway (green arrows), chorismate is converted to phenylalanine which is further processed in the cytosol by PAL and AIM1 to benzoic acid. Enzymes facilitating the final conversion to SA are unknown (dotted arrow). Modified from Huang *et al.* (2020).

et al., 2001; Garcion *et al.*, 2008; Macaulay *et al.*, 2017; Yokoo *et al.*, 2018). Isochorismate is then transported to the cytosol, most likely via the membrane transporter ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) (Serrano *et al.*, 2013; Yamasaki *et al.*, 2013; Rekhter *et al.*, 2019). In the cytosol, avrPphB SUSCEPTIBLE 3 (PBS3) converts isochorismate to isochorismate-9-glutamate, which is then converted to SA by the acetyltransferase ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1 (EPS1) or by spontaneous decomposition (Rekhter *et al.*, 2019; Torrens-Spence *et al.*, 2019).

In Arabidopsis, the PAL pathway only plays a minor role in pathogen-triggered accumulation of SA. In plastids, chorismate is converted to phenylalanine, which is exported into the cytosol, where PAL converts it to trans-cinnamic acid (Mobley *et al.*, 1999; Cho *et al.*, 2007; Lefevere *et al.*, 2020). Trans-cinnamic acid is further converted into benzoic acid by ABNORMAL INFLORESCENCE MERISTEM 1 (AIM1) and finally to SA via yet unknown enzymes (Ribnicky *et al.*, 1998; Richmond and Bleecker, 1999; Zhang and Li, 2019).

2.1.2 Transcriptional control of SA biosynthesis

Since the SA pathway is a major defence response, SA biosynthesis has to be tightly controlled. Foremost, SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1) and CALMODULIN-BINDING PROTEIN 60G (CBP60g) are recognised as master regulators of *ICS1* induction. In *sard1 cbp60g* double mutants pathogen-triggered SA accumulation is almost completely abolished (Wang *et al.*, 2009; Zhang *et al.*, 2010; Wang *et al.*, 2011). *ICS1* is not the only SA biosynthesis gene targeted by SARD1 and CBP60g. *PBS3* and *EDS5* are also positively regulated by these transcription factors (Sun *et al.*, 2015). SARD1 and CBP60g, therefore, positively regulate all major enzymes of the SA biosynthesis pathway. In addition, a number of WRKY transcription factors, WRKY 8, 28, 46 and 48, and two transcription factors of the Teosinte branched1/ Cycloidea/ Proliferating cell factor (TCP) family, TCP8 and TCP9, have been shown to contribute to positive regulation of *ICS1* expression (Gao *et al.*, 2013)

Negative regulators of SA biosynthesis include three transcription factors of the NAC family, ANAC019, ANAC055, and ANAC072, which target *ICS1* during JA-crosstalk (see section 2.3 Crosstalk between the SA and the JA/ET defence pathway) (Zheng *et al.*, 2012). CBP60a, WRKY18 and WRKY40 also negatively regulate the *ICS1* promoter (Truman *et al.*, 2013; Birkenbihl *et al.*, 2017). Moreover, WRKY18 and WRK40 negatively regulate *EDS5* and *PBS3*, therefore downregulating expression of all important enzymes of SA biosynthesis (Birkenbihl *et al.*, 2017). Furthermore, DIMERIZATION PARTNER (DP)-E2F-LIKE 1 (DEL1) directly targets *EDS5* under non-stressed conditions to repress SA production (Chandran *et al.*, 2014).

Due to their role as master regulators of SA production, SARD1 and CBP60g are themselves tightly regulated. Two transcription factors of the TGACG-binding factor (TGA) family, TGA1 and TGA4 are redundantly involved in the induction of *SARD1* and *CBP60g* in *Psm*-triggered responses (Sun *et al.*, 2018). *SARD1* and *CBP60g* are negatively regulated by three proteins of the Calmodulin-binding transcription factor (CAMTA) family, CAMTA1, CAMTA2, and CAMTA3 (Kim *et al.*, 2013; Sun *et al.*, 2019; Kim *et al.*, 2020). CAMTA3 directly binds to the promoter of *CBP60g* for repression, whereas the negative effect of CAMTA transcription factors on *SARD1* is still to be mechanistically shown (Sun *et al.*, 2019). In non-triggered conditions, WRKY70 binds to the *SARD1* promoter as means of repression of the SA defence (Zhou *et al.*, 2018).

2.1.3 SA perception

After pathogen-triggered SA production defence outputs are generated by massive transcriptional reprogramming. SA reception is still a topic extensively investigated with many open questions. NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1), NPR1-LIKE PROTEIN 3 (NPR3) and NPR4 are to date discussed as the main players in SA defence responses, but many more SA binding proteins exist, highlighting the fact that our current knowledge of SA perception might be restricted (Pokotylo *et al.*, 2019).

In 1997, NPR1 was found to be the main signalling hub for downstream transcriptional reprogramming after SA accumulation (Cao *et al.*, 1997). NPR1 has been shown to bind SA, however, the consequences of the assumed conformational alteration are not known (Wu *et al.*, 2012; Ding *et al.*, 2018). NPR1 is thought to be mainly present in oligomeric form in the cytosol in unstressed conditions, stabilised by disulphide bonds (Tada *et al.*, 2008). SA accumulation leads to redox state changes in the cell and THIOREDOXIN H3 (TRX-h3) and TRX-h5 catalyse NPR1 monomerisation (Mou *et al.*, 2003; Tada *et al.*, 2008). Monomeric NPR1 then translocates to the nucleus where it interacts with TGAs to activate defence gene expression (Zhang *et al.*, 1999).

Independently of NPR1, NPR3 and NPR4, which also bind SA, act as negative regulators of the SA defence response under basal conditions (Ding *et al.*, 2018). For their repressive activity, NPR3 and NPR4 require TGAs, suggesting that they directly act as repressors on promoters of SA-responsive genes (Ding *et al.*, 2018). Accumulation of SA abolishes the

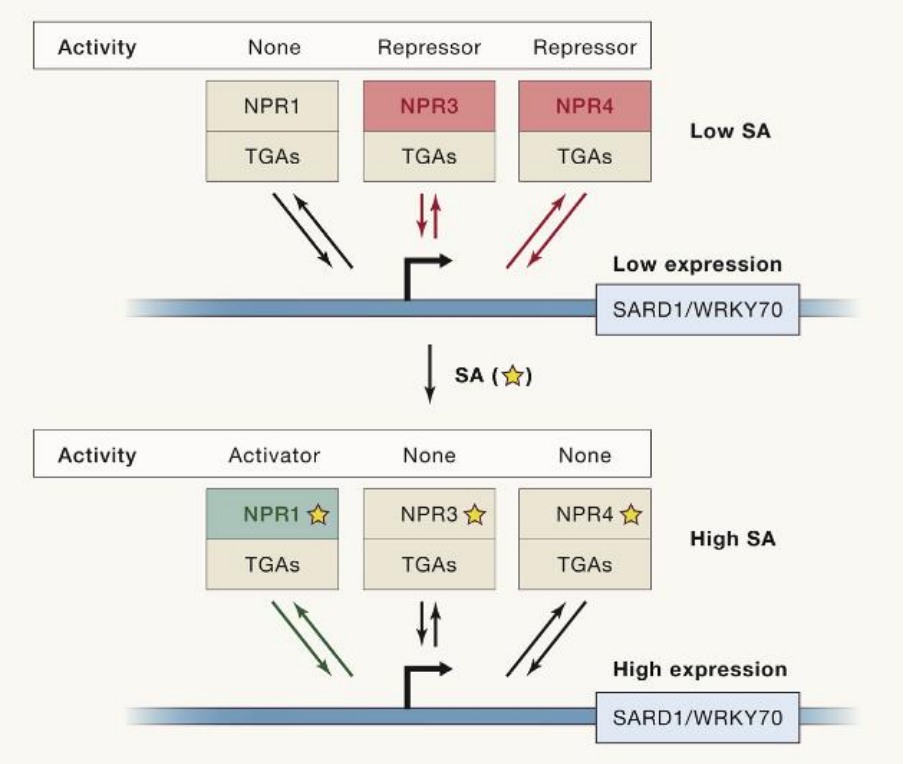

Figure 2. Current model of gene expression regulation in the SA defence response.
During low SA levels (upper panel) NPR3 and NPR4 repress promoters of SA-responsive genes via interaction with TGA factors. NPR1 cannot regulate gene expression in the absence of SA. SA accumulation (lower panel) activates NPR1 which induces defence gene expression. Binding of SA to NPR3 and NPR4 abolishes their repressor activity. Figure taken from Innes (2018).

repressive effect of NPR3 and NPR4 on downstream gene expression (Ding *et al.*, 2018). Figure 2 summarises the current model of transcriptional regulation of SA-responsive gene expression (Ding *et al.*, 2018; Innes, 2018). In unstressed conditions NPR3 and NPR4 repress SA-responsive gene expression and NPR1 is inactive. Accumulation of SA simultaneously leads to defence gene induction by activating NPR1 and decreases repressive effects of NPR3 and NPR4.

2.1.4 Systemic acquired resistance

Despite inducing local defence gene expression, SA is also a crucial component of systemic acquired resistance (SAR). SAR describes a process in which local activation of immune responses by pathogen infection leads to defence priming of distal foliar tissues. If these systemic tissues then encounter pathogen attack, they mount defences more robustly. Plants unable to synthesise SA, as well as SA signalling mutants, show greatly attenuated SAR responses (Gaffney *et al.*, 1993; Cao *et al.*, 1997; Nawrath and Métraux, 1999; Bernsdorff *et al.*, 2016). Nevertheless, grafting experiments of tobacco plants showed that SA, even though crucial for proper defence mounting in distal tissues, is not itself the mobile signal that travels to distal leaves to induce priming (Vernooij *et al.*, 1994). Recent studies suggest that, instead, N-hydroxypipecolic acid (NHP) is the mobile signal (Hartmann *et al.*, 2018; Yildiz *et al.*, 2021). Nevertheless, NHP and SA orchestrate SAR synergistically, with heavily intertwined gene regulation processes (Bernsdorff *et al.*, 2016; Hartmann and Zeier, 2019; Yildiz *et al.*, 2021).

2.2 The JA/ET-mediated defence

2.2.1 JA-Ile biosynthesis

In response to stimuli like wounding and pathogen attack JA-Ile is rapidly synthesised. Figure 3 gives an overview of JA biosynthesis. In plastids, 18:3 and 16:3 fatty acids are converted into *cis*-12-oxo-phytodienoic acid (OPDA) and *dinor*-oxo-phytodienoic acid (*dn*OPDA) (Brash

et al., 1988; Wasternack and Hause, 2013). This consecutively involves lipoxygenases (LOXs), ALLENE OXIDE SYNHASE (AOS) and ALLENE OXIDE CYCLASE (AOC). Subsequently, in peroxisomes, OPDA REDUCTASE 3 (OPR3) converts OPDA and *dn*OPDA to 3-oxo-2-(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC-8) and OPC-6, which are beta-oxidised to yield jasmonic acid (Breithaupt *et al.*, 2001; Breithaupt *et al.*, 2006; Wasternack and Hause, 2013). Jasmonic acid is exported from peroxisomes to the cytosol where JASMONOYL ISOLEUCINE CONJUGATE SYNTHASE 1 (JAR1) converts it into the bioactive form jasmonoyl-isoleucine (JA-Ile) (Staswick and Tiryaki, 2004; Fonseca *et al.*, 2009; Westfall *et al.*, 2012). JA-Ile is translocated to the nucleus via JASMONATE TRANSPORTER 1 (JAT1) where its perception leads to massive transcriptional programming (Li *et al.*, 2017). JA does not only play a major role in defence responses but is also required to coordinate developmental processes such as growth, fertility, senescence and responses to abiotic stress (Huang *et al.*, 2017).

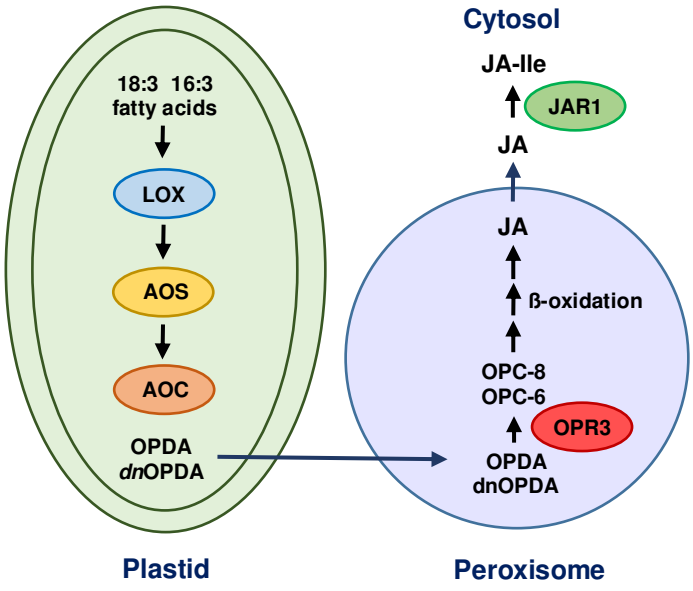

Figure 3. Biosynthesis of JA-Ile in plastids, peroxisomes and the cytosol.
Tri unsaturated fatty acids are the starting point of synthesis and are converted to OPDA and *dn*OPDA in plastids. In peroxisomes, OPDA and *dn*OPDA are converted to JA. The final step of conversion takes place in the cytosol where JA is modified to the bioactive form JA-Ile. Modified from Dhakarey *et al.* (2016).

2.2.2 Perception of JA-Ile by COI1 and JAZs

JA-Ile is perceived by CORONATINE INSENSITIVE 1 (COI1) together with co-receptors of the JASMONATE ZIM-domain (JAZ) family. JAZ proteins are repressors of transcription factors inducing JA-Ile-responsive genes, and their degradation upon JA-Ile perception leads to activation of the JA-defence pathway.

COI1 has two domains, a small N-terminal F-box domain and a large horseshoe shaped domain formed by 18 tandem LRRs (Sheard *et al.*, 2010). The C-terminal LRR domain contains the surface binding pocket for JA-Ile (Sheard *et al.*, 2010). Additionally to JA-Ile binding, inositol pentakisphosphate is required for complex formation between COI1 and JAZs (Sheard *et al.*, 2010). Through association with either ARABIDOPSIS SKP1-LIKE 1 (ASK1) or ASK2, CULLIN 1 (CUL1) and RING-BOX 1 (RBX1), COI1 forms a functional E3 ubiquitin ligase complex; SKP1/CULLIN/F-BOX (SCF^{COI1}) (Dai *et al.*, 2002; Devoto *et al.*, 2002; Xu *et al.*, 2002). COI1 interacts with ASK1/2 via its F-box motif (Sheard *et al.*, 2010). As a scaffolding protein, CUL1 links COI1 and ASK1/2 to RBX1, which binds the E2 ubiquitin-conjugating enzyme (Gray *et al.*, 2002). Upon binding of JA-Ile, COI1 and JAZ interaction is facilitated, leading to polyubiquitination of JAZ proteins and their degradation via the 26S proteasome (Chini *et al.*, 2007; Thines *et al.*, 2007). Via its F-box motif, COI1 also interacts with MEDIATOR 25 (MED25), a protein of the multisubunit mediator complex crucial for transcriptional initiation, at JA-Ile responsive promoters (An *et al.*, 2017). The ability of JA-Ile to promote the interaction of COI1 and JAZs is reduced in *med25* mutants, suggesting that recruitment of COI1 to target promoters by MED25 is crucial for bringing together COI1 and JAZs (An *et al.*, 2017). Activation of the JA pathway by perception of JA-Ile is displayed in Figure 4.

The JAZ proteins are a family of 13 repressors. JAZs contain two important domains; the ZINC-FINGER EXPRESSED IN INFLORESCENCE MERISTEM (ZIM) domain and the C-terminal JA-associated (Jas) domain. The Jas domain is important for JAZ degradation upon JA-Ile sensing. It contains the highly conserved 20 amino-acid long Jas-degron consisting of an α-helix for interaction with COI1 and a loop region important for trapping JA-Ile in its binding

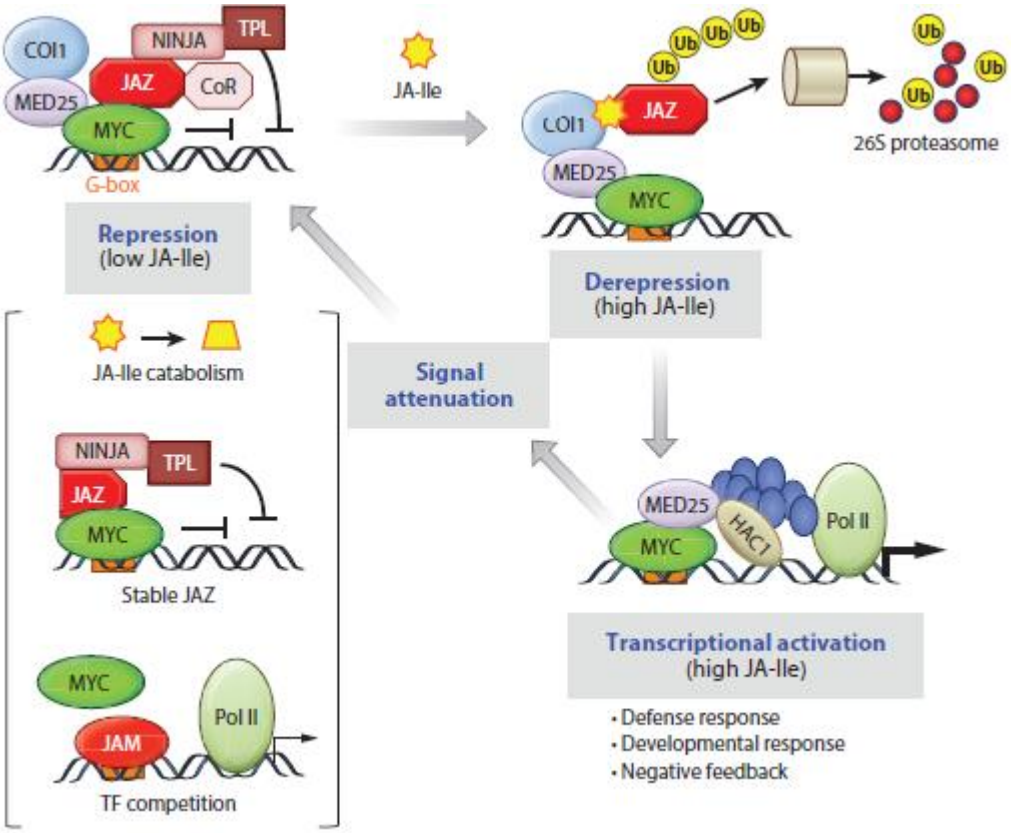

Figure 4. Induction of JA-signalling by JA-Ile.
In basal conditions, transcription factors of JA-responsive genes, like MYCs, are repressed by JAZ proteins with their co-repressors TPLs and potentially other co-repressors (CoR). JA-Ile facilitates binding of COI1 and JAZs which leads to JAZ polyubiquitination and degradation via the 26S proteasome. MYC and other transcription factors activate expression of target genes via the Polymerase II Transcription-Initiation Complex involving MED25 and HISTONE ACETYLTRANSFERASE OF THE CBP FAMILY 1 (HAC1). Activation of JA-Ile catabolic genes, alternative spliced JAZs and negative competitors of MYCs reconstitutes negative feedback after JA-defence activation. Figure taken from Howe *et al.* (2018).

pocket (Sheard *et al.*, 2010). Via their Jas domain JAZs also bind to and repress transcription factors of the JA response such as the basic helix-loop-helix (bHLH) transcription factors MYC2, 3 and 4 (Zhang *et al.*, 2015).

Different mechanisms by which JAZs repress transcription factor activity have been demonstrated. In roots, JAZs recruit the adapter protein NOVEL INTERACTOR OF JAZ (NINJA) through their ZIM domain (Pauwels *et al.*, 2010; Acosta *et al.*, 2013). NINJA facilitates recruitment of the Groucho/Tup1-type co-repressor TOPLESS (TPL) and TPL-related proteins (Pauwels *et al.*, 2010). Direct binding of TPL has also been observed for JAZ8 and is thought to occur with JAZ5, 6 and 7 as well (Kagale *et al.*, 2010; Shyu *et al.*, 2012). Moreover, JAZs

have been shown to repress JA-signalling by blocking interaction of MYC3 and MED25, thereby hindering recruitment of the Polymerase II Transcription-Initiation Complex (Zhang *et al.*, 2015; Zhang *et al.*, 2017). JAZs have also been suggested to employ chromatin structure condensation for repression by interacting with histone modifying proteins HISTONE DE-ACETYLASE 6 (HDA6), LIKE HETEROCHOMATIN PROTEIN 1 (LHP1) and EMBRYONIC FLOWER 2 (EMF2) (Zhu *et al.*, 2011; Li *et al.*, 2021).

2.2.3 JA defence branches

JAZ proteins repress of a number of different transcription factors regulating various JA-Ile-mediated plant processes (Song *et al.*, 2011; Hu *et al.*, 2013; Jiang *et al.*, 2014). Two branches of JAZ-repressed JA-responses are specifically important in defence: the MYC branch and the ETHYENE RESPONSE FACTOR (ERF) branch.

Activation of the MYC2-, 3-, 4-regulated pathway leads to accumulation defence compounds such as glucosinolates and anthocyanins, launched in response to herbivores and wounding (Niu *et al.*, 2011; Schweizer *et al.*, 2013). A typical marker gene for activation of the MYC-branch is *VEGETATIVE STORAGE PROTEIN 2 (VSP2).*

The ERF branch of the JA-defence is co-regulated by JA and ethylene (ET) which are often induced simultaneously in response to necrotrophic pathogen attack (De Vos *et al.*, 2007). The JA and ET pathway converge at the transcription factors ETHYLENE INSENSITIVE 3 (EIN3) and ETHYLENE INSENSITIVE 3-LIKE 1 (EIL1) which are repressed by JAZ proteins (Zhu *et al.*, 2011). Downstream of their perception, both hormones synergistically activate EIN3/EIL1. Activation of EIN3/EIL1 in turn induces the transcription factor OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2 59 (ORA59) that regulates downstream defences against necrotrophic pathogen attack (Pré *et al.*, 2008). A marker gene for activation of the ERF-branch is *PLANT DEFENSIN 1.2* (*PDF1.2*) (Penninckx *et al.*, 1996; Zarei *et al.*, 2011; Zhu *et al.*, 2011).

2.2.4 Negative regulation of the JA defence

The JA pathway is negatively regulated by the SA pathway, which will be discussed in 2.3 Crosstalk between the SA and the JA/ET defence pathway. Moreover, JA signalling is regulated via negative feedback loops. JA-Ile-induced turnover of JAZ proteins induces genes involved in JA catabolism and negative regulators of JA signalling such as JA-associated MYC2-like (JAM) proteins and MYC2-Targeted BHLH (MTB) proteins that interfere with MYC activity (Koo *et al.*, 2011; Nakata *et al.*, 2013; Sasaki-Sekimoto *et al.*, 2013; Song *et al.*, 2013; Fonseca *et al.*, 2014; Liu *et al.*, 2019). JA-Ile perception also leads to activation of *JAZs* themselves (Chung *et al.*, 2010). Alternative splicing of these newly made *JAZ* transcripts can create JAZ proteins resistant to degradation via SCF^{COI1} desensitising the cell for JA-Ile (Chung *et al.*, 2010; Moreno *et al.*, 2013; Zhang *et al.*, 2017).

2.3 Crosstalk between the SA and the JA/ET defence pathway

The SA and JA pathway reciprocally antagonise each other. This is thought to occur to cost-effectively launch the most efficient defence against the invading pathogen (Huot *et al.*, 2014).

The JA pathway negatively regulates the SA pathway by both inhibiting SA biosynthesis and promoting inactivation of SA. Upon release from JAZ repression, MYC2 induces the transcription factors *ANAC019*, *ANAC055* and *ANAC072*, which repress the *ICS1* promoter (Zheng *et al.*, 2012). Moreover, MYC2 induces BENZOIC ACID/SA CARBOXYL METHYLTRANSFERASE 1 (BSMT1) which converts SA to inactive methyl salicylate (MeSA) (Zheng *et al.*, 2012).

The mechanisms by which the SA pathway negatively influences the JA pathway are less clear. The majority of SA-mediated crosstalk seems to happen downstream of JA biosynthesis (Leon-Reyes *et al.*, 2010). Even though some biosynthetic genes of the JA pathway, such as *AOS*, *AOC* and *OPR3*, are reduced by SA treatment, *PDF1.2* can still be repressed in the *aos* mutant, suggesting repressive effects downstream of JA biosynthesis (Leon-Reyes *et al.*, 2010). Spoel *et al.* (2003) showed that NPR1 is required to repress JA-induced expression of

PDF1.2 after SA treatment. This, however, does not require NPR1 in the nucleus so the mechanism of the repression is unclear (Spoel *et al.*, 2003).

Moreover, SA-induced glutaredoxins (GRXs) have been shown to be able to repress *PDF1.2* through suppression of *ORA59*, which requires TGA transcription factors (Ndamukong *et al.*, 2007; Zander *et al.*, 2012; Zander *et al.*, 2014).

Li *et al.* (2004 and 2006) showed that SA-induced WRKY70 is involved in SA-JA crosstalk. Overexpression of WRKY70 has been shown to repress JA-induced *PDF1.2* expression (Li *et al.*, 2006). Nevertheless, SA is still able to repress *PDF1.2* in *wrky70* mutants, suggesting that WRKY70 might be competent but not necessary for SA-JA crosstalk or different WRKYs show redundancy for this role (Li *et al.*, 2006).

3. The vascular pathogen *Verticillium longisporum*

3.1 Infection cycle of *V. longisporum*

The genus of the soil-borne ascomycete fungus *Verticillium* comprises ten species, several of which are plant pathogens (Inderbitzin, Davis, *et al.*, 2011). Originally, *V. longisporum* was described as a subspecies of its close relative *V. dahliae* (Stark, 1961). However, in 1997, *V. longisporum* was recognised as a separate species (Karapapa *et al.*, 1997). *V. longisporum* is unique within the *Verticillium* genus as it is the only allodiploid species among its haploid relatives (Ingram, 1968; Inderbitzin, Bostock, *et al.*, 2011). For laboratory studies this makes *V. longisporum* harder to genetically manipulate and often the haploid *V. dahliae* is used to study plant-*Verticillium* interactions.

V. longisporum has a narrow host range, mostly restricted to Brassicaceae (Depotter *et al.*, 2016). In particular, it is a threat to rapeseed (*Brassica napus*) production, especially in Europe (Depotter *et al.*, 2016). In the field, an observable symptom of *V. longisporum* infection in *B. napus* is the appearance of dark unilateral stripes on the stems towards the end of the growing season (Depotter *et al.*, 2016). *A. thaliana* is also a suitable host to study *V. longisporum* interactions with. Under laboratory conditions, *V. longisporum* causes similar symptoms in *B.*

napus and *A. thaliana*, which include stunting, leaf chlorosis and premature senescence. Another observable symptom is vein clearing in infected foliage. Vein clearing is the result of trans-differentiation of chloroplast-rich bundle sheath cells into functional xylem vessels (Reusche *et al.*, 2012).

V. longisporum is a vascular pathogen that enters hosts through the root and spreads through colonisation of the xylem. Figure 5 depicts the infection cycle of *V. longisporum* (Berlanger and Powelson, 2000). *V. longisporum* can produce melanised microsclerotia, thick-walled fungal cells that are long lasting resting structures (Stark, 1961). These can remain in the soil for many years (Depotter *et al.*, 2016). Upon sensing root exudates of potential host plants these microsclerotia germinate and hyphae grow towards the host root (Berlanger and Powelson, 2000). Hyphae grow along root hairs towards the root surface, where the fungus penetrates preferentially lateral roots (Eynck *et al.*, 2007). Before penetration, hyphae swell up and then form a thin penetration peg for breaching the plant cell wall (Eynck *et al.*, 2007). Once hyphae have penetrated cell walls, they grow intercellularly and intracellularly towards the central cylinder and into the xylem (Eynck *et al.*, 2007). In the xylem of *B. napus*, *V. longisporum* produces conidia that can be transported shootward by the transpiration stream (Depotter *et al.*, 2016). Conidia can get trapped and germinate to colonise other xylem vessels throughout the plant (Depotter *et al.*, 2016). Eynck *et al.* (2007) reported that *V. longisporum* never manages full colonisation of *B. napus* roots but remains within a few heavily colonised vessels. In the narrow roots of Arabidopsis, *V. longisporum* might grow in hyphal form though the root xylem to the shoot. At later stages of infection *V. longisporum* grows out of the xylem and starts feeding on the senescing plant matter (Eynck *et al.*, 2007; Depotter *et al.*, 2016). Therefore, *V. longisporum* is classed as a hemibiotrophic pathogen. At the vessel colonising phase *V. longisporum* employs a biotrophic lifestyle feeding from the relatively nutrient poor xylem sap, the later feeding of the foliage is the necrotrophic life stage (Depotter *et al.*, 2016). During its necrotrophic phase *V. longisporum* produces microsclerotia that are released into the soil with the decomposed plant foliage (Heale and Karapapa, 1999).

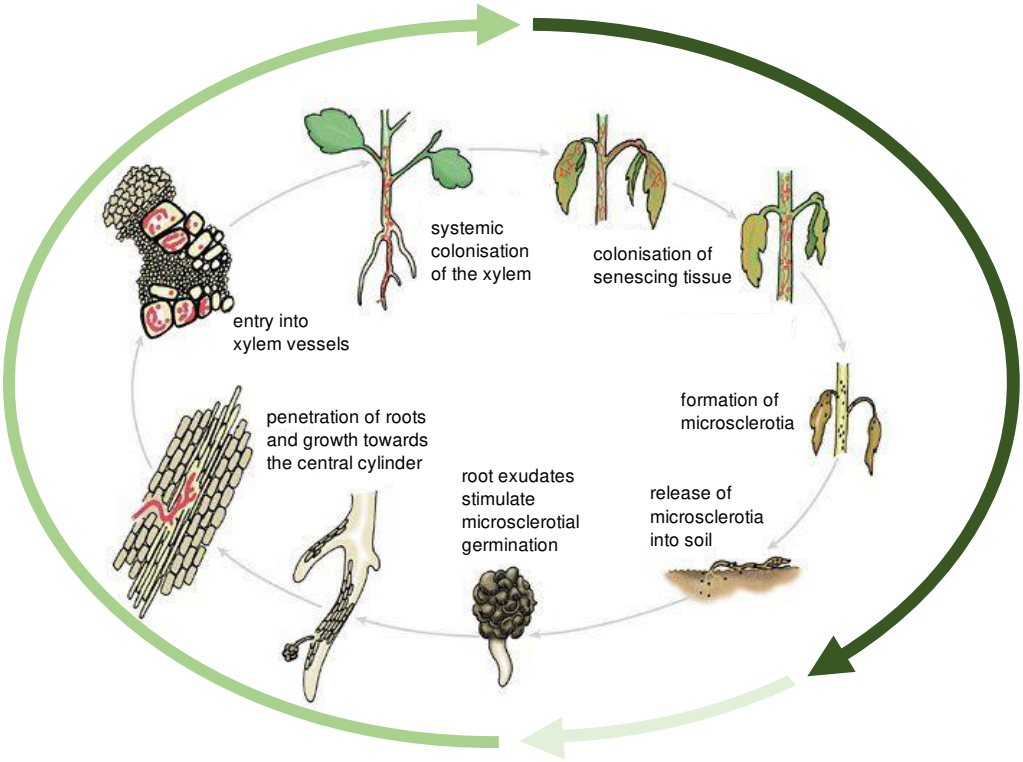

Figure 5. Infection cycle of *V. longisporum*.
Microsclerotia in the soil germinate upon sensing root exudates and penetrate host roots. Once hyphae have entered the root they grow towards the central cylinder and into the xylem. In the xylem, *V. longisporum* spreads though hyphal growth and production of conidia, reaching and colonising also upper parts of the host plant. During host senescence, *V. longisporum* leaves the xylem and starts feeding on plant material. Microsclerotia are formed and released in to the soil, where they rest until germination is stimulated. Pale green arrow: resting stage; light green arrow: biotrophic stage; dark green arrow: necrotrophic stage. Drawing by Vickie Brewster, coloured by Jesse Ewing. Figure modified from Berlanger and Powelson (2000).

3.2 Disease control measures against *V. longisporum* are insufficient

The vascular lifestyle of *V. longisporum* makes it inaccessible for fungicides during its residency in the plant. Hence, measures of reducing the primary inoculum in the soil have been concentrated on for disease control. The persistence of microsclerotia makes these measures difficult. As many fumigation techniques used to minimise the microsclerotia load in the soil have been banned for ecological reasons, crop rotations are one of the few effective measures to control infection events (Powelson and Carter, 1973; Depotter *et al.*, 2016). The disease control of choice would be the use of resistant plants, however, to date these remain scarce. Moreover, monocultural farming promotes selection pressure for pathogens to overcome resistance, making the sustainability of this approach questionable (Lo Presti *et al.*, 2015).

Resistance genes have been identified for *a* few *Verticillium* spec*ies* and the similar hemibiotrophic, soil-borne, vascular pathogen *Fusarium oxysporum,* not however*,* for *V. longisporum.*

In tomato, for example, *Ve1* has been identified as a resistance gene against certain strains of *V. dahliae*, *V. albo-atrum* and *F. oxysporum* but not *V. longisporum* (Kawchuk *et al*., 2001; Fradin *et al*., 2009; De Jonge *et al*., 2012). *Ve1* encodes a plasma membrane-localised RLP that detects Ave1, a fungal effector secreted during host colonisation (Kawchuk *et al*., 2001; Fradin *et al*., 2009; De Jonge *et al*., 2012).

Also, six *RESISTANCE TO FUSARIUM OXYSPORUM (RFO1-6*) genes have been identified against different races of *F. oxysporum* (Diener and Ausubel, 2005; Cole and Diener, 2013). Cole and Diener (2013) showed that induced expression of *FMO3*, which encodes an RLK, in roots upon infection hinders the spread of *F. oxysporum* in the vascular tissue.

Even though no resistance gene against *V. longisporum* has been identified, a few tolerant cultivars of *B. napus* exist. For example, the *B. napus* cultivar SEM 05-500256 produces higher constitutive and induced amounts of cell wall-bound and soluble phenolics as well as enhanced lignin deposition in roots and hypocotyl than susceptible cultivars (Eynck *et al*., 2009). These processes hinder the spread of *V. longisporum* to the shoot and convey tolerance (Eynck *et al*., 2009).

3.3 Plant defences against *V. longisporum*

Globally, rapeseed is the second largest oil seed crop, being important for production of oil for human nutrition, lubricants and biofuels (FAO Database, 2018). In 2017, rapeseed constituted 63% of all oil seed production in Europe, and demand is yet increasing (FAO Database, 2018). To improve the meagre disease management of *V. longisporum* infection on rapeseed, it is crucial to enhance our understanding of plant-*V. longisporum* interactions.

A number of plant defences have been shown to be somewhat effective in combating infection by *V. longisporum* including barrier construction and production of antifungal compounds.

As described above, enhanced lignification to combat fungal spread can be effective (Eynck *et al.*, 2009). In accordance, Fröschel *et al.* (2021) showed that *V. longisporum* targets endodermal barriers to get access into the central cylinder. Translating ribosome affinity purification (TRAP)-translatome analysis showed that genes involved in the formation of the endodermal barriers, casparian strip and suberin lamellae, were downregulated after infection with *V. longisporum* (Fröschel *et al.*, 2021). Moreover, they claim that mutants impaired in proper casparian strip and suberin lamellae formation are more susceptible to *V. longisporum* (Fröschel *et al.*, 2021). Hence, the structural barriers in differentiated plant roots constitute a basal defence to infection, that needs to be overcome by *V. longisporum* to reach the xylem.

The production of tryptophan-derived indole glucosinolates (IGs) against *V. longisporum* has been shown to be important in plant defence. Arabidopsis *cyp79b2 cyp79b3* double mutants, carrying mutations in two CYTOCHROME P450 coding family members impaired in production of camalexin and IGs, showed enhanced susceptibility to *V. longisporum* infection (Iven *et al.*, 2012). In accordance, Fröschel *et al.* (2019) showed that overexpression of different ERF transcription factors leads to decreased susceptibility against *V. longisporum* by induction of *CYP81F2,* another CYTOCHROME P450 family member involved in IG synthesis (Fröschel *et al.*, 2019).

Analysis of apoplastic wash fluid from leaves of *A. thaliana* infected with *V. longisporum* at 25 dpi, showed enrichment of GERMIN-LIKE PROTEIN 3 (GLP3) (Floerl *et al.*, 2012). Germins and Germin-like proteins (GLPs) are glycoproteins of the cupin superfamily, which have been shown to possess direct antifungal activities as well as defence signalling capabilities (Dunwell *et al.*, 2008). Indeed, Germin-like-proteins from cotton *(Gossypium hirsutum)* have been shown to inhibit growth of *V. dahliae* and *F. oxysporum* (Pei *et al.*, 2019*;* Pei *et al.*, 2020). Overexpression of *GLP1* from sugar beet (*Beta vulgaris*) in *A. thaliana* has been shown to

reduce susceptibility to *V. longisporum* infection, by reducing fungal growth on the root surface and inside the root (Knecht *et al*., 2010).

The role of phytohormones in defences against *V. longisporum* is less clear. Ratzinger *et al.* (2009) reported accumulation of SA and SA-glucoside (SAG) in root and hypocotyl xylem sap as well as shoot extracts of *B. napus* at 14, 21, 28 and 35 dpi with *V. longisporum Vl43*. No differences in JA and abscisic acid (ABA) accumulation between mock-treated and infected plants at these time points were detected.

Zheng *et al.* (2019) showed that *B. napus* plants ectopically expressing the bacterial salicylate hydroxylase *NahG*, which degrades SA to catechol, accumulated higher fungal loads in the hypocotyl at 14 and 21 dpi with *V. longisporum Vl43*. At 7 dpi, a resistant cultivar had higher levels of SA in the hypocotyl compared to a susceptible cultivar, however, no differences in SA levels were detected at 14 and 21 dpi anymore.

In contrast, Johansson *et al.* (2006) reported that the Arabidopsis JA and ET biosynthesis/signalling mutants *jar1-1*, *coi1-16* and *ein3-1* showed no difference in susceptibility to *V. longisporum VD11* compared to wild-type (WT) plants. SA-biosynthesis deficient *NahG* and *sid2-1* plants also showed no difference in susceptibility. In contrast, *npr1-1* mutants were more susceptible to *V. longisporum* infection than WT plants. Moreover, SA treatment caused no phenotypic differences to infection, whereas pre-treatment with MeJA or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) increased tolerance to fungal infection. Similarly, Ralhan *et al.* (2012) showed no difference in susceptibility of JA and SA biosynthesis mutants *aos* and *sid2-2*, however, reported increased tolerance of *coi1-t* plants infected with *V. longisporum Vl43*.

On the other hand, genes that promote susceptibility to *V. longisporum* have been found. Pröbsting *et al.* (2020) identified the susceptibility gene CALRETICULIN 1A *(CRT1a*) in *B. napus* and *A. thaliana*. *CRT1a* is induced after infection in *A. thaliana* and *B. napus* but when mutated, plants did not show severe loss of leaf area after infection anymore (Pröbsting *et al*., 2020).

4. Preliminary work

Another susceptibility gene identified in *A. thaliana* for successful infection with *V. longisporum* is *COI1* (Ralhan *et al.*, 2012). After infection, the JA-Ile-receptor mutant *coi1* shows greatly reduced infection symptoms compared to WT plants (Figure 6a) (Ralhan *et al.*, 2012). The JA biosynthesis mutant *aos* shows WT-like symptoms (Figure 6a), hence, the tolerance of *coi1* plants is not due to a disruption in the JA signalling pathway. Initial fungal colonisation of *coi1* roots is not compromised, and the progression of infection is unaltered in *coi1* plants compared to *aos* and WT until about 10 days past inoculation (dpi) (Ralhan *et al.*, 2012). However, at later stages of infection (15-19 dpi) lower amounts of fungal biomass are found in shoots of *coi1* as compared to WT and *aos* (Ralhan *et al.*, 2012).

Some biotrophic pathogens like *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 produce coronatine which interacts with COI1 and activates the JA pathway to supress SA defences (Kloek *et al.*, 2001). *coi1* mutants, which are unable to perceive coronatine, are more tolerant to infection as they do not experience a suppression of the SA pathway (Kloek *et al.*, 2001).

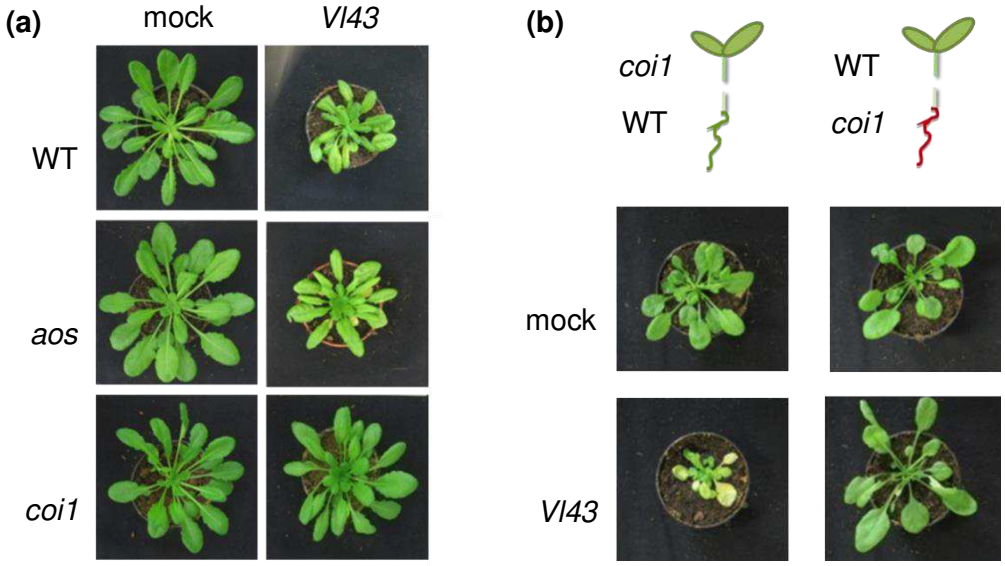

Figure 6. *coi1* plants are tolerant against infection with *V. longisporum*.
(a) Disease symptoms in WT (Col-0), *aos* and *coi1* shoots 15 days after mock treatment or inoculation with *V. longisporum Vl43.* (b) Reciprocal grafts between WT (Col-0) and *coi1* plants 21 days after mock treatment or inoculation with *V. longisporum Vl43.* Figures modified from Ralhan *et al.* (2012).

In the interaction with *V. longisporum*, the possibility of a fungal-derived JA-Ile mimic activating COI1-mediated signal transduction in *aos* plants but not *coi1* plants, was excluded by the observation that marker genes for neither the JA nor the JA/ET pathway were induced in *aos* plants after infection (Ralhan *et al.*, 2012). In line with this, *coi1* plants do not show high levels of *PATHOGENESIS-RELATED PROTEIN 1 (PR1)* after infection, which would hint at a hyper-activation of the SA pathway (Ralhan *et al.*, 2012).

In grafting experiments, reciprocal grafts between Col-0 and *coi1* plants were created (Ralhan *et al.*, 2012). Infection of these chimeric plants showed that only plants which had *coi1* roots would stay tolerant to infection (Figure 6b) (Ralhan *et al.*, 2012).

To investigate gene expression patterns that could explain the observed tolerance mediated by *coi1* roots, two RNA-Sequencing (RNA-seq) experiments were performed by a previous PhD student, Johanna Schmitz. The first RNA-seq data set was generated from axenically grown *coi1*, *aos* and WT roots at 4 dpi. The transcriptome data revealed basal de-repression of defence related genes specifically in mock-treated *coi1* roots, however, no notable responses to fungal infection were observed on transcriptome level in any genotype. Therefore, to gain insight into the role of COI1 after infection with *V. longisporum*, a second RNA-seq analysis was performed in a more natural soil-based infection system at 10 dpi. Here, besides *coi1*, *aos* and WT roots, additionally the SA biosynthesis-impaired *sid2* mutant was included to assess the contributions of both the JA and the SA defence to the root response triggered by *V. longisporum*. Again, mock-treated *coi1* roots showed constitutive de-repression of defence-related genes. In contrast to the first RNA-seq data set, this time pronounced changes in the root transcriptomes were detectable after infection.

II. Thesis aims

The de-repression of a large number of genes in mock-treated *coi1* roots showed that COI1 has a role in gene repression. As the *aos* mutant shows WT-like expression of those genes, the new COI1 repressor function must be JA-Ile-independent. The first aim of this thesis was to investigate if the novel COI1 repressor function works independently of components of the canonical JA-signalling pathway. Findings regarding this objective are described in Article 1. In the second RNA-seq dataset from the soil-based infection system, clear responses to the fungus were seen in root transcriptomes. The second aim of this thesis was to explore the role of COI1 in gene expression regulation upon infection with *V. longisporum*. Furthermore, potential reasons for the tolerance of *coi1* plants were addressed. Article 2 presents the findings achieved in understanding these processes.

III. Article 1

The jasmonoyl-isoleucine receptor CORONATINE INSENSITIVE1 suppresses defense gene expression in Arabidopsis roots independently of its ligand

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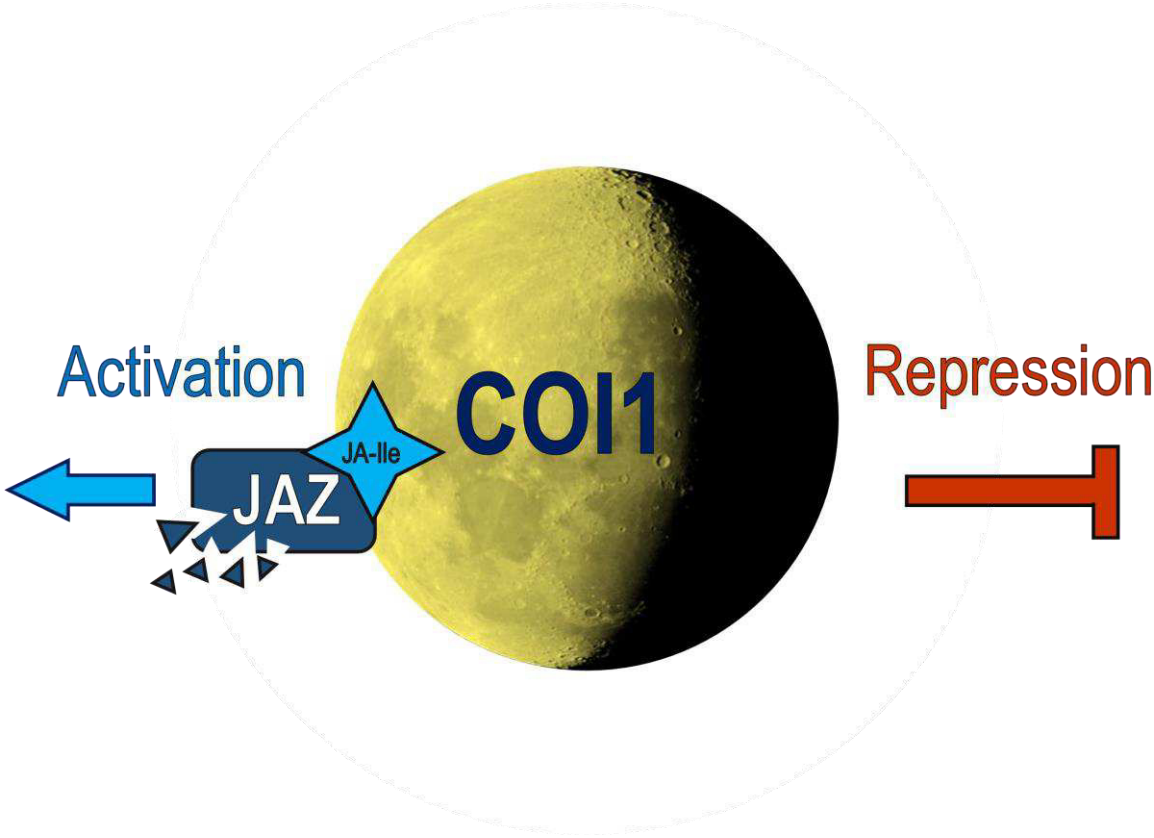
Detailed contributions by the PhD candidate:

Louisa Ulrich prepared, performed and analysed the following experiments: gene expression analyses displayed in Figure 3a, Figure 5a and 5c, Figure 6, Figure S4 and Figure S9; western blot analyses displayed in Figure 5b and Figure S12; yeast two hybrid analysis displayed in Figure S11 (right panel); the transient reporter assay displayed in Figure S12 and documentation of seed pod production displayed in Figure S13. Louisa Ulrich prepared clones and generated the transgenic *coi1-t/COI1* and *coi1-t/COI1$_{AA}$* lines. Louisa Ulrich performed all statistical analysis for the article with exception of the RNA-seq data analysis. Louisa Ulrich created all Figures with the exception of Figure 1a, Figure 2a, Figure S1 and Figure S10.

Graphical Abstract



Significance Statement

Phenotypic differences of hormone receptor and corresponding hormone biosynthesis mutants are unexpected. Such an unusual scenario was discovered for COI1 which affects the root transcriptome even when disconnected from its signaling pathway.

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The jasmonoyl-isoleucine receptor CORONATINE INSENSITIVE1 suppresses defense gene expression in Arabidopsis roots independently of its ligand

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SUMMARY

The F-box protein CORONANTINE INSENSITIVE1 (COI1) serves as the receptor for the plant hormone jasmonoyl-isoleucine (JA-Ile). COI1, its co-receptors of the JASMONATE ZIM-domain (JAZ) protein family, and JA-Ile form a functional unit that regulates growth or defense mechanisms in response to various stress cues. Strikingly, COI1, but not JA-Ile, is required for susceptibility of *Arabidopsis thaliana* towards the soil-borne vascular pathogen *Verticillium longisporum*. In order to obtain marker genes for further analysis of this JA-Ile-independent COI1 function, transcriptome analysis of roots of *coi1* and *allene oxide synthase* (*aos*) plants (impaired in JA biosynthesis) was performed. Intriguingly, nearly all of the genes that are differentially expressed in *coi1* versus *aos* and wild type are constitutively more highly expressed in *coi1*. To support our notion that COI1 acts independently of its known downstream signaling components, *coi1* plants were complemented with a COI1 variant ($COI1_{AA}$) that is compromised in its interaction with JAZs. As expected, these plants showed only weak induction of the expression of the JA-Ile marker gene *VEGETATIVE STORAGE PROTEIN2* after wounding and remained sterile. On the other hand, genes affected by COI1 but not by JA-Ile were still strongly repressed by $COI1_{AA}$. We suggest that COI1 has a potential moonlighting function that serves to repress gene expression in a JA-Ile- and JAZ-independent manner.

Keywords: CORONATINE INSENSITIVE1, JASMONATE ZIM-domain, jasmonoyl-isoleucine, moonlighting, repression, root.

INTRODUCTION

Hormones serve as signaling molecules that are crucial for the regulation of development, growth, and anti-stress programs. It is generally accepted that internal or external cues lead to increased cellular hormone concentrations. Binding of hormones to their cognate receptors is crucial for the activation of signaling cascades resulting in cellular responses like transcriptional re-programming. Consistent with this concept, hormone receptor mutants usually have similar phenotypes as the corresponding hormone biosynthesis mutants.

The jasmonoyl-isoleucine (JA-Ile) receptor CORONATINE INSENSITIVE1 (COI1) acts as an adaptor protein within the E3 ubiquitin ligase complex SCF^{COI1} and forms – upon hormone binding – a transient ternary complex with JASMONATE ZIM-domain (JAZ) proteins, resulting in their ubiquitination and subsequent degradation through the 26S proteasome (Chini *et al.*, 2007; Thines *et al.*, 2007). JAZs interfere with the activity of various transcription factors, including MYC2, MYC3, MYC4 (Fernandez-Calvo *et al.*, 2011), ETHYLENE INSENSITIVE3 (EIN3), and EIN3-LIKE1 (EIL1) (Zhu *et al.*, 2011). Reduced JAZ protein levels thus lead to the activation of promoters controlled by these factors. In *Arabidopsis thaliana*, the pathway is initiated during stamen development (Jewell and Browse, 2016) and after various stress cues like wounding and insect feeding (McConn *et al.*, 1997), pathogen infection (Vijayan *et al.*, 1998), and salt treatment (Geng *et al.*, 2013). All these processes are impaired in both the *coi1* mutant and the JA-Ile biosynthesis mutant *allene oxide synthase* (*aos*).

However, *coi1* and *aos* do not always show the same phenotype. For instance, root growth of the JA biosynthesis mutant *aos* was as sensitive to 4 μM of the ethylene (ET) precursor 1-aminocyclopropane-1-carboxylic acid (Adams and Turner, 2010) or phytoprostane PPA1 (Stotz *et al.*, 2013)

as the wild type, while the *coi1-16* mutant was less affected. More recently, COI1, but not JA-Ile, was shown to be required for the extracellular adenosine 5′-triphosphate (eATP)-mediated reinforcement of plant defense against the necrotrophic fungus *Botrytis cinerea*. Here, eATP treatment of *aos* plants led to the degradation of a transgenic JAZ1: GUS fusion protein (Tripathi *et al.*, 2018).

An unexpected difference between *coi1* and *aos* plants has also been observed after infection with the soil-borne vascular pathogens *Fusarium oxysporum* (Thatcher *et al.*, 2009) and *Verticillium longisporum* (Ralhan *et al.*, 2012). In both pathosystems, *coi1* was more tolerant than wild type and *aos*. It was hypothesized that *F. oxysporum* or *V. longisporum* might synthesize JA-Ile or a JA-Ile mimic to induce susceptibility through COI1, a strategy that has been demonstrated for the coronatine-producing bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 (Kloek *et al.*, 2001). Like JA-Ile, coronatine interacts with COI1, which results in the degradation of JAZ repressor proteins (Chini *et al.*, 2007; Thines *et al.*, 2007). Activation of the JA pathway antagonizes SA-dependent defense responses, which explains the observed higher resistance of *coi1* (Brooks *et al.*, 2005). Hence, infection of *aos* plants with coronatine-producing *Pseudomonas syringae* pv *maculicola* (*Psm*) ES4326 leads to the induction of the expression of JA-responsive genes (Wang *et al.*, 2008). In contrast, after *V. longisporum* infection, expression of neither the JA marker gene *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) nor the JA/ET marker gene *PLANT DEFENSIN1.2* (*PDF1.2*) was induced in the *aos* mutant. This indicates that fungal compounds that would activate the known COI1-dependent signal transduction chain are not produced by *V. longisporum* (Ralhan *et al.*, 2012). Consistently, the tolerance observed in *coi1* is not associated with hyper-activation of the SA-induced gene *PATHOGENESIS-RELATED1* (*PR1*) (Ralhan *et al.*, 2012), which was observed after infection with *Pst* DC3000 (Kloek *et al.*, 2001). Together, our results have unraveled a COI1 activity which acts independently from JA-Ile or any JA-Ile mimic (Ralhan *et al.*, 2012). Grafting experiments between *coi1* rootstocks and wild-type scions (and *vice versa*) revealed that the JA-Ile-independent COI1-mediated susceptibility towards *F. oxysporum* and *V. longisporum* requires the wild-type *COI1* allele in roots (Ralhan *et al.*, 2012; Thatcher *et al.*, 2009).

Here, we approached the question whether known components of the JA-Ile signaling pathway are required for the JA-Ile-independent COI1 function. Since *coi1*-mediated tolerance is a complex phenotype, we aimed to find a simpler proxy for our analysis. Therefore, we performed transcriptome analysis of roots of *coi1*, *aos*, and wild-type plants. We found that the transcriptome of *coi1* roots is characterized by a set of constitutively expressed genes. Using selected marker genes, we show that COI1 can function as a repressor even when the interaction between COI1 and JAZ proteins is severely impaired. Moreover, other known components of the COI1 signaling cascade, like JAZ-regulated transcription factors MYC2, MYC3, and MYC4 or EIN3 and EIL1, do not contribute to COI1-mediated repression of the marker genes. It is concluded that either COI1 facilitates degradation of yet unknown substrates or, alternatively, it is a moonlighting protein.

RESULTS

Segregating plants from heterozygous *COI1*/*coi1* and *AOS*/*aos* populations were used for RNA-seq analysis

Previous grafting experiments have shown that the *coi1* allele has to be present in roots to confer tolerance against either *F. oxysporum* or *V. longisporum* (Ralhan *et al.*, 2012; Thatcher *et al.*, 2009). Therefore, the transcriptomes of roots from *V. longisporum*-infected and uninfected wild-type, *coi1-t*, and *aos* plants were analyzed. Since defects in JA perception or synthesis lead to male sterility (von Malek *et al.*, 2002; Park *et al.*, 2002; Xie *et al.*, 1998), plants with strong *coi1* alleles can only be maintained as a heterozygous population. In contrast, the *aos* phenotype is rescued by methyl jasmonate (MeJA) treatment during flower development. To avoid differences in the history of the seed batches, we generated heterozygous *AOS/aos* plants by back-crossing the homozygous *aos* mutant with wild-type Col-0. Individual plantlets of the segregating *AOS/aos* and *COI1/coi1-t* (Mosblech *et al.*, 2011) populations were infected with *V. longisporum*. After genotyping, RNA was extracted from 30 to 33 roots per segregating wild type and homozygous *coi1* or *aos* mutants (mock and infected) and replicates from three independent experiments were used to construct libraries for Illumina sequencing.

COI1 suppresses gene expression in the absence of jasmonoyl-isoleucine

To obtain a first impression of the global structure of the transcriptome dataset, principal component analysis was performed (Figure 1a). Surprisingly, clusters representing mock-treated versus infected plants of one genotype showed overlapping datasets. This result indicates that the plantlets did not strongly respond to the fungal infection under our conditions. In contrast, the datasets of the four genotypes showed a clear separation, with those representing the transcriptomes of the two segregating wild types ($WT_{coi1\text{-}t}$ and WT_{aos}) being most related, though still distinct. The transcriptome of the *aos* mutant was more related to that of its segregating WT_{aos} than the transcriptome of the *coi1-t* mutant to its segregating $WT_{coi1\text{-}t}$. Moreover, the *coi1-t* transcriptome was clearly different from the *aos* transcriptome.

Since our main aim was to explore the JA-Ile-independent function of COI1, we focused on those genes

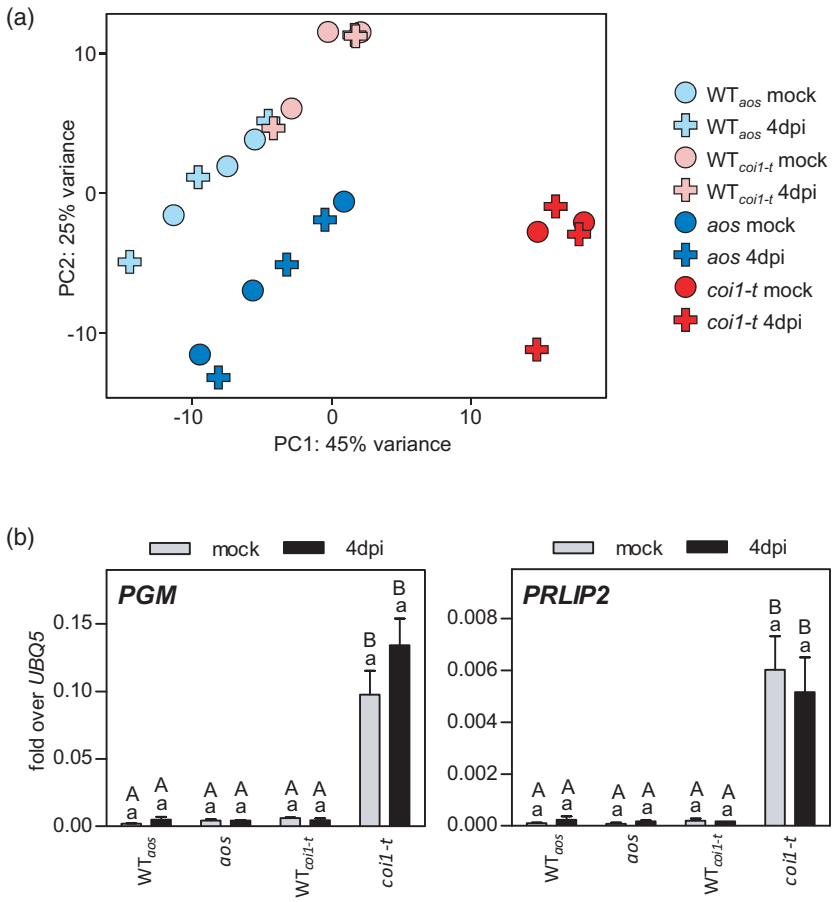

Figure 1. The *coi1-t* transcriptome differs from the transcriptomes of *aos* and the respective segregating wild types.
(a) Principal component analysis of the normalized transcriptome data obtained from RNA-seq analysis. Symbols represent biological replicates resulting from three independent experiments. Note that only two mock samples (*coi1-1*) were processed. Wild-type (WT$_{aos}$ and WT$_{coi1\text{-}t}$) samples originated from the segregating offspring of the heterozygous *aos* and *coi1-t* populations.
(b) *PHOSPHOGLYCERATE MUTASE* (*PGM*) and *PATHOGENESIS-RELATED LIPASE 2* (*PRLIP2*) expression, measured by qRT-PCR. The same RNA samples as in (a) were used. For statistical analysis, two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 4 days post-infection ($P < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($P < 0.05$).

that were differentially (> twofold; $P < 0.05$) expressed in *coi1-t* as compared to both wild types and the *aos* mutant (Table S1 and sub-tables). In the mock-treated samples, only 12 genes were more lowly expressed in *coi1-t* than in the other genotypes, while 222 genes were more highly expressed. Analysis of the infected samples yielded the same pattern, with only nine genes being expressed at lower levels and 199 being de-repressed in *coi1-t*. In infected and mock-treated *coi1-t* plants, 167 genes were more highly expressed, indicating that increased expression of this set of genes is robust (Figure S1). Only two genes were expressed at lower levels in *coi1-t* irrespective of the treatment.

Figure 1b displays the expression patterns of two representative genes from the group of 167 genes that were highly de-repressed in *coi1-t*. Quantitative reverse transcription PCR (qRT-PCR) analysis of the material subjected to RNA sequencing (RNA-seq) analysis indicated that these genes (AT3G60415 [*PHOSPHOGLYCERATE MUTASE* {*PGM*}] and AT5G24200 [*PATHOGENESIS-RELATED LIPASE2* {*PRLIP2* }]) (Jakab *et al.*, 2003) were about 50-fold more highly expressed in *coi1-t* than in the two wild-type lines and the *aos* mutant (Figure 1b). In contrast, primary target genes of the canonical COI1-dependent pathway (*JAZ1*, *JAZ9*, and *JAZ10*) are expressed at lower levels in both *coi1-t* and *aos* (Figure S2). As expected, the expression pattern was independent of whether plants were mock-treated or infected. Furthermore, increased expression of *PGM* and *PRLIP2* was confirmed in *coi1-1* (Xie *et al.*, 1998) and the temperature-sensitive *coi1-16* mutant (Ellis and Turner, 2002) (Figure S3). Higher transcript levels of *PGM* and *PRLIP2* were also observed in uninfected roots of soil-grown plants (Figure S4a). Under these conditions, one of the two genes with lower expression levels in roots

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of axenically grown seedlings was not affected by *coi1* (AT2G05420) (Figure S4b). For the other (AT5G54450), expression was so low that no specific PCR product was detected. Hence, these genes are unlikely to act as repressors of the large number of de-repressed genes in *coi1*. These analyses indicate that COI1 can interfere with the expression of specific genes when acting independently of JA-Ile.

Functional enrichment analysis of the 167 genes with elevated expression levels in *coi1-t* demonstrated that Gene Ontology (GO) terms associated with immune responses were more than fivefold enriched (Figure 2a). In particular, processes connected to the defense hormone salicylic acid (SA) were overrepresented. The expression of the SA biosynthesis gene *ISOCHORISMATE SYNTHASE1* (*ICS1*) (Wildermuth *et al.*, 2001) was 2.8-fold higher in *coi1-t* than in *aos* (Figure 2b). Enhanced expression of *ICS1* was also observed in *coi1-1*, but not in *coi1-16* (Figure S3).

Next, we analyzed whether elevated expression of *ICS1* was the primary reason for the increased transcript levels of SA-related genes. To this end, we crossed *coi1-1* and the SA biosynthesis mutant *sid2-2*. Analysis of the resulting *coi1-1 sid2-2* double mutant showed that enhanced expression of *PGM* and *PRLIP2* in *coi1-1* occurred in the absence of ICS1-derived SA (Figure 3a; Figure S5). This correlates with the tolerance phenotype after infection with *V. longisporum* which was observed in *coi1-1* and *coi1-1 sid2-2* but not in wild type, *aos*, and *sid2-2* (Figure 3b).

We have shown previously that – similar to *coi1-1 sid2-2* – the JA-Ile-deficient *coi1-t aos* double mutant is as tolerant as *coi1-t* (Ralhan *et al.*, 2013). Likewise, *PGM* and *PRLIP2* expression was as high in *coi1-t aos* as in *coi1-t*, demonstrating that JA-Ile does not induce the expression of these genes in the absence of its receptor (Figure S6).

COI1-mediated repression is apparently independent of its interaction with JAZ proteins

JA-Ile-facilitated interaction of COI1 with JAZ repressor proteins leads to their degradation (Chini *et al.*, 2007; Thines *et al.*, 2007). *In vitro*, recombinant COI1 does not interact with the JAZ1 degron in the absence of the ligand

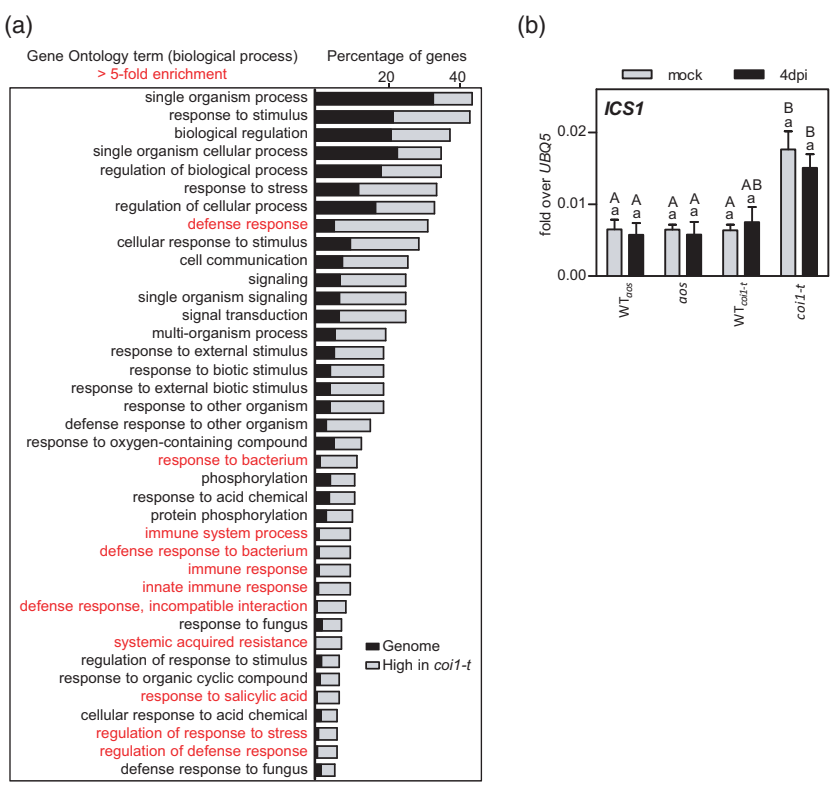

Figure 2. Genes related to salicylic acid-mediated immune responses are de-repressed in *coi1-t* roots.
(a) Gene Ontology (GO) overrepresentation analysis of 167 genes that were more highly expressed (> twofold; $P < 0.05$) in *coi1-t* as compared to *aos* and the respective segregating wild types. Black bars indicate the percentage of genes of each GO term found within the group of all annotated genes of the Arabidopsis genome. Gray bars indicate the percentage of genes of each GO term found within the group of 167 genes de-repressed in *coi1-t*.
(b) *ISOCHORISMATE SYNTHASE 1* (*ICS1*) transcript levels, measured by qRT-PCR. The same RNA samples as for the RNA-seq experiment were used. For statistical analysis, two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 4 days post-infection ($P < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($P < 0.05$). WT_{aos} and $WT_{coi1\text{-}1}$ are the two wild-type lines obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds.

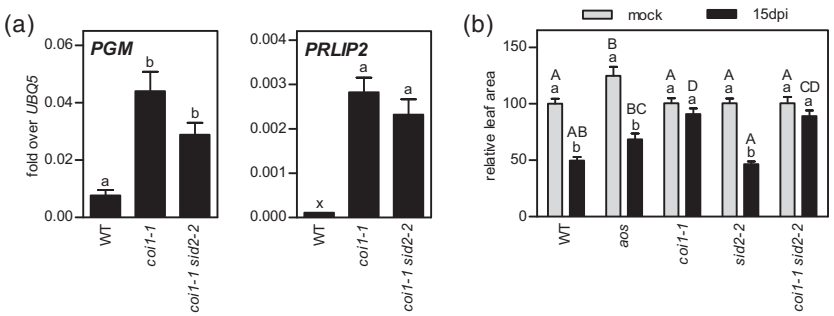

Figure 3. ICS1-derived SA is not responsible for de-repression of *PGM* and *PRLIP2* or the tolerant disease phenotype of *coi1-1*.
(a) *PGM* and *PRLIP2* transcript levels, measured by qRT-PCR. RNA was extracted from roots of sand–soil-grown *coi1-1*, *coi1-1 sid2-2*, and Col-0 plants 10 days after mock treatment and subsequent transfer to soil. Bars show the mean ± SEM of six roots per genotype. For statistical analysis, one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($P < 0.05$), x indicates that for expression of *PRLIP2* in wild type only one value was obtained, while the other five fell below the detection threshold in our analysis. Thus, an unpaired two-tailed Student *t*-test was performed between *coi1-1* and *coi1-1 sid2-2* samples.
(b) Leaf area of mock-treated and *V. longisporum*-infected plants at 15 days post-infection (dpi). Plants were grown on sand–soil mixture and transferred to soil after treatment. Bars show the mean ± SEM of 48 plants from three independent experiments. Values from mock-treated wild-type plants are set to 100. For statistical analysis, two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 15 dpi ($P < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($P < 0.05$).

(Yan *et al.*, 2018). Still, COI1 functions as a repressor of gene expression in roots of the *aos* mutant, suggesting that JAZ proteins are not involved in this process. Nevertheless, to explore potential ligand-independent degradation of specific JAZ proteins *in vivo*, we tested for COI1-mediated activation of the *JAZ1* promoter in transiently transformed protoplasts of the *coi1-1 aos* mutant. This experimental system allows to repress a $JAZ1_p$*:luciferase* reporter construct upon expression of specific JAZs as effector proteins. Upon additional expression of COI1 and incubation of protoplasts in the presence of coronatine, luciferase activity is induced (Li *et al.*, 2019). In the absence of coronatine, none of the repressors were degraded as deduced from the absence of any positive effect of COI1 on luciferase activity (Figure S7). Thus, at least in protoplasts, no ligand-independent degradation of specific JAZ proteins by COI1 was observed.

To obtain further evidence for the JAZ-independent COI1 function, we analyzed transgenic plants constitutively expressing the non-degradable JAZ1Δ3A-GUS fusion protein which mimic the *coi1* phenotype in various aspects (male sterility, JA-insensitive root growth, severely compromised wound-induced expression of JA marker genes in leaves) (Thines *et al.*, 2007). For reasons that have remained unknown, the *coi1* phenotype was only partially mimicked with respect to reduced expression of *JAZ10* in MeJA-treated roots (Figure S8). With this positive control not fully functioning, we did not further draw any conclusion from our result that *PRLIP2* expression was not affected in *35S:JAZ1Δ3A:GUS* plants.

Alternatively, we used the *jaz* decuple (*jazD*) mutant, which is defective in *JAZ1*–*7*, *9*, *10*, and *13*, resulting in constitutive activation of both JA and ET responses (Guo *et al.*, 2018). In this mutant, *PGM* expression was not significantly reduced (Figure S9). Since *PRLIP2* transcript levels are already low in wild-type roots grown in soil, we chose *SYSTEMIC ACQUIRED RESISTANCE DEFICIENT1 (SARD1)* as a second gene and again found no influence of the *jazD* genotype.

To obtain further evidence that might support our preliminary results that JAZ proteins are not required for the repressive action of COI1 on *PGM* and *PRLIP2* expression, we designed an alternative strategy. The idea was to complement *coi1-t* with a mutant COI1 protein that would be hampered in its interaction with JAZ proteins. To this aim, we made use of the known crystal structure of the complex formed between COI1 and the 20-amino acid (aa) JAZ1 degron in the presence of JA-Ile (Sheard *et al.*, 2010). The JAZ degron, which is shared between all JAZ proteins, has a bipartite structure with a six-aa loop region trapping the hormone in its binding pocket and a short helix that serves as a low-affinity anchor for docking the JAZ degron on COI1. Since the data obtained with the *aos* mutant already showed that hormone-mediated stabilization of the interaction between COI1 and the loop region of JAZs is not required for the repressive COI1 function, we decided to mutate amino acids interacting with the docking helix. It is shown that mutation of Tyr302 results in reduced COI1–JAZ interactions in yeast (Sheard *et al.*, 2010). To disturb the interaction more efficiently, we additionally mutated Glu203, which forms a hydrogen bridge to Lys215 in the JAZ1 docking helix ($COI1_{AA}$; Figure S10 shows the wild-type situation). As expected, the coronatine-induced interaction of $COI1_{AA}$ with JAZ1, 2, 3, 9, and 12 fell below the level of detection in a yeast two-hybrid system (Figure S11).

Next, we tested for COI1-mediated activation of the *JAZ1* promoter in transiently transformed *coi1-t*

protoplasts, which are competent to produce JA-Ile (Li *et al.*, 2019). As expected, expression of $COI1_{WT}$ led to the induction of luciferase activity. In contrast, $COI1_{AA}$ was far less efficient. This supports the notion that the interaction between $COI1_{AA}$ and JAZs is severely impaired (Figure S12).

Subsequently, we generated transgenic *coi1-t* plants constitutively expressing *COI1* cDNA with an HA tag preceding the open reading frame. Unexpectedly, the wild-type HA-COI1 protein did not efficiently repress *PGM* and *PRLIP2* expression. The expression of *PRLIP2*, for instance, was still 26-fold higher in complementation line #2 than in the wild type, while being only 2.6-fold lower as compared to the empty vector control. In contrast, basal *JAZ10* expression was 22-fold higher in this line than in plants transformed with the empty vector (Figure 4). This result indicates that HA-COI1 can efficiently activate *JAZ10* expression, but that it can only barely fulfill the repressive function of COI1. This finding already indicates that COI1 functions in a manner that is different from its known mechanism of action when operating as a JA-Ile-independent repressor.

Taking into account that the N-terminal tag or expression from a cDNA sequence could compromise the JA-Ile-independent COI1 function, we generated genomic *COI1* clones with C-terminal tags. At least with regard to male fertility, the functionality of such a construct has been reported before (Jewell and Browse, 2016). $COI1_{WT}$ and $COI1_{AA}$ constructs were transformed into the *coi1-t* mutant and transgenic lines were selected based on similar $COI1_{WT}$ and $COI1_{AA}$ protein levels. First, we tested wound-induced activation of *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*), which is often used as a marker gene representing the response to JA-Ile- and COI1-dependent signaling processes. As expected, *VSP2* expression was not induced in the segregating *coi1-t* lines and a significant induction was observed in the two $COI1_{WT}$ complementation lines (Figure 5a). Plants harboring $COI1_{AA}$ barely responded to the wounding stimulus. $COI1_{AA}$ expression lines #24 and #55 had somewhat lower COI1 protein levels than the two control lines (Figure 5b), which might contribute to the weaker induction of *VSP2* expression. Still, line #44, which has similar or slightly higher COI1 levels as compared to the two control lines, showed lower *VSP2* expression. We therefore conclude that $COI1_{AA}$ complements the canonical COI1 functions less efficiently than $COI1_{WT}$. This is supported by the observation that fertility is only restored in plants expressing $COI1_{WT}$, while *coi1-t*/$COI1_{AA}$ plants resemble sterile *coi1-t* plants and do not produce seed pods (Figure S13).

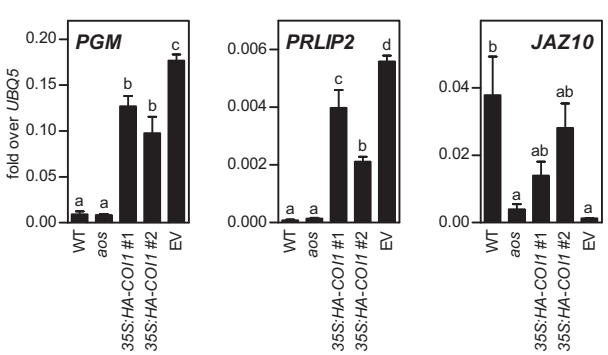

Figure 4. A *35S:HA-COI1* construct complements the canonical COI1 function more efficiently than the JA-Ile-independent function.
PGM, *PRLIP2*, and *JAZ10* transcript levels, measured by qRT-PCR, in wild-type, aos, *coi1-t/35S:HA-CO1*, and *coi1-t/EV* (EV = empty vector) plants. RNA was extracted from untreated roots of seedlings grown on ½ MS plates for 20 days with subsequent (5 days) cultivation on agarose in the absence of any added nutrients. Bars show the mean ± SEM of three to four replicates with 40 (23 for EV) roots per replicate. For statistical analysis, one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($P < 0.05$).

In roots, differences between COI1 protein levels were less pronounced than in shoots (Figure 5b). In both types of complementation lines ($COI1_{WT}$ and $COI1_{AA}$), expression of *PGM* and *PRLIP2* was as low as in the segregating wild-type plants, while expression was high in the segregating *coi1-t* plants (Figure 5c). Altogether, our results show that $COI1_{AA}$ is able to repress the two marker genes in roots almost as efficiently as $COI1_{WT}$, but that it is far less efficient in the activation of canonical COI1 functions as part of the JA signaling cascade.

Having established that the interaction between COI1 and JAZs is most likely not important for repression of *PGM* and *PRLIP2*, we expected that the JAZ-regulated transcription factors MYC2, MYC3, and MYC4 (Fernandez-Calvo *et al.*, 2011) would not be involved in the regulation of *PGM* and *PRLIP2* expression. Indeed, transcript levels of these genes were not altered in the *myc2 myc3 myc4* triple mutant (Figure S14). In contrast, the *myc2 myc3 myc4* mutant phenocopied the *coi1* mutant with respect to *JAZ10* expression. Likewise, EIN3 and EIL1, which are repressed by at least JAZ1 (Zhu *et al.*, 2011), did not influence expression of the marker genes that are de-repressed in *coi1* (Figure S14).

MED25 is required for *PGM* and *PRLIP2* expression

Recently, it has been shown that COI1 is recruited to target promoters through its interaction with subunit 25 of the mediator complex (MED25) (An *et al.*, 2017). In a similar fashion, MED25 might be involved in the JA-Ile-independent repressor function by recruiting COI1 to promoters of genes such as *PGM* and *PRLIP2*. To address this option, we assessed the expression of two marker genes in the *med25* mutant and its outcrossed wild type. Due to very low expression levels in the wild type, we tested *SARD1* rather than *PRLIP2*. The expression of *PGM* and *SARD1* was even lower in *med25*, indicating that MED25 is

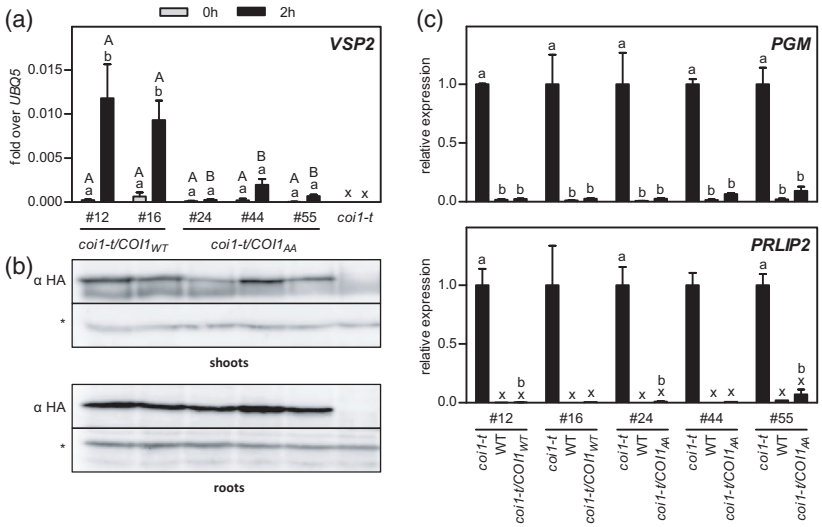

Figure 5. $COI1_{AA}$ is less efficient than $COI1_{WT}$ with respect to wound-induced *VSP2* expression but is similarly effective as a repressor of *PGM* and *PRLIP2*.
(a) *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) transcript levels were measured by qRT-PCR. RNA was extracted from untreated leaves at 0 h and at 2 h after wounding. Complementation lines are homozygous for the *coi1-t* allele and carry at least one copy of transgenic $COI1_{WT}$ or $COI1_{AA}$. *coi1-t* controls are a total of four plants with one plant segregated from each of the lines #12, #16, #24, and #55. Bars show the mean ± SEM of two separately harvested leaves for each time point from three to four plants. For statistical analysis, two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each line between 0 h and 2 h ($P < 0.05$), uppercase letters denote significant differences between lines at the same time point ($P < 0.05$), and x denotes that statistical analysis was not possible due to too many values falling below the detection threshold.
(b) Western blot of protein extracts obtained from shoots and roots from 39 to 40 2-week-old seedlings of the segregating T2 generation of *coi1-t*/$COI1_{WT}$ or *coi1-t*/$COI1_{AA}$ complementation lines (extracts are loaded as indicated in (a)). C-terminally 3×HA-StrepII-tagged COI1 ($COI1_{WT}$ or $COI1_{AA}$) protein levels were detected using an anti-HA antibody. The asterisk (*) depicts an unspecific band shown as loading control.
(c) *PGM* and *PRLIP2* transcript levels, measured by qRT-PCR. RNA was extracted from roots of sand–soil-grown plants 10 days after mock treatment and subsequent transfer to soil. Three genotypes were obtained from the segregating offspring of each transgenic line: *coi1-t* mutants carrying the respective *COI1* construct, wild type, and *coi1-t* controls without the transgene. Values (normalized to reference gene *UBQ5*) from *coi1-t* were set to 1.0. Bars show the mean ± SEM of two to seven outcrossed *coi1-t* roots, two to four outcrossed WT roots, and seven to 13 *coi1-t*/$COI1_{WT}$ or *coi1-t*/$COI1_{AA}$ roots per transgenic line. For statistical analysis, one-way ANOVA was performed between the three genotypes segregated from one transgenic line each, followed by Tukey's multiple comparison test. In case of too many values falling below the detection limit as for *PRLIP2* transcript levels (marked with x), an unpaired two-tailed Student *t*-test was performed between *coi1-t*/$COI1_{WT}$ or *coi1-t*/$COI1_{AA}$ complementation lines and the respective outcrossed *coi1-t* samples. Lowercase letters denote significant differences between samples ($P < 0.05$).

involved in their activation (Figure 6). Whether it also contributes to repression by recruiting COI1 can therefore not be concluded.

DISCUSSION

The plant hormone JA-Ile controls both developmental and anti-stress programs (Wasternack and Hause, 2013). JA-Ile facilitates the interaction of the receptor COI1 with transcriptional repressors (JAZs), which leads to JAZ degradation and activation of gene expression. Consistently, JA-Ile-controlled processes like wound- or pathogen-induced gene expression, fertility, and growth are affected in the receptor mutant *coi1* and the biosynthesis mutant *aos*. In contrast, tolerance of Arabidopsis against the vascular pathogens *V. longisporum* and *F. oxysporum* is observed in *coi1* but not in *aos* plants (Ralhan *et al.*, 2012; Thatcher *et al.*, 2009). In this study we identified target genes of the JA-Ile-independent COI1 function and we used these to demonstrate that COI1 can negatively affect a set of genes through a mechanism that does not seem to require known components of the JA-Ile signaling pathway (Figure 7).

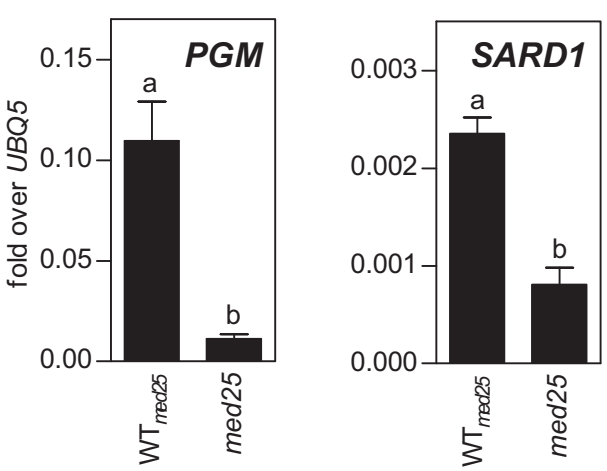

Figure 6. MED25 is required for expression of *PGM* and *SARD1*.
PGM and *SARD1* transcript levels, measured by qRT-PCR. RNA was extracted from sand–soil-grown roots 21 days after mock treatment and subsequent transfer to soil. Bars show the mean ± SEM of seven to eight roots per genotype. WT_{med25} is the wild type obtained from the segregating offspring of heterozygous *med25* seeds. For statistical analysis, an unpaired two-tailed Student *t*-test was performed; lowercase letters denote significant differences between samples ($P < 0.05$).

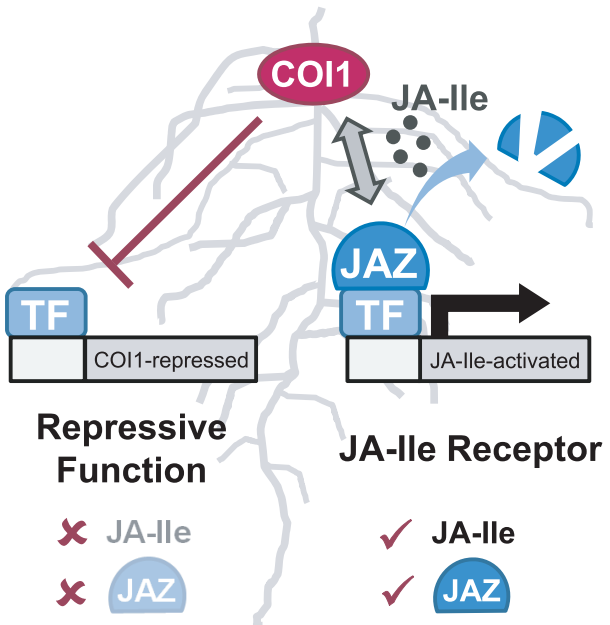

Figure 7. COI1 represses genes through a mechanism that does not involve JA-Ile and most likely no JAZ repressor proteins.'

Constitutive de-repression of SA-related genes in *coi1* roots is different from the JA-mediated repression of the SA pathway observed in leaves

The most conspicuous difference between the transcriptomes of *coi1* versus *aos* or wild-type roots is the large number of SA-related genes that are de-repressed in *coi1* (Table S1). From the two SA biosynthesis pathways known to operate in *A. thaliana* (Huang *et al.*, 2010; Wildermuth *et al.*, 2001), only genes of the isochorismate pathway (*ICS1* and *avrPphB SUSCEPTIBLE3* [*PBS3*]) (Rekhter *et al.*, 2019; Wildermuth *et al.*, 2001) are more highly expressed in *coi1*, leading to the hypothesis that increased SA synthesis through the activation of the isochorismate pathway is instrumental for increased expression of the whole group of SA-related genes. However, analysis of gene expression in the *coi1 sid2* double mutant demonstrated that de-repression of the two marker genes was detected even in the absence of elevated *ICS1* transcript levels.

The negative effect of COI1 on SA-related genes in roots is different from the well-known inhibition of the SA pathway that occurs in leaves. In leaves, repression of the SA pathway is only observed when COI1 is activated by the bacterial JA-Ile mimic coronatine (Kloek *et al.*, 2001). Reduction of pathogen-induced SA levels is brought about by a mechanism that requires coronatine, COI1, and MYC2 (Zheng *et al.*, 2012). Hence, in contrast to the situation in roots, the SA pathway in leaves is not constitutively activated, but it is hyper-activated after induction. Both activation of the SA pathway by *Psm* ES4326 and reduced growth of the pathogen were reverted to wild-type levels in *coi1* expressing the SA-degrading enzyme NahG (Kloek *et al.*, 2001). When we interfered with elevated SA synthesis in *coi1 sid2* plants, increased expression of the COI1-repressed marker genes and increased tolerance towards *V. longisporum* was still observed (Figure 3).

Nevertheless, it is notable that COI1 constitutively represses SA-related genes in roots. Many of these genes (e.g., *ENHANCED DISEASE SUSCEPTIBILITY1* [*EDS1*], *PHYTOALEXIN DEFICIENT4* [*PAD4*], *SARD1*, *ICS1*, *PBS3*, *FLAVIN MONOOXYGENASE1* [*FMO1*], and *AGD2-LIKE DEFENSE RESPONSE PROTEIN 1* [*ALD1*]) play crucial roles in the immune response systemic acquired resistance (Navarova *et al.*, 2012). The transcription factor SARD1 is essential for the biosynthesis of the two signaling molecules *N*-hydroxy-pipecolic acid and SA (Sun *et al.*, 2015), with ICS1/PBS3 and ALD1/FMO1 being important enzymes in the respective biosynthesis pathways (Hartmann and Zeier, 2018; Navarova *et al.*, 2012; Rekhter *et al.*, 2019; Wildermuth *et al.*, 2001). Transcript profiling placed the SARD1-dependent section of genes downstream of the EDS1/PAD4 immune complex (Wagner *et al.*, 2013; Wang *et al.*, 2008). It can be speculated that inappropriate upregulation of this pathway might interfere with the composition of the microbiome in the rhizosphere, making an extra layer of repression necessary. It remains to be explored whether COI1 is a constitutive repressor or whether repression can be lifted on demand.

Since growth of *V. longisporum* or *F. oxysporum* is not inhibited in the root (Ralhan *et al.*, 2013; Thatcher *et al.*, 2009) and since the pathway is not constitutively activated in *coi1* shoots (Kloek *et al.*, 2001), a contribution to tolerance can only be assumed under the premises that extracellular defense compounds travel from the root to the shoot, where they might accumulate to interfere with fungal growth. It remains to be elucidated whether the tolerance phenotype can be reverted to susceptibility by suitable mutations of the above-mentioned regulators in the *coi1* background.

The repressive COI1 function is most likely independent of JAZ repressor proteins

The high expression of 167 genes in *coi1* roots might be explained by the accumulation of (certain) JAZs which would interfere with the action of a transcriptional repressor of this group of genes. As discussed below, the following pieces of evidence suggest that the repressive COI1 function is not due to the accumulation of JAZ proteins and thus acts through a different mechanism. (i) JA-Ile, which is required for mediating the interaction between COI1 and JAZs, is not required for the repression (Figure 1). (ii) None of the JAZ proteins can be inactivated by COI1 in the *aos* background, indicating that ligand-independent degradation of specific JAZs is unlikely

(Figure S7). (iii) The repression is mediated by a $COI1_{AA}$ mutant protein that can only weakly interact with JAZs (Figure 5). (iv) An N-terminal HA-tag interferes more strongly with the repressive than with the canonical function (Figure 4).

Although JA-Ile is required for the interaction between COI1 and JAZs, a JAZ1-GUS fusion protein can be degraded in the eATP-treated *aos* mutant. eATP treatment consequently activates known genes of the JA pathway (Tripathi *et al.*, 2018). We consider this scenario to be unlikely in untreated roots since the affected target genes are different from those of the classical response. Furthermore, upon using a functional assay to assess COI1 activity in protoplasts, we did not get any evidence for ligand-independent degradation of specific JAZs (Figure S7).

$COI1_{AA}$ has a weaker affinity to JAZs due to mutations in amino acids that stabilize the interaction between the docking helix of the JAZ degron and COI1 (Figures S10 and S11). Complementation of the *coi1* mutant with this protein resulted in plants showing reduced *VSP2* expression after wounding (Figure 5a). According to the accepted model of JA signaling through COI1, lower *VSP2* expression is due to inefficient degradation of JAZs by SCF^{CO1AA}. Since JA-Ile levels are elevated upon wounding, residual $COI1_{AA}$/JAZ interactions might occur. It is likely that at low JA-Ile levels in non-wounded roots, complex formation between $COI1_{AA}$ and JAZs is more affected. Hence, JAZ proteins might accumulate to similar or to only slightly lower levels in roots of *coi1-t*/$COI1_{AA}$ lines as compared to *coi1-t*. Still, transcription of *PGM* and *PRLIP2* was strongly repressed despite the fact that JAZ proteins are stabilized. In combination with the data obtained with the *aos* mutant, we take this result as further evidence that JAZs do not take part in the regulation of COI1-repressed genes.

Plants expressing the non-degradable JAZ protein JAZ1Δ3A-GUS turned out to be not valuable for our research since even *JAZ10* expression, which should be as low as in *coi1*, was not strongly affected (Figure S8). In *jazD*, which lacks 10 out of the 13 *JAZs* (Guo *et al.*, 2018), strong repression would be expected if repressor activity was enhanced in the absence of JAZs (Figure S9). However, no significant repression was detected, leaving the only option that JAZ8, JAZ11, or JAZ12 might be JA-Ile-independent substrates of COI1. However, no evidence for this was found in transient assays (Figure S7).

Is COI1 a moonlighting protein?

Moonlighting proteins perform multiple functions, which differ mechanistically (Huberts and van der Klei, 2010). Well-known examples are glycolytic enzymes. Arabidopsis glycerin aldehyde 3-phosphate dehydrogenase (GAPDH), for example, promotes transcriptional activation by interacting with the transcription factor nuclear factor Y subunit C10 (NF-YC10) and enhancing binding to its target promoters (Kim *et al.*, 2020). A decisive criterion for a moonlighting protein is the independency of both functions, meaning that inactivation of one of the functions should not affect the second function and *vice versa*. Adding an N-terminal tag to COI1 might have disturbed the potential moonlighting function (Figure 4) but not the JA-Ile receptor function, while mutating amino acids Glu203 and Tyr302 interfered with the receptor function but not with the potential moonlighting function (Figure 5). When acting as a JA-Ile receptor, COI1 operates as a ligand-dependent F-box protein in an E3 ligase complex. The mechanism of action of its potential moonlighting activity remains to be elucidated. It can be envisioned that COI1 is recruited to the chromatin where it might act as a scaffold for the assembly of a repressive complex. Alternatively, COI1 might act as a JA-Ile-independent F-box protein that mediates the degradation of, e.g., a transcriptional activator. In this case, the label moonlighting would be debatable. Further studies are required to solve this question.

EXPERIMENTAL PROCEDURES

Plant material

All plants used in this study are in the *A. thaliana* Col-0 background. Mutant *Arabidopsis* lines were obtained from the following sources: *aos* (SALK_017756) and *pft1-3* (*med25*, SALK_059316) (Kidd *et al.*, 2009) from the Nottingham Arabidopsis Stock Centre (NASC); *coi1-t* (SALK 035548) (Mosblech *et al.*, 2011) from I. Heilmann (Martin-Luther-University, Halle, Germany); *coi1-1* (Xie *et al.*, 1998) and *coi1-16* (Ellis and Turner, 2002) from J. Turner (University of East Anglia, Norwich, UK); *sid2-2* (Wildermuth *et al.*, 2001) from F. M. Ausubel (Harvard University, Boston, USA); *jazD* (Guo *et al.*, 2018) from G. Howe (Michigan State University, Michigan, USA); *myc2 myc3 myc4* (Fernandez-Calvo *et al.*, 2011) from R. Solano (National Centre for Biotechnology, Madrid, Spain); and *eil1-1 ein3-1* (Alonso *et al.*, 2003) from R. Vierstra (University of Wisconsin, Madison, USA). The *coi1-t aos* (Koster *et al.*, 2012) and *coi1-1 sid2-2* (this work) double mutants were generated by crossing the respective genotypes. Primers used for genotyping of the different alleles are given in Table S2. The identity of the *eil1-1 ein3-1* mutant was verified by the lack of the triple response (Alonso *et al.*, 2003) and the identities of *coi1-16* and *myc2 myc3 myc4* by compromised *JAZ10* expression.

Plant growth conditions and treatments

For RNA-seq analysis, each experiment started with 320 seeds obtained from heterozygous *aos* and *coi1-t* plants, respectively. Surface-sterilized seeds were sown onto vertical agar plates (10 × 10 cm, 20 seeds per plate) containing half-strength Murashige–Skoog (MS) medium supplemented with 1% sucrose and kept at 4°C in darkness for 48–72 h. Subsequently, plates were transferred to short day conditions (8-h day/16-h night cycle) at 22°C, 60% relative humidity, and a photon flux density of 80–100 μmol m^{-2} s^{-1}. The lower parts of the plates were covered with aluminum foil to keep roots in semi-darkness. After 3 weeks, plantlets were transferred for 24 h onto vertical plates containing agarose (1% in water) in order to reduce saprophytic growth of the fungus. Roots were sprayed with 10^5 spores/mL tap water or only with water (mock). After 4 more days under the

conditions mentioned above, roots and shoots were individually harvested with shoots yielding DNA for determination of the genotype and roots yielding RNA for transcriptome analysis. In detail, 33–36 roots of the two wild types, the homozygous *aos*, and the homozygous *coi1-t* plants, respectively, were combined for one RNA preparation. This experimental setup was repeated twice to obtain three independent biological replicates per genotype per treatment. RNAs from in total 24 samples (four genotypes, two treatments, three replicates) were used to construct libraries for Illumina sequencing. The same setup was used for qRT-PCR analysis (Figures 1, 2, and 4; Figures S3, S5, S6, S8, and S14). Here, between 20 and 50 roots were combined for one replicate.

For analysis of gene expression (Figures 3, 5, and 6; Figure S9) and for fungal infection (Figure 3), surface sterilized seeds were sown onto horizontal MS agar plates supplemented with 2% sucrose and grown in the same short day conditions described above. After 14 days, plantlets were transferred onto a 1:1 mixture of sand (white, 1–2 mm grain size, Rosnerski, Königslutter, Germany) and steamed soil (Fruhstorfer Erde, Spezial Substrat, Typ T, Str. 1 fein, HAWITA, Vechta, Germany) on a thin layer of Seramis (Westland Deutschland, Mogendorf, Germany) and grown for another 14 days under short day conditions at 120–140 μmol photons m^{-2} s^{-1}. The sand–soil mixture was initially watered with 0.1% Wuxal Super (Manna, Ammerbuch-Pfäffingen, Germany) in dH_2O. For the first week, plants were kept under a transparent hood. If genotyping was required, a single leaf was clipped from each plant during the first week of growth on the sand–soil mixture. Plants were carefully uprooted from the sand–soil mixture and washed in tap water. Roots were then dipped in spore suspension (1×10^6 spores/mL tap water) or tap water (mock) for 45 min, after which plants were planted into individual pots containing steamed soil (Fruhstorfer Erde, Spezial Substrat, Typ T, Str. 1 fein, HAWITA, Vechta, Germany) soaked with 0.2% Wuxal Super, where plants were kept for a final 10 to 21 days in short day conditions at 120–140 μmol photons m^{-2} s^{-1} until harvest. During the first 2 days after transfer of plants to soil, they were kept under transparent hoods. A rootstock of one single plant was harvested for one biological replicate. For gene expression analysis in roots of untreated soil-grown plants (Figure S4), seeds were directly placed on soil, subjected for 2 days to cold treatment, and cultivated in a growth cabinet at 22°C in short day conditions at 120 μmol photons m^{-2} s^{-1} and 60% relative humidity. After 5 weeks, plants were uprooted and roots were washed in tap water, after which they were frozen in liquid nitrogen. For wounding experiments, two leaves of 4.5-week-old plants grown under long day conditions (16-h day/8-h night cycle, 22°C/18°C, 100 μmol photons m^{-2} s^{-1}) were cut at the petiole and immediately frozen in liquid nitrogen. Two further leaves of the same plant were wounded with forceps without damaging the mid rib. Leaves were collected separately after 2 h. Subsequently, plants were further grown to assess their capacity to develop seed pods (Figure S8).

Other methods

For RNA-seq analysis (Methods S1), qRT-PCR (Methods S2), Western blot analysis (Methods S2), construction of recombinant plasmids (Methods S3), generation of transgenic plants (Methods S3), fungal culture (Methods S4), leaf area measurement (Methods S5), protoplast preparation/transfection (Methods S6), and yeast two-hybrid analysis (Methods S7), see detailed protocols in the Supporting Information. Appendix 1 displays the sequence of pB-GW-HAS7 used to express COI1 and COI_{AA} in transgenic plants.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). In order to not distort the statistical analysis for *VSP2* expression in leaves (two-way analysis of variance [ANOVA]) by disregarding values that fell below the detection threshold, we corrected the values to ones orientated around the lowest value measured for that line and time point (two values for #16 [0 h]; three values for #24 [0 h]; three values for #24 [2 h]; four values for #44 [0 h]; and five values for #55 [0 h]).

Accession numbers

Sequence data from this article can be found in The Arabidopsis Information Resource (http://www.arabidopsis.org/) under the following accession numbers: *AOS* (AT5G42650), *COI1* (AT2G39940), *ICS1* (AT1G74710), *JAZ1* (AT1G19180), *JAZ9* (AT1G70700), *JAZ10* (AT5G13220), *MED25* (AT1G25540), *PGM* (AT3G60415), *PRLIP2* (AT5G24200), *SARD1* (AT1G73805), *UBQ5* (AT3G62250), *VSP2* (AT5G24770).

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CONFLICT OF INTEREST

None of the authors has declared a conflict of interest.

AUTHOR CONTRIBUTIONS

LU and JS performed the experiments; CT designed and supervised the research and analyzed the RNA-seq data; CG designed the experiments and wrote the manuscript with input from all authors.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Venn diagrams showing the numbers and overlap of genes differentially expressed in mock- and *V. longisporum*-infected *coi1-t* roots (> twofold; $P < 0.05$) as compared to *aos* and the two segregating wild-type lines.

Figure S2. Known genes regulated by the JA pathway are lowly expressed in *aos* and *coi1-t*.

Figure S3. *PGM* and *PRLIP2* are de-repressed in *coi1-1* and *coi1-16* roots.

Figure S4. *PGM* and *PRLIP2* are de-repressed in *coi1-t* in untreated roots of soil-grown plants.

Figure S5. *PGM* and *PRLIP2* are de-repressed in *coi1* in the absence of ICS1-derived SA.

Figure S6. *PGM* and *PRLIP2* are de-repressed in *coi1* in the absence of AOS-derived JA-Ile.

Figure S7. No COI1-dependent degradation of any JAZs is observed in the absence of JA-Ile.

Figure S8. *JAZ1Δ3A:GUS* plants only partially mimic the *coi1-16* phenotype with respect to *JAZ10* expression in roots.

Figure S9. *PGM* and *SARD1* are not affected in *jaz* decuple plants.

Figure S10. Top view of the JAZ1 docking helix bound to COI1.

Figure S11. $COI1_{AA}$ is impaired in mediating induction of the *JAZ1* promoter in protoplasts.

Figure S12. *coi1-t* plants expressing $COI1_{AA}$ remain sterile.

Figure S13. COI1-mediated repression of *PGM* and *PRLIP2* does not involve known JAZ-interacting transcription factors.

Figure S14. COI1-mediated repression of *PGM* and *PRLIP2* does not involve known JAZ-interacting transcription factors.

Table S1. RNA-seq analysis.

Table S2. Primers for genotyping.

Table S3. Primers for qRT-PCR analysis.

Table S4. Primers for cloning.

Methods S1. RNA-seq analysis.

Methods S2. Quantitative reverse transcription PCR (qRT-PCR) and Western blot analysis.

Methods S3. Construction of recombinant plasmids and generation of transgenic plants.

Methods S4. Fungal culture.

Methods S5. Leaf area measurement.

Methods S6. Assessment of COI1 activity in transiently transformed protoplasts.

Methods S7. Yeast two-hybrid analysis.

Appendix 1. Sequence of pB-GW-HAS7.

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Figure S1

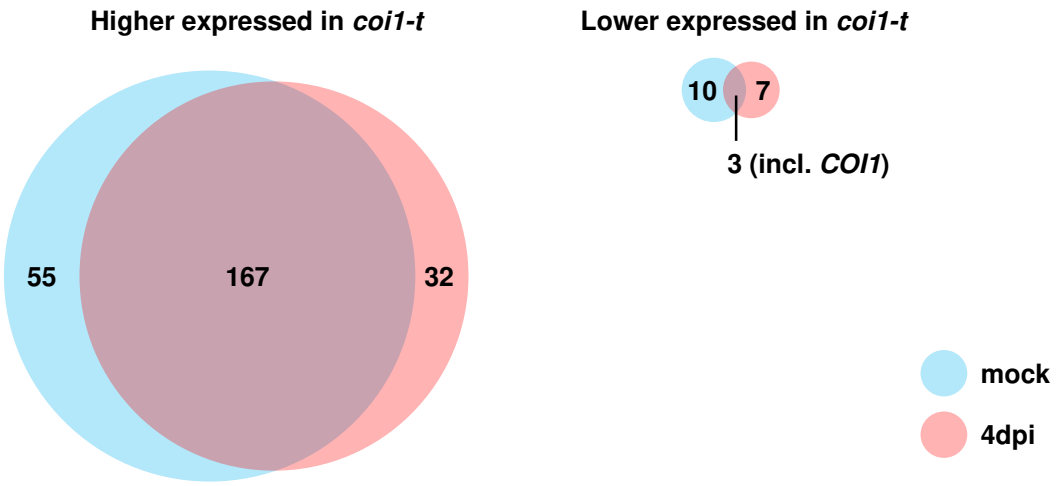

Figure S1. Venn diagrams showing the numbers and overlap of genes differentially expressed in mock- and *V. longisporum*-infected *coi1-t* roots (> 2-fold; p < 0.05) as compared to *aos* and the two segregating wild-type lines.

Expression data were obtained by RNAseq analysis of RNA extracted from roots of four genotypes (*aos*, *coi1-t*, and the two wild-types lines obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds) after mock treatment or infection with *V. longisporum*. Circles are drawn to scale with respect to the number of genes.

Figure S2

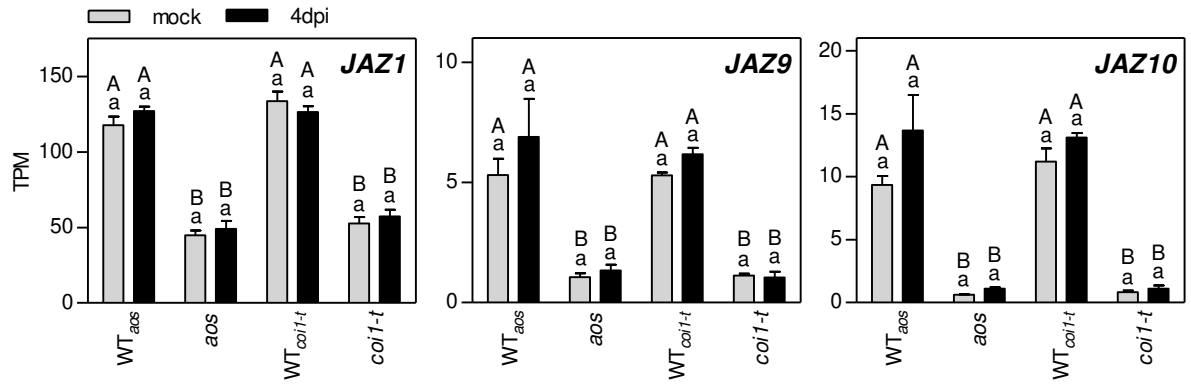

Figure S2. Known genes regulated by the JA pathway are less expressed in *aos* and *coi1-t.*

Relative expression of *JAZ1*, *JAZ9* and *JAZ10* transcript levels as quantified by RNAseq analysis. Bars represent the average of Transcripts Per Million (TPM) ± SEM of three biological replicates of each genotype, with each replicate representing 33 to 36 roots from one independent experiment. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 4 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). WT_{aos} and $WT_{coi1\text{-}t}$ are the two wild-types lines obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds.

Figure S3

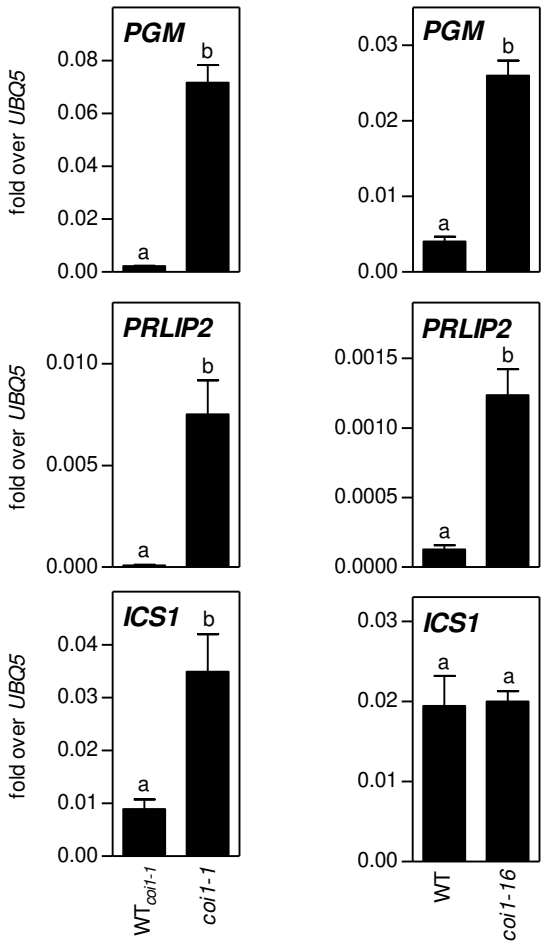

Figure S3. *PGM* and *PRLIP2* are de-repressed in *coi1-1* and *coi1-16* roots.

PGM, *PRLIP2* and *ICS1* transcript levels, measured by qRT-PCR. RNA was extracted from mock-treated roots of seedlings grown on ½ MS plates with subsequent cultivation on agarose in the absence of any added nutrients. Bars are means ± SEM of three to four replicates with 20-23 roots per replicate. For statistical analysis, an unpaired Student's t-test (two-tailed) was performed between *coi1* and the respective WT samples; lowercase letters denote significant differences between samples ($p < 0.05$). WT_{coi1-1} is the wild-type obtained from the segregating offspring of heterozygous *coi1-1* seeds.

Figure S4

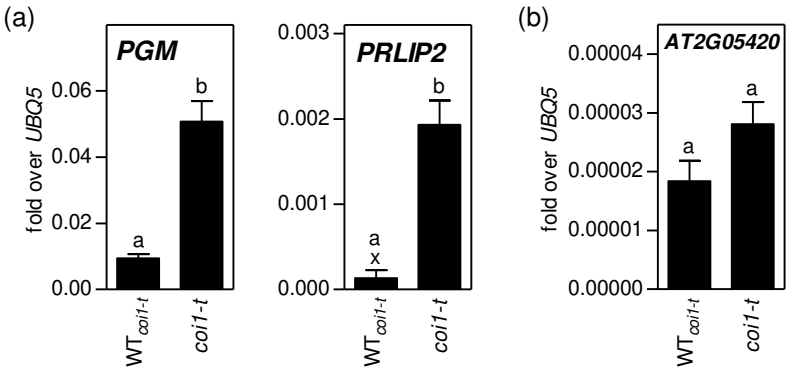

Figure S4. *PGM* and *PRLIP2* are de-repressed in *coi1-t* in untreated roots of soil-grown plants.

(a) *PGM* and *PRLIP2* transcript levels, measured by qRT-PCR. RNA was extracted from untreated roots of soil-grown 5-week old plants. Bars are means ± SEM of twelve roots per genotype. x indicates that for *PRLIP2* expression in WT only three values are shown as the other nine fell below the detection threshold in our analysis. For statistical analysis, an unpaired Student's t-test (two-tailed) was performed between *coi1-t* and WT samples; lowercase letters denote significant differences between samples ($p < 0.05$). WT_{coi1-t} is the wild-type obtained from the segregating offspring of heterozygous *coi1-t* seeds. **(b)** *AT2G05420* transcript levels, measured by qRT-PCR. RNA was extracted from untreated roots of soil-grown 5-week old plants. Bars are means ± SEM of ten to twelve roots per genotype. For statistical analysis, an unpaired Student's t-test (two-tailed) was performed between *coi1-t* and WT samples; lowercase letters denote significant differences between samples ($p < 0.05$). WT_{coi1-t} is the wild-type obtained from the segregating offspring of heterozygous *coi1-t* seeds.

Figure S5

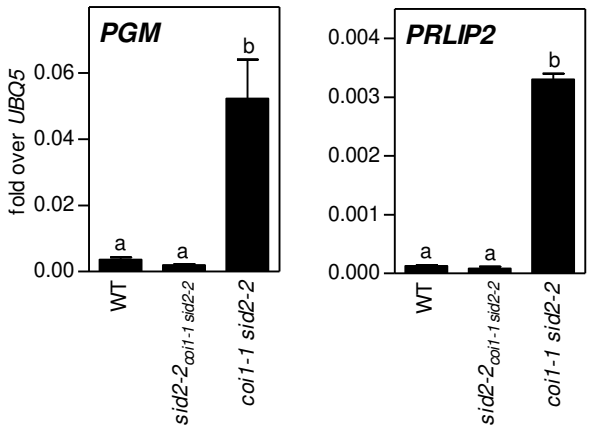

Figure S5. *PGM* and *PRLIP2* are de-repressed in *coi1* in the absence of ICS1-derived SA.

PGM and *PRLIP2* transcript levels, measured by qRT-PCR. RNA was extracted from mock-treated roots of seedlings grown on ½ MS plates with subsequent cultivation on agarose in the absence of any added nutrients. Bars are means ± SEM of three to five replicates with 20-23 roots per replicate. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$). *sid2-2*$_{coi1\text{-}1\ sid2\text{-}2}$ are *sid2-2* plants obtained from the segregating offspring of *coi1-1 sid2-2* plants, which are heterozygous for the *coi1-1* allele and homozygous for the *sid2-2* allele.

Figure S6

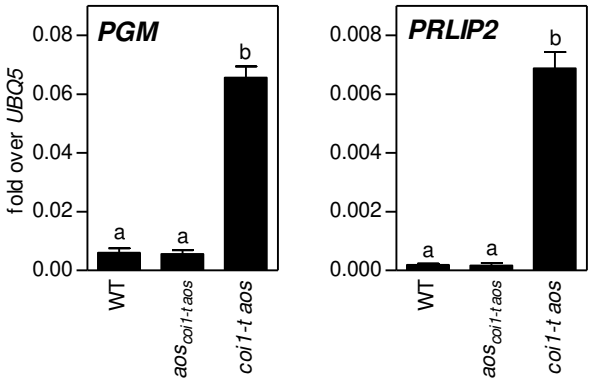

Figure S6. *PGM* and *PRLIP2* are de-repressed in *coi1* in the absence of AOS-derived JA-Ile.

PGM and *PRLIP2* transcript levels, measured by qRT-PCR. RNA was extracted from mock-treated roots of seedlings grown on ½ MS plates with subsequent cultivation on agarose in the absence of any added nutrients. Bars are means ± SEM of three to four replicates with at least ten roots per replicate. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$). $aos_{coi1\text{-}t\ aos}$ are *aos* plants obtained from the segregating offspring of *coi1-t aos* plants, which are heterozygous for the *coi1-t* allele and homozygous for *aos* allele.

Figure S7

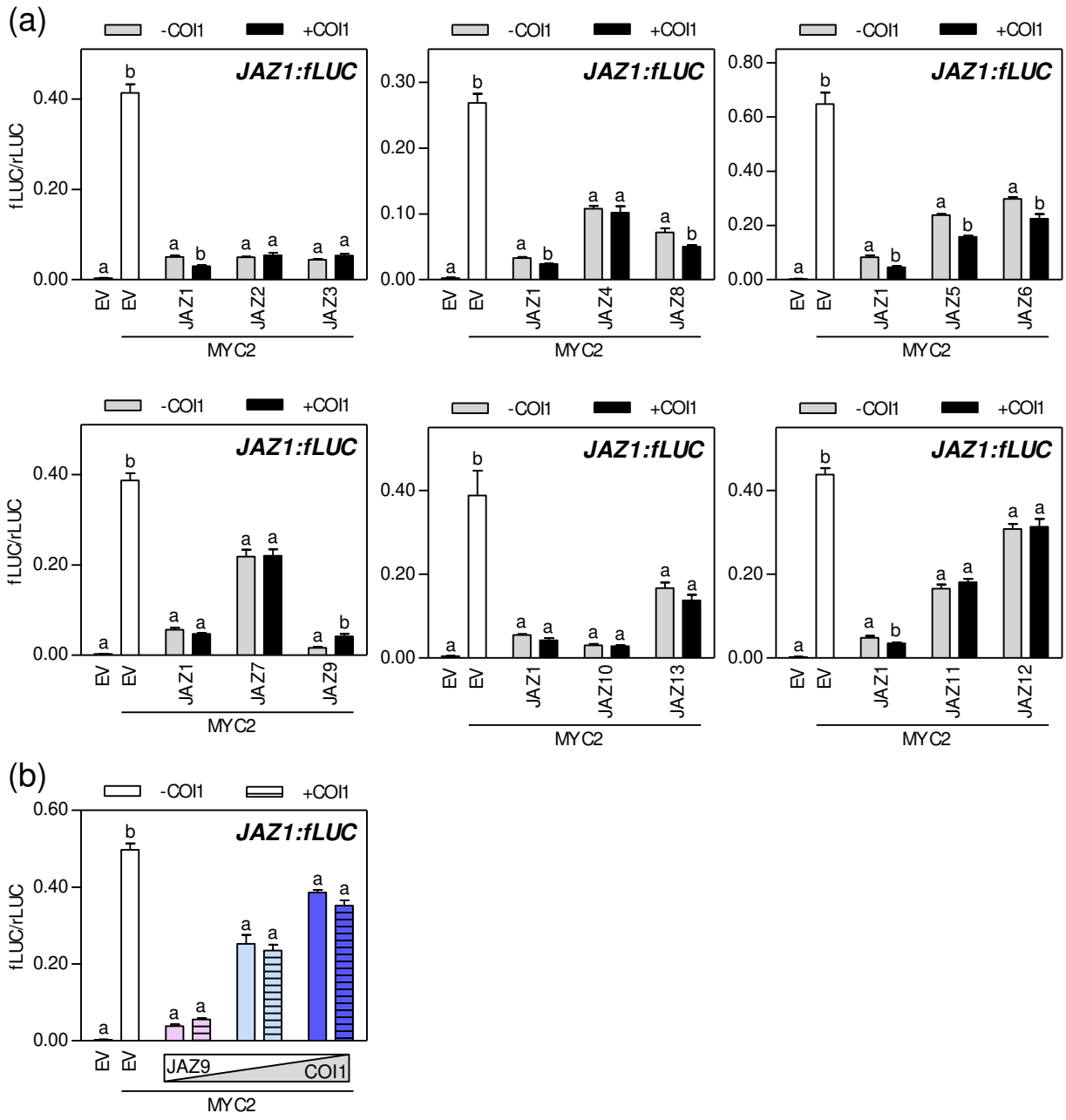

Figure S7. No COI1-dependent degradation of any JAZ is observed in the absence of Coronatine/JA-Ile.

Full Legend on next page.

F igure S7. No COI1-dependent degradation of any JAZs is observed in the absence of JA-Ile.

(**a**/**b**) Luciferase activities yielded by MYC2-activated *JAZ1:fLuc* in the presence or absence of co-transfected JAZs and COI1. Since JAZ9-mediated repression seemed to be relieved by COI1, the assay was repeated with different JAZ9/COI1 ratios in b.

Arabidopsis leaf protoplasts prepared from *coi1-t aos* mutant plants were cotransfected with the reporter plasmid (3.5 µg) containing the firefly *LUCIFERASE* coding region (*fLUC*) driven by the *JAZ1* promoter. Effector plasmids (3.5 µg each per sample) contained the coding regions of *MYC2*, *JAZ1-13*, and *COI1* driven by the *UBQ10* promoter. In b, different JAZ9/COI1 ratios were used (pink: 3.5 µg JAZ9 + 3.5 µg EV or COI1; light blue: 1 µg JAZ9 + 6.1 µg EV or COI1; blue: 0.5 µg JAZ9 + 6.6 µg EV or COI1). Each sample contained 0.7 µg of the plasmid pUBQ10-HA-rLUC encoding the *Renilla LUCIFERASE* (*rLUC*) gene driven by the *UBQ10* promoter The empty vector plasmid (EV) was added so that the amounts of transfected DNA was always 14.7 µg. Firefly luciferase (fLUC) activities were normalized to *Renilla* luciferase (rLUC) activities. Values represent means (±SE) of four independently transformed batches of protoplasts. For statistical analysis, an unpaired Student's t-test (two-tailed) was performed between -COI1 and +COI1 values for each *JAZ* construct and between EV and EV + MYC2 controls; letters denote significant differences between samples ($p < 0.05$).

Figure S8

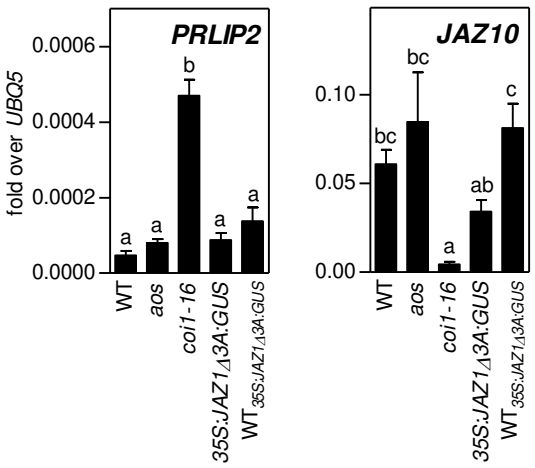

Figure S8. *JAZ1Δ3A:GUS* plants only partially mimic the *coi1-16* phenotype with respect to *JAZ10* expression in roots.

PRLIP2 and JAZ10 transcript levels, measured by qRT-PCR. RNA was extracted from roots of seedlings grown vertically on ½ MS plates for three weeks in short day conditions with subsequent cultivation on agarose for 5 days in the absence of any added nutrients. Seedlings were then sprayed with with 10 µM MeJA (in H_20 with 0.0018% EtOH) and incubated for two hours. Bars are means ± SEM of two to four replicates with 20-44 roots per replicate. $WT_{35S:JAZ1\Delta3A:GUS}$ plants were obtained from the segregating population derived from the cross between the male sterile *35S:JAZ1Δ3A:GUS* with pollen from wild-type plants. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$).

Figure S9

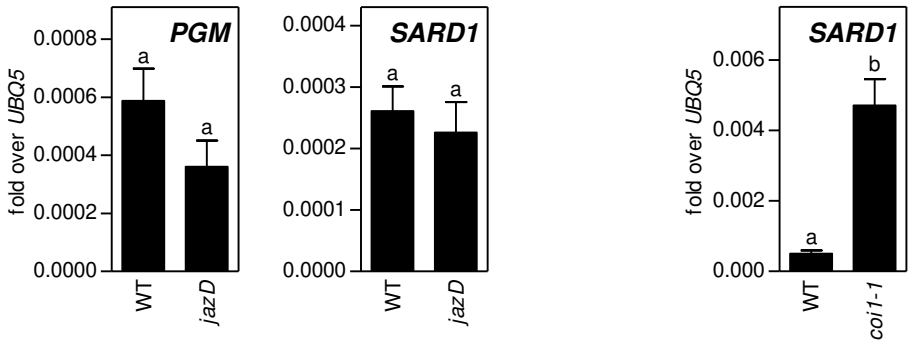

Figure S9. *PGM* and *SARD1* are not affected in *jaz* decuple plants.

PGM and *SARD1* transcript levels, measured by qRT-PCR. RNA was extracted from roots of sand-soil grown plants 10 days after mock treatment and subsequent transfer to soil. Bars are means ± SEM of seven to eight replicates. The right panel demonstrates that *SARD1* is de-repressed in *coi1-1* under these experimental conditions. For statistical analysis, an unpaired Student's t-test (two-tailed) was performed between WT and mutant samples; lowercase letters denote significant differences between samples ($p < 0.05$).

Figure S10

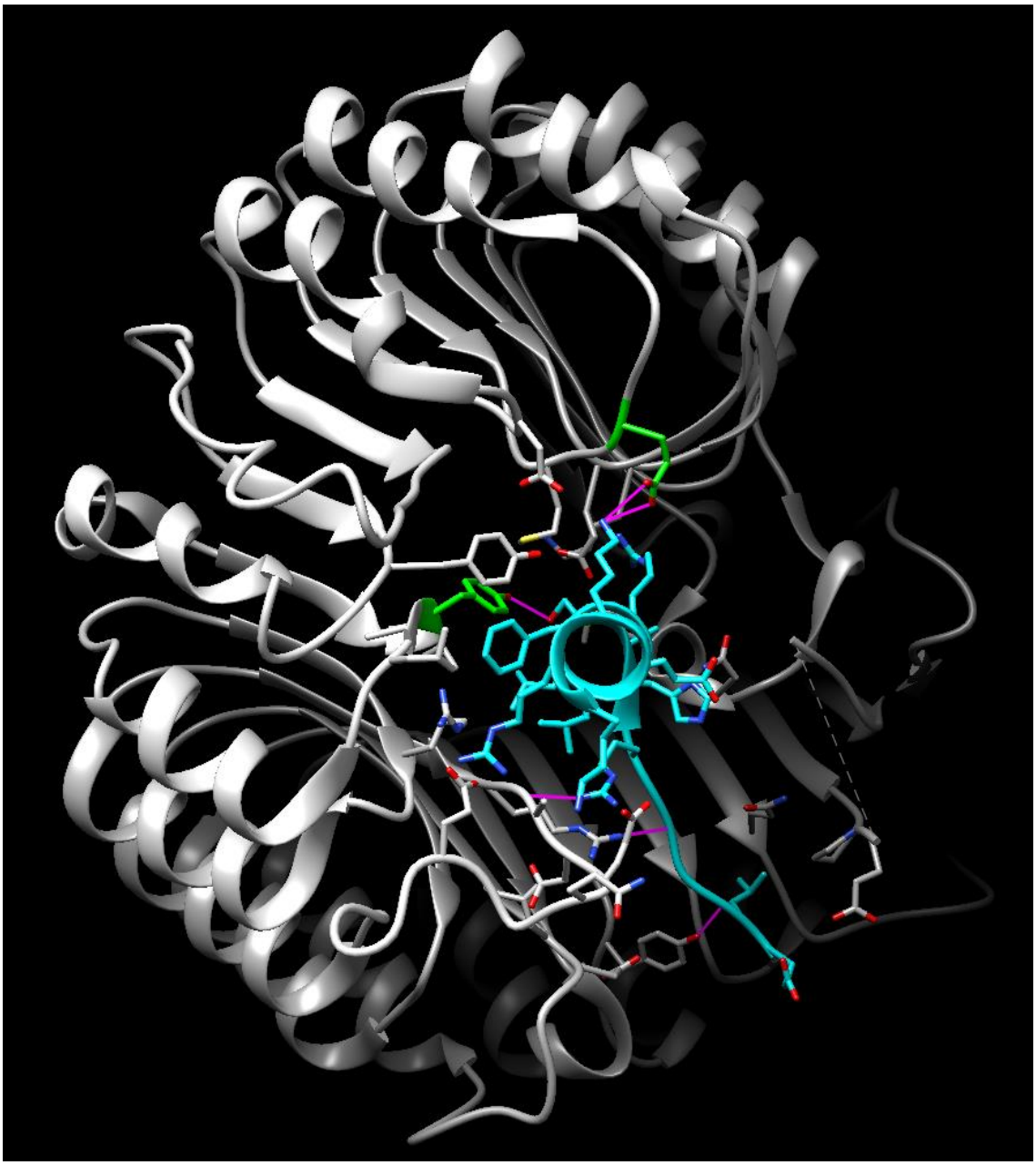

Figure S10. Top view of the JAZ1 docking helix bound to COI1.

Relevant amino acid residues of JAZ1 (cyan blue) and COI1 (grey) are shown in stick representation. Side chains of Glu203 and Tyr302 in COI1 are shown in green. Hydrogen bonds between the docking helix and COI1 are shown in magenta.

Figure S11

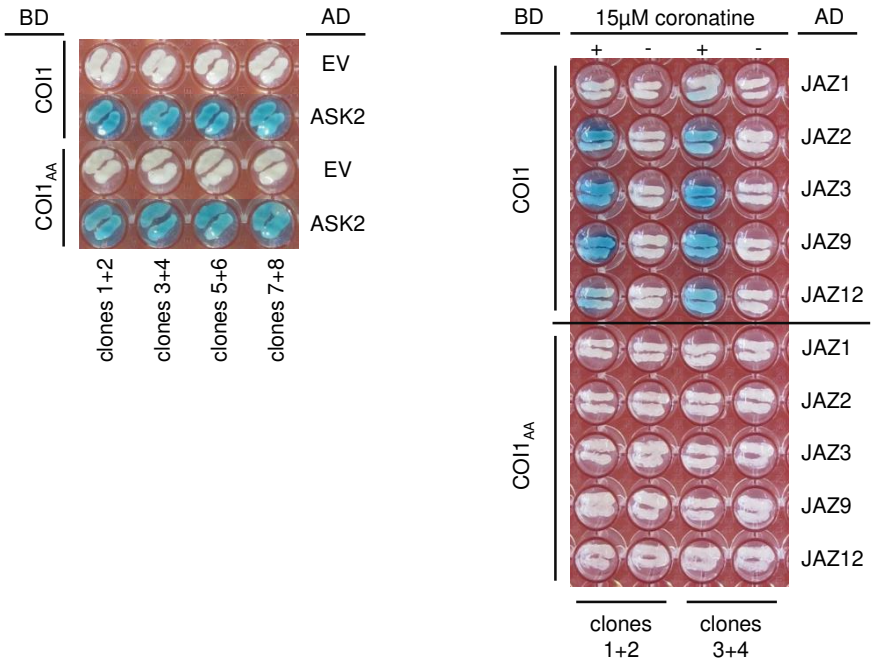

Figure S11. Coronatine-induced interaction of mutant $COI1_{AA}$ with JAZs is impaired.

Yeast strains co-expressing hybrid proteins composed of the LexA DNA binding domain (BD) fused to $COI1_{WT}$ and $CO1_{AA}$ proteins, and the B42 activation domain (AD) fused to different JAZs or ASK2, or without fusion protein as empty vector control (EV), were streaked on media supplemented with X-Gal. Coronatine (15 µM) was added to the media as indicated. As controls (-) the same volume of the solvent (H_2O) was added. Blue color indicates protein interaction.

Figure S12

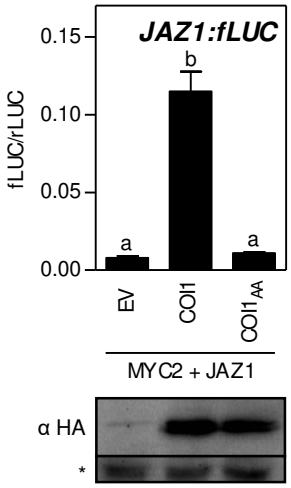

Figure S12. COI1$_{AA}$ is Impaired in mediating induction of the *JAZ1* promoter in protoplasts.

Upper panel: Mesophyll protoplasts from the *Arabidopsis thaliana coi1-t* mutant were co-transfected with a reporter construct expressing firefly *LUCIFERASE* under control of the COI1-dependent *Arabidopsis thaliana JAZ1* promoter (*JAZ1:fLUC*) (5 µg) and plasmids enabling constitutive expression of MYC2 (1.5 µg), JAZ1 (5 µg) and HA-tagged COI1 or COI1$_{AA}$ or empty vector (EV) (5 µg). Firefly luciferase (fLUC) activities were normalized to *Renilla* luciferase (*r*LUC) activities. Values represent means ± SEM of four independently transformed batches of protoplasts. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$). Lower panel: Expression of HA-COI1 proteins was assessed by Western blot analysis. * depicts an unspecific band shown as loading control.

Figure S13

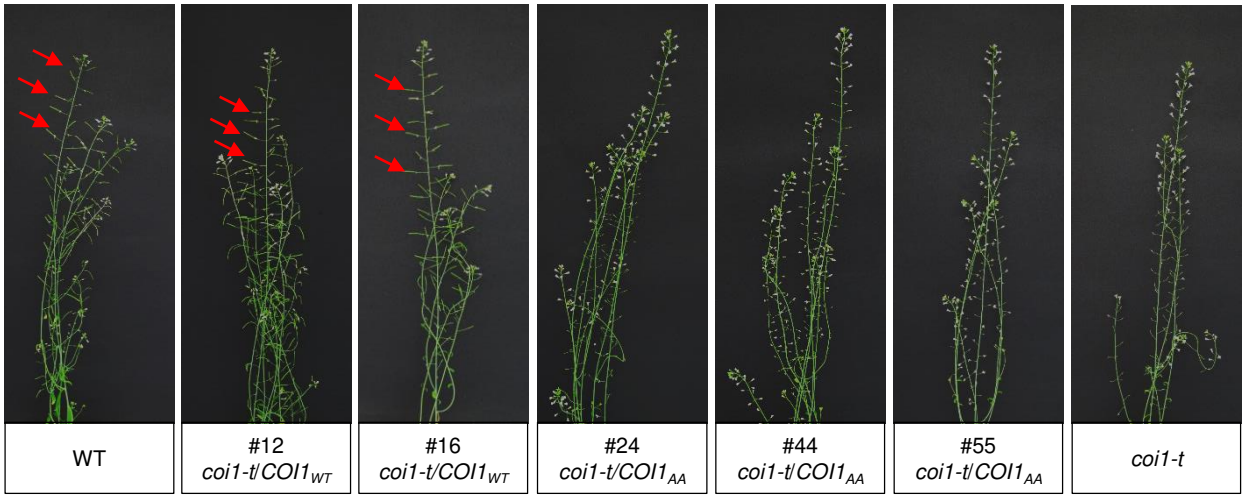

Figure S13. *coi1-t* plants expressing $COI1_{AA}$ remain sterile.

Assessment of seed pod production in eight-week-old wild-type plants, two $COI1_{WT}$ complementation lines in the *coi1-t* background, three $COI1_{AA}$ complementation lines in the *coi1-t* background and *coi1-t* plants. Plants are from the T2 generation and are homozygous for the *coi1-t* allele and carry at least one copy of transgenic $COI1_{WT}$ or $COI1_{AA}$. Three to four plants from each line were monitored for seed pod production. Red arrows point at seed pods.

Figure S14

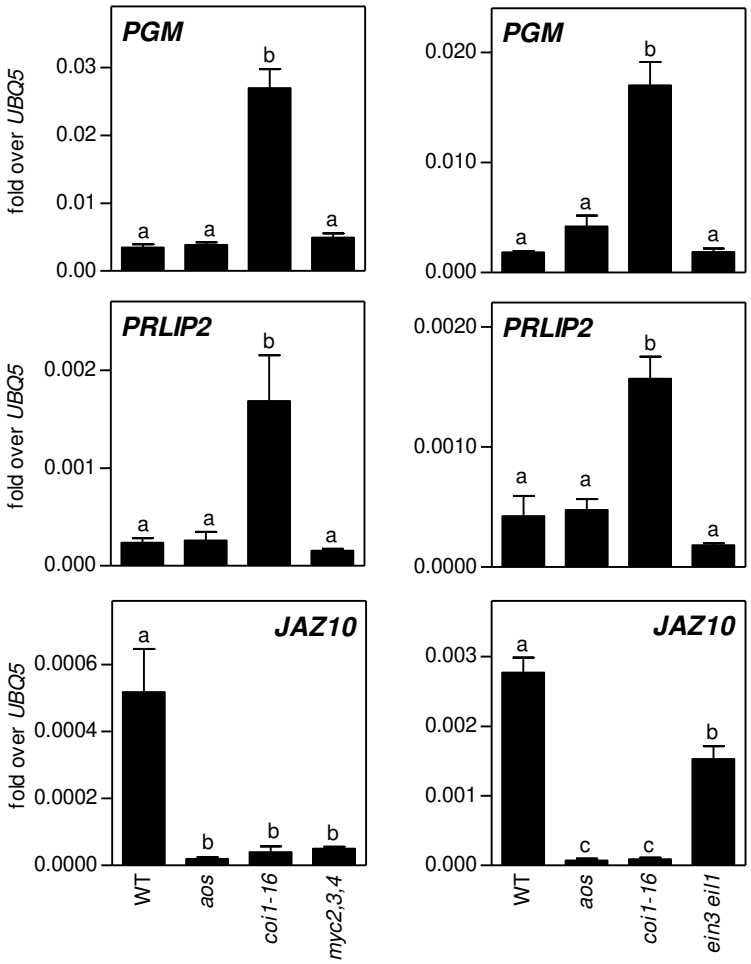

Figure S14. COI1-mediated repression of *PGM* and *PRLIP2* does not involve known JAZ-interacting transcription factors.

PGM, *PRLIP2*, and *JAZ10* transcript levels, measured by qRT-PCR. RNA was extracted from mock-treated roots of wild-type, *aos*, *coi1-16*, *myc 2,3,4* and *ein3 eil1* seedlings grown on ½ MS plates with subsequent cultivation on agarose in the absence of any added nutrients. Bars are means ± SEM of three to five replicates with 30 roots per replicate. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$).

Supporting Tables

Table S2: Primers for genotyping

	Primer ID	**Sequence 5'-3'**
aos	aos-fwd	AATCGTAGGACCAATCAAAGACCG
	aos-rev	CAGATCCTTCTTCGCTCTACCGTA
bar	BAR-fwd	GGTCTGCACCATCGTCAACCAC
	BAR-rev	CAGCTGCCAGAAACCCACGTC
coi1-1	coi1-1 up	GTAATCGGAGATAGGGGTCTAGAGG
	coi1-1 low	TGTACCCACAAGTATCTCAGTGAAGG
		Subsequent digestion with *Mva*1296I
coi1-16	coi1-16 fwd (Gutierrez *et al.*, 2012)	AACTTCTACATGACGGAGTTTGC
	coi1-16 rev (Gutierrez *et al.*, 2012)	GGAGCCACCACAAAATTCTTCTA (dCAPS primer introducing an *Xba*I cleavage site into the wildtype PCR product)
coi1-t	COI1gen-1936fwd	CATCTTCTGGCTTTTCTGAAACAGCTG
	COI1gen1115rev	CACCAATTTCATTAAGGACAAAAAGTATCCAC
	LBb1	GCGTGGACCGCTTGCTGCAACT
EV (*HA-COI1*)	pB2GW7-fwd (HA-COI1, empty vector=pB2noHA, 35Sprom)	CACAATCCCACTATCCTTCGCA
	pB2GW7-rev (HA-COI1, empty vector=pB2noHA, 35Sterm)	CATGAGCGAAACCCTATAAGAACC
med25	SALK_059316.56.00LP (*pft1-3*)	CATGGCGACGATCGAGTTGACCAAAGAAG
	SALK_059316.56.00_RP (*pft1-3*)	CCTGACTTTGCATCAGGCAATATGTTGGC
sid2-2	sid2-2 fwd1	TTCTTCATGCAGGGGAGGAG
	sid2-2 fwd2	CAACCACCTGGTGCACCAGC
	sid2-2 rev	AAGCAAAATGTTTGAGTCAGCA
COI1-HA-Strep	ecoi-LPnew	TGGACCATATAAATTCATGCAGTCAACAAC
	ecoi-RPnew	CTGCAGTGTGTAACGATGCTCAAAAGTC
	LBb1.3	ATTTTGCCGATTTCGGAAC

Table S3: Primers for qRT-PCR analysis

Primer ID	Sequence 5'-3'
COI1-HA-Strep-RT fwd	AGTCCTGAAGGAGCCAATAGACCC
COI1-HA-Strep-RT rev	TGAGACCAAGCGTAATCTGGAAC
ICS1	QuantiTect QT00893473 (Qiagen)
JAZ10	QuantiTect QT00828401 (Qiagen)
PGM	QuantiTect QT00795879 (Qiagen)
PRLIP2	QuantiTect QT01833671 (Qiagen)
SARD1 fwd RT	TCAAGGCGTTGTGGTTTGTG
SARD1 rev RT	CGTCAACGACGGATAGTTTC
UBQ5 fwd RT	GACGCTTCATCTCGTCC
UBQ5 rev RT	GTAAACGTAGGTGAGTCCA
VSP2 fwd RT	CAAACTAAACAATAAACCATACCATAA
VSP2 rev RT	GCCAAGAGCAAGAGAAGTGA

Table S4: Primers for cloning

	Primer ID	Sequence 5'-3'
P1	COI1GW-fwd	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAGGATCCTGATATCAAGAGG
P2	COI1GW-rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATATTGGCTCCTTCAGGACTC
P3	COI1gGW-fwd	GGGGACAAGTTTGTACAAAAAAGCAGGCTATTCCTCCTCGAGTGCATCATC
P4	COI1gnostopGW-rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTGGCTCCTTCAGGACTCTAACAG
P5	coi1out-fwd	GGAGGATCCTGATATCAAGAGGTG

P6	coi1E203-rev	ATTTTGGCAAAAGCCGTCATGTAGAAGTTTAAAACCTCAAG
P7	coi1E203-fwd	CATGACGGCTTTTGCCAAAATCAGTCCCAAAG
P8	coi1Y302-rev	GCAATGCAGCAAGCAAATCCAGCTTTCGG
P9	coi1Y302-fwd	GCTGGATTTGCTTGCTGCATTGCTAGAAACTGAAGACC
P10	coi1out-rev	GGATGCTCCATCTCTCTTATCTCTCC

Supporting Experimental Procedures

Methods S1. RNAseq analysis

For RNAseq analysis, 33 (*coi1-t* and WT_{coi1-t}) or 36 (*aos* and WT_{aos}) single homozygous roots were combined for one replicate; replicates per genotype and treatment (two for *coi1-t* mock) were obtained from three independent infection experiments. RNA was extracted using the Trizol method (Chomczynski and Mackey, 1995) and RNA quality was controlled with an AGILENT BIOANALYZER 2100. Single-end 50-bp raw reads from mRNA sequencing were generated with the Illumina HiSeq 2000 platform and sequence images were transformed with the Illumina BaseCaller software to BCL files, which were subsequently demultiplexed to FASTQ files with CASAVA (v1.8.2). Using a Galaxy platform (Afgan *et al.*, 2018), mapping of reads to the *Arabidopsis thaliana* genome reference sequence (TAIR10 release-39, ftp://ftp.ensemblgenomes.org/pub/plants/release-39) was carried out with RNA STAR (Galaxy version 2.5.2b-2 (Afgan *et al.*, 2018)) and aligned reads were quantified using HTSeq-count (Galaxy version 0.9.1 (Anders *et al.*, 2015)). Normalization and differential expression analysis was performed with DESeq2 (Galaxy version 2.11.40.6+galaxy1 (Love *et al.*, 2014)) to obtain log2-fold changes and adjusted *p* values (Benjamini-Hochberg-corrected). The agriGO v2.0 program was used for the functional classification of differentially expressed genes (Tian *et al.*, 2017).

Methods S2. Quantitative reverse transcription (qRT)-PCR and Western blot analysis

Total RNA from frozen ground plant material was extracted with Trizol (Chomczynski and Mackey,1995). cDNA was synthesized from 1 µg of total RNA. First, RNA was treated with 1 U DNase (Thermo Scientific, Vilnius, Lithuania) in 1x DNase I-Buffer with $MgCl_2$ (Thermo Scientific, Vilnius, Lithuania) in a total volume of 10 µL. The mixture was incubated at 37°C for 30 min and the reaction was stopped by the addition of 1 µL 25 mM EDTA and further 10 min incubation at 65°C. Next, 20 pmol of oligo(dT) (20-mer and water were added to a total volume of 12.2 µL and the mixture was incubated at 70°C for 10 min. Finally, cDNA synthesis was completed by adding 20 pmol deoxynucleotide triphosphate, 4 µL of 5x RT reaction buffer (Thermo Scientific, Vilnius, Lithuania), 60 U of RevAid H-Minus Reverse Transcriptase (Thermo Scientific, Vilnius,

Lithuania), topping up to 20 µL total volume with water and incubating the mixture at 42°C for 70 min. The reaction was stopped by incubation at 70°C for 10 min. qRT-PCR analysis set up was as described (Fode *et al.*, 2008) with SYBR Green from Lonza (Rockland, ME, USA). PCR consisted of a 90 s denaturation step at 95°C followed by 39 cycles of 20 s at 95°C, 20 s at 55°C, and 40 s at 72°C.Calculations were done according to the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001) using the *UBQ5* (*AT3G62250*) transcript as a reference (Kesarwani *et al.*, 2007). Primers serving to amplify and quantify transcript levels are listed in Table S3.

Expression of HA-tagged proteins in stably transformed plants was monitored by Western blot analysis. Protein extracts were prepared in 250 µl extraction buffer (4 M urea, 16.6% glycerol, 5% SDS, 5% β-mercaptoethanol) per 100 mg plant material. Protein concentrations were determined using the Pierce 660 nm assay kit (Thermo Scientific, Rockford, IL USA). 50 µg were loaded onto a 10% SDS gel. Proteins were detected using the αHA-antibody (Abcam, Cambridge, United Kingdom) and Super Signal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL USA).

Methods S3. Construction of recombinant plasmids and generation of transgenic plants

The GATEWAY technology (Invitrogen, Karlsruhe, Germany) was used to generate recombinant plasmids. The *COI1* coding region was amplified from cDNA using primers that add GATEWAY recombination sites (P1–P2, Table S4) and inserted into pDONR201. The *COI1* insert was subsequently recombined into pB2HAGW7. pB2HAGW7 originates from the binary vector pB2GW7.0 (http://www.psb.ugent.be/gateway/), but contains the expression cassette of pE-35S-HA-GW7 (Weiste *et al.*, 2007). After confirming the sequence, the construct pB2-HA-COI1 and the empty vector were first introduced into *coi1-16* using *Agrobacterium tumefaciens*-mediated gene transfer (Clough and Bent, 1998). Plants expressing HA-COI1 were identified by Western blot analysis using the αHA antiserum. Later, the *coi1-16* allele was replaced by the *coi1-t* allele by fertilization of *coi1-t* with pollen derived from *35S:HA-COI1* expressing *coi1-16* plants. Plants homozygous for the transgene and the *coi1-t* allele were used for further analysis. The plants transformed with the empty vector were maintained as a heterozygous population with respect to

the *coi1-t* allele and had to be genotyped before analysis (see Table S2 for primers used for genotyping).

To create the C-terminally 3xHA-StrepII tagged genomic *COI1* (*COI1g*) constructs, the genomic *COI1* region comprising 2287 bps upstream of the annotated transcriptional start site and the last amino acid of the coding region was amplified from Arabidopsis DNA (P3–P4, Table S4) and inserted into pDONR207. Generation of $COI1g_{AA}$ was achieved by amplification of three fragments using primer pairs P5/P6, P7/P8 and P9/P10 with pDONR207/COI1g as a template. Primers P6 and P7 served to introduce the E203A mutation, while primers P8 and P9 served to introduce the Y302A mutation. The resulting three fragments served as templates for overlapping PCR with primers P5 and P10. The fragment was cut with *Hind*III and *Eco*RI and ligated into the pDONR207-COI1g, also cut with *Hin*dIII and *Eco*RI. This step yielded pDONR207-$COI1g_{AA}$. Wild-type and mutant *COI1g* sequences were inserted into the destination vector pB-GW-HAS7 using the LR recombination reaction. pB-GW-HAS7 is a pB-GW derivative that carries an 3xHA and a Strep tag downstream of the Gateway cassette. The sequence of the vector is given in Appendix 1. The resulting plasmids pB-COI1g-HAS7 and pB-$COI1g_{AA}$-HAS7 were introduced into heterozygous *coi1-t* plants which had been selected from the segregating population by pre-growth on MS medium containing 50 µM MeJA (Reymond *et al.*, 2000) to discard homozygous *coi1-t* plants and subsequent genotyping to discard wild-type plants. BASTA-selected plants were genotyped and plants heterozygous for *coi1-t* were further characterized by Western blot analysis of leaf material using an αHA antiserum. Plants expressing comparable amounts of wild-type and mutant COI1 were chosen for further analysis. Since plants were not homozygous with respect to the *coi1-t* allele and the transgene, they were genotyped directly before the experiment. After identifying homozygous *coi1-t* and $WT_{coi1\text{-}t}$ plants (Table S2), the selected plants underwent another round of genotyping. Homozygous *coi1-t* plants underwent PCR with ecoi-LPnew, ecoi-RPnew and LBb1.3 primers. A pattern of WT and homozygous bands together in this second PCR indicated at least one copy of transgenic $COI1_{AA}$/$COI1_{WT}$. A homozygous mutant band pattern alone meant an absence of transgenic $COI1_{AA}$/$COI1_{WT}$. The latter plants were used as *coi1-t* controls. $WT_{coi1\text{-}t}$ plants underwent a second round of genotyping using *bar* primers (BASTA resistance) to identify plants not carrying the $COI1_{AA}$ or $COI1_{WT}$ construct, which were used as WT controls. Additionally, qRT-PCR using COI1-HA-

Strep-RT fwd and COI1-HA-Strep-RT rev primers was used for all plants to confirm the presence or absence of $COI1_{AA}$ or $COI1_{WT}$.

Methods S4. Fungal culture

Verticillium longisporum Vl43 was grown in Potato Dextrose Medium with 0.5 mg/L Cefotaxim for 14 days at 21°C, 90 rpm, in the dark. Spores were harvested by straining through a filter (Nucleo Bond Folded filters, Macherey-Nagel, Düren, Germany). Spores were washed in sterile tap water, the spore concentration determined with a hemocytometer and finally spores were diluted to $1x\ 10^6$ spores/mL for sand-soil infections or $1x\ 10^5$ spores/mL for plate infections.

Methods S5. Leaf area measurement

Photographs of individual plants were taken and the surface area of the whole rosette was determined using 'BlattFlaeche' Software (Datinf GmbH, Tübingen, Germany) (Ralhan *et al.*, 2012).

Methods S6. Assessment of COI1 activity in transiently transformed protoplasts

Construction of plasmids and transient assays were performed essentially as described (Li *et al.*, 2019b). *JAZ* sequences were amplified and inserted into pDONR207 and subsequently recombined into the destination vector *UBQ10pro*:HA-GW.

Methods S7. Yeast two-hybrid analysis

COI1, ASK2 and *JAZ* sequences were transferred to the GATEWAY-compatible vectors for the LexA yeast two-hybrid system pGILDA-GW and pB42AD-GW described earlier (Li *et al.*, 2019a). Yeast two-hybrid experiments were performed as described previously (Zhang *et al.*, 2015). Plasmids were transformed into yeast strain *Saccharomyces cerevisiae* EGY48 (Estojak *et al.*, 1995) harboring the LexA reporter plasmid p8opLacZ (pSH18-34, Invitrogen, Thermo Fisher Scientific, Dreieich,

Germany) using the PEG-LiAc method (Gietz *et al.*, 1992). Transformants were selected on Synthetic Defined (SD) medium supplemented with glucose and –Ura/–His/–Trp drop-out solution. To assess the interaction, preselected yeast transformants were streaked onto SD medium supplemented with galactose, raffinose, –Ura/–His/–Trp drop-out solution, containing 80 µg/ml X-Gal. To trigger COI1-JAZ interactions, 15 µM coronatine was added.

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Appendix 1, Sequence of pB-GW-HAS7

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GGGGTCATAACGTGACTCCCTTAATTCTCATGTATGATAATTCGAGGGTACCCGGGGATCCTCTAGAGGGCC

IV. Article 2

Induction of salicylic acid-related defence genes in Arabidopsis roots upon infection with *Verticillium longisporum* requires transcription factor SARD1 and the inactivation of COI1-mediated repression

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This manuscript is ready for submission.

Detailed contributions by the PhD candidate:

Louisa Ulrich prepared, performed and analysed all experiments presented in this manuscript with exception of the RNA-seq experiment, the Motif Mapper analysis and the SA measurements. Louisa Ulrich generated the *coi1-t sard1-1 cbp60g-1* lines and characterised the *SARD1* overexpression lines. Louisa Ulrich created all Figures with the exception of Figure 1a and wrote a complete first draft of the manuscript.

Table 1 (RNA-seq data) will be deposited at the department of Plant Molecular Biology and Physiology, Georg-August University Göttingen, until the manuscript is published. For access, please contact Prof. C. Gatz (cgatz@gwdg.de).

Induction of salicylic acid-related defence genes in Arabidopsis roots upon infection with *Verticillium longisporum* requires transcription factor SARD1 and the inactivation of COI1-mediated repression

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Running title: The repressive effect of COI1 on SA-related defence genes in roots is overcome by *V. longisporum* infections in the absence of JA.

Abstract

Verticillium longisporum is a soil-borne fungal pathogen causing vascular disease predominantly in oilseed rape. The pathogen enters hosts through the root and subsequently entertains a parasitic life stage in the xylem before invading other tissues late in the infection cycle. Using *Arabidopsis thaliana* wild-type and mutants in major defence pathways, we have analysed the root transcriptomes at 10 days after inoculation (dpi). At this time point, nearly all of the 661 induced genes were expressed independently of the defence hormones jasmonic acid (JA) and salicylic acid (SA). Intriguingly, over 25% of these genes were constitutively expressed in mock-treated *coronatine insensitivie1 (coi1)* plants, which are deficient in JA perception. Since constitutive expression levels in *coi1* were in a similar range as in *V. longisporum*-infected plants, we postulate that induction of these genes is mediated by the

systemic inactivation of COI1. *V. longisporum*-induced/COI1-repressed genes were related to SA-dependent defence responses and included the master regulator of SA signalling, SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1). Mutating *SARD1* and its homolog *CALMODULIN-BINDING PROTEIN 60-LIKE G* interfered with *Verticillium*-induced expression and with constitutive expression of these genes in the *coi1* background. In contrast, overexpression of *SARD1* did not lead to enhanced expression of target genes, most likely because the negative effect of COI1 was not overcome.

Introduction

Plant roots are in close contact with a plethora of commensal, mutualistic and pathogenic microorganisms densely populating soil environments. Interactions with commensals and mutualists are beneficial for plant health, whereas pathogenic microorganisms can cause severe damage to plants (Raaijmakers *et al.*, 2009; Berendsen *et al.*, 2012; Mauchline and Malone, 2017).

All microorganisms carry some form of microbe-associated molecular pattern (MAMP). MAMPs are essential conserved molecules like flagellin, chitin or NLPs (Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins) that are perceived by pattern recognition receptors (PRRs) on the plant's plasma membrane (Bittel and Robatzek, 2007; Boller and Felix, 2009; Newman *et al.*, 2013; Oome *et al.*, 2014). Upon detection of these MAMPs, plant defences are activated including the synthesis of plant hormones. The corresponding hormone-mediated signalling pathways lead to massive transcriptional reprogramming to generate appropriate defence outputs against pathogen attack. The two main defence pathways are the salicylic acid (SA)-mediated defence and the jasmonic acid (JA)/ethylene (ET)-mediated defence.

Crucial in activating the SA pathway are the two transcription factors SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1) and CALMODULIN-BINDING PROTEIN 60-LIKE G (CBP60g), which start SA synthesis by inducing expression of the enzymes ISOCHORISMATE

SYNTHASE 1 (ICS1) and avrPphB SUSCEPTIBLE 3 (PBS3) (Strawn *et al.*, 2007; Wang *et al.*, 2009; Zhang *et al.*, 2010; Sun *et al.*, 2015). Downstream of SA biosynthesis, an indispensable component of the SA signalling cascade is NONEXPRESSER OF PATHOGENESIS-RELATED GENES 1 (NPR1), which interacts with transcription factors of the TGACG-motif binding (TGA) family to coordinate massive transcriptional reprogramming (Cao *et al.*, 1994; Cao *et al.*, 1997; Zhang *et al.*, 2003; Rochon *et al.*, 2007). Transcriptional reprogramming in the JA defence pathway is initiated by degradation of repressors of the JASMONATE ZIM-domain (JAZ) family, which block transcription factors like MYC2, 3 and 4, or ETHYLENE-INSENSITIVE3 (EIN3) on promoters of JA- and JA/ET-responsive genes (Chini *et al.*, 2007; Thines *et al.*, 2007; Fernández-Calvo *et al.*, 2011). The bioactive JA conjugate jasmonoyl-isoleucine (JA-Ile) facilitates binding of the F-box protein CORONATINE INSENSITIVE 1 (COI1) as part of the SCF^{COI1} complex to the JAZ repressors (Sheard *et al.*, 2010). The SCF^{COI1} complex constitutes a functional E3 ligase (Xu *et al.*, 2002). Upon co-reception of JA-Ile by COI1 and JAZs, JAZs are polyubiquitinated and degraded via the 26S proteasome.

Initiation of the SA or JA/ET pathway is based on the type of pathogen invading. Pathogens can be divided into different classes depending on their lifestyle; biotrophic pathogens feed off living hosts, whereas necrotrophic pathogens kill hosts to feed on the dead plant matter (Glazebrook, 2005). Hemibiotrophs employ a transitional lifestyle; they start off as biotrophs, often spreading widely in the host plant during this initial phase, and then turn necrotrophic feeding off dead host tissue (Horbach *et al.*, 2011). Generalised, SA-mediated defence responses are deployed against biotrophic pathogens and the JA/ET defence pathway is launched against necrotrophic pathogens (Glazebrook, 2005).

The ascomycete fungus *Verticillium longisporum* is a hemibiotrophic soil-borne pathogen with a host range largely restricted to Brassicaceae (Depotter *et al.*, 2016). Mainly *Brassica napus* is an economically important host crop in Europe, to whose production *V. longisporum* poses an increasing threat (Depotter *et al.*, 2016). *V. longisporum* penetrates roots and uses xylem vessels to spread systemically in its host. Infection with *V. longisporum* causes stunted growth,

vein clearing, leaf chlorosis and premature senescence (Reusche *et al.*, 2012; Depotter *et al.*, 2016).

V. longisporum, as well as another soil-born vascular ascomycete fungus, *Fusarium oxysporum*, have been shown to require the JA-Ile receptor COI1 for successful infection of Arabidopsis plants (Thatcher *et al.*, 2009; Ralhan *et al.*, 2012). Infected *coi1* mutants show less severe disease symptoms in shoots where lower fungal amounts were detected at late stages of infection. In contrast, the JA biosynthesis mutant *allene oxide synthase (aos)* shows wild-type (WT)-like symptoms (Ralhan *et al.*, 2012). Reciprocal grafts between scions and roots of *coi1* and WT revealed that COI1 is required in roots to cause susceptibility to *F. oxyposum* and *V. longisporum* (Thatcher *et al.*, 2009; Ralhan *et al.*, 2012).

Prompted by these findings, we have recently shown that in Arabidopsis roots, COI1 acts as repressor of defence gene expression (Ulrich *et al.*, 2021). RNA-seq analysis revealed that in *coi1* roots a number of SA defence-related genes are basally de-repressed. Again, *aos* mutant roots behave like WT roots and show no such de-repression. This repressor function of COI1 is uncoupled from its role in the JA pathway as it does not require JA-Ile and most likely no interaction with JAZ proteins (Ulrich *et al.*, 2021). It remains to be elucidated how this COI1-mediated repression is mechanistically achieved. In the setup used for our previous RNA-seq analysis, however, we could not gain any information on how *coi1* and susceptible plant roots react to infection with *V. longisporum* as axenically grown plantlets did not show significant responses to infection on the transcriptional level.

Here we present data from a new RNA-seq analysis of *V. longisporum*-infected *coi1*, *aos*, WT and SA biosynthesis-impaired *sid2* (*salicylic acid induction-deficient 2*) roots. This time, plants were grown in soil during the infection and a number of genes were induced in all genotypes. Intriguingly, roughly 25% of these genes were pre-induced in *coi1*. We furthermore show that transcription factor SARD1 is the master regulator of this group of genes, facilitating their increased expression in both *coi1* roots and after infection. However, overexpression of SARD1 in WT roots could not induce gene expression on its own. Hence, we conclude that, besides SARD1 binding, induction of these genes in response to *V. longisporum* infection

additionally requires a second yet unknown mechanism, potentially the inactivation of COI1 as a repressor. This inactivation of COI1 does not seem to be required in shoot tissue, correlating with the fact that in shoots COI1 does not act a repressor on the same set of genes. It is tempting to speculate that COI1 might have a role in attenuating SA responses in roots until a strong defence response becomes indispensable.

Results

A subgroup of COI1-repressed genes is induced in roots after infection with *V. longisporum*

As the *coi1* mutant is tolerant against infection with *V. longisporum,* we were interested to see how the root transcriptome of *coi1* plants differs from that of susceptible plants. Since the phenotypes of the JA biosynthesis mutant *aos* and the JA receptor mutant *coi1-t* differ after infection, we also included *aos* plants in the analysis. We also analysed the SA biosynthesis mutant *sid2-2* in order to identify potential effects resulting from interactions between the defence pathways. As *coi1-t* and *aos* plants are male sterile, we genotyped the plantlets resulting from heterozygous seed batches before experiments and also included the respective outcrossed WT plants from each population in the RNA-seq analysis. RNA was derived from four experiments, each comprising combined roots from twelve plants per genotype and treatment. Tissue was harvested at 10 dpi or 10 days after mock treatment. We chose this time point as we expected that at 10 dpi the root was sufficiently colonised by the fungus to observe robust responses. At this time point no differences in fungal load in the shoot can be detected between susceptible WT and *aos* plants and tolerant *coi1* plants (Ralhan *et al.*, 2012). Thus, differences in infection-specific transcript levels in the different genotypes are unlikely to be due to different fungal loads.

We obtained a first impression of the root transcriptomes by principal component analysis (Figure 1a). Mock-treated samples of *aos*, WT_{aos}, $WT_{coi1\text{-}t}$ and *sid2-2* are closely grouped with clear separation from their infected counterparts, which also group together. Mock-treated

coi1-t roots are clearly different from the other four mock-treated genotypes. This difference between *coi1-t* and the other genotypes is less pronounced at 10 dpi.

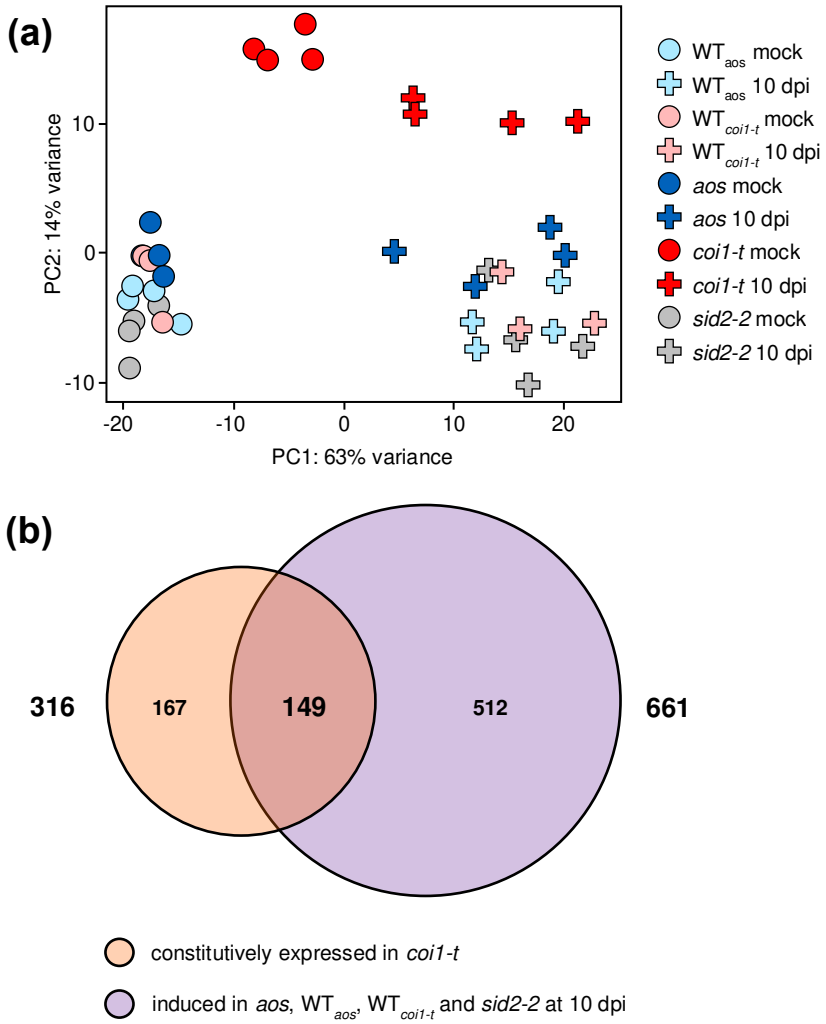

Figure 1. A group of 149 genes is de-repressed in *coi1-t* roots and induced in *aos*, WT_{aos}, WT_{coi1-t}, and *sid2-2* at 10 dpi.

(a) Principal component analysis of the normalised root transcriptome data acquired by RNA-seq analysis 10 days after mock treatment or inoculation with $1x10^6$ spores/mL sGFP-expressing *V. longisporum*. Biological replicates from four independent experiments are symbolised by circles (mock) or plus signs (10 dpi). For WT_{coi1-t} only three replicates were analysed for both mock and 10 dpi treatments. WT_{aos} and WT_{coi1-t} are the wild-types obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds. **(b)** Venn diagram showing the overlap between 316 genes constitutively upregulated in mock-treated coi1-t roots vs mock-treated aos, WT_{aos}, WT_{coi1-t} and *sid2-2* (> 2-fold, $p < 0.05$) and 661 genes induced in *aos*, WT_{aos}, WT_{coi1-t} and *sid2-2* at 10 dpi (> 2-fold, $p < 0.05$). Expression data was obtained by RNA-seq analysis from root material 10 days after mock treatment or inoculation with $1x10^6$ spores/mL sGFP-expressing *V. longisporum*. Circles are drawn to scale with respect to the number of genes represented in each group.

After infection, gene induction is similar in both WTs (> 2-fold, $p < 0.05$) (Figure S1a, Table 1 and subtables). When intersecting gene induction responses in both WTs there is 12.4% and 18.6% drop out on either side, mostly of genes that closely miss the threshold (Figure S1a). Generally, the responses induced in WT plants are related to cell wall biogenesis, xylem development and SA defence responses (Figure S2). Gene induction after infection in WT_{aos} shows 84.8% overlap with gene induction in the *aos* mutant (Figure S1b). Moreover, both WTs show largely overlapping gene induction patterns with the *sid2-2* mutant after infection (Figure S1c and S1d). This indicates that there are no major differences between the WTs and either of the JA or SA biosynthesis mutants, as suggested by the PCA analysis (Figure 1a). Hence, we continued our analysis with the genes most robustly differentially regulated, i.e. those that are induced in all four genotypes WT_{aos}, $WT_{coi1\text{-}t}$, *aos* and *sid2-2.* After infection a total of 661 genes are upregulated in these genotypes (Table 1 and subtables). Together in *aos, sid2-2* and the WTs, 91 genes are downregulated after infection (> 2-fold, $p < 0.05$) (Table 1 and subtables).

At 10 dpi, eleven genes were lower expressed in *coi1-t* roots compared to all other genotypes (> 2-fold, $p < 0.05$) (Table 1 and subtables). Of these, only three genes are induced after infection in *aos*, both WTs and *sid2-2*. At 10 dpi, 71 genes are higher expressed in *coi1-t* compared to the other four genotypes, of which seven genes are inducible in WT_{aos}, $WT_{coi1\text{-}t}$, *aos* and *sid2-2* (Table 1 and subtables).

The greatest difference observed between *coi1-t* roots and the other genotypes is a set of 316 genes higher expressed in mock-treated *coi1-t* roots (> 2-fold, $p < 0.05$) (Table 1 and subtables). As these genes are not de-repressed in *aos,* this expression pattern seems to be specific for *coi1* roots and not due to disruptions in the JA signalling pathway. Only eight genes are lower expressed in mock-treated *coi1-t* roots compared to *aos*, both outcrossed WTs and *sid2-2* (> 2-fold, $p < 0.05$).

The 661 genes induced after infection in *aos*, WT_{aos}, $WT_{coi1\text{-}t}$ and *sid2-2* share an overlapping 149 genes with the group of 316 genes de-repressed in *coi1-t* roots (Figure 1b and Table 1

and subtables). Of these 149 genes, only 21 genes are higher expressed in *coi1-t* at 10 dpi compared to mock treated *coi1-t* roots (Table 1 and subtables).

The genes found to be de-repressed here are largely overlapping with the set of genes we have previously reported to be suppressed by COI1 in roots (Ulrich *et al.*, 2021). This interesting group of 149 genes identified here raises the question whether activation of gene expression after infection with *V. longisporum* is achieved via the inactivation of the COI1-mediated repression.

The subgroup of COI1-suppressed and *V. longisporum*-responsive genes is related to SA-mediated defence responses and is significantly enriched with the 'GAAATTT' motif

GO term analysis showed that the 149 genes de-repressed in *coi1-t* and induced in *aos*, both WTs and in *sid2-2* roots after infection are associated with immune defence responses, in particular SA-mediated defence responses (Figure 2). Similarly, the 167 genes under negative control of COI1 and not induced after infection, are also associated with SA defence responses (Figure S3a). In contrast, the 512 genes induced after infection in *aos*, both WTs and *sid2-2* but not under control of COI1 are not associated with SA-mediated defences but with various processes in cell wall production (Figure S3b).

Using motif enrichment analysis, we aimed to determine if a certain transcription factor binding motif was overrepresented in the regulatory regions of the different clusters of genes we had identified in the RNA-seq, specifically in the 149 genes de-repressed in *coi1* and induced after infection. The Motif Mapper cis-element analysis tool scans 1-kb sequences upstream of predicted transcriptional start sites (Berendzen *et al.*, 2012). The average number of detected binding motifs in a specific gene set is compared to the average number of found binding motifs randomly obtained 1000 times from a chosen control set. We screened for enriched motifs in the five clusters of genes defined from the RNA-seq data (shown in Figure 1b): The two main groups of 316 genes de-repressed in *coi1* roots and 661 genes induced after infection in susceptible genotypes, along with the subgroups identified; the 149 genes de-repressed in

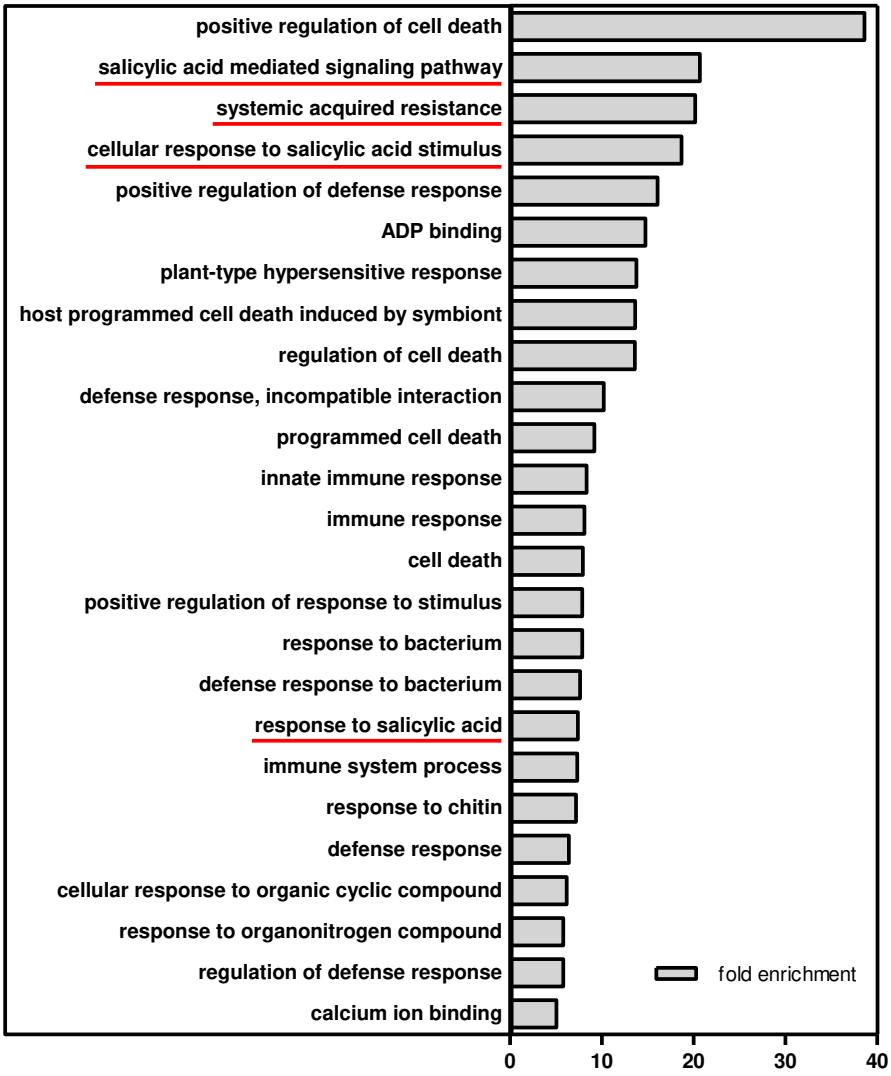

Figure 2. The 149 genes de-repressed in *coi1* and induced after infection are related to defence responses.

Gene Ontology (GO) term enrichment analysis of the 149 genes basally upregulated in *coi1-t* compared to *aos,* WT_{aos}, $WT_{coi1\text{-}t}$ *and sid2-2* (> 2-fold; $p < 0.05$) and induced in *aos*, WT_{aos}, $WT_{coi1\text{-}t}$ *and sid2-2* at 10 dpi (> 2-fold, $p < 0.05$). Bars represent the fold enrichment of the number of genes found per GO term in the group of 149 genes against the number of genes found within the Arabidopsis genome associated with that GO term. Only GO terms with > 5-fold enrichment against the genome are shown. SA defence related GO terms are underlined in red.

coi1 and induced after infection, the 167 genes de-repressed in *coi1* but not induced after infection and the 512 genes induced after infection but not under control of COI1.

Analysis of the regulatory regions showed that the 'GAAATTT' motif was significantly enriched in the promoters of the 149 genes de-repressed in *coi1* and induced after infection compared to promoter sequences randomly drawn from the entire genome (Figure 3a). 'GAAATTT' is the

binding motif for SARD1 and CBP60g. This binding motif was not enriched in any of the other groups of genes.

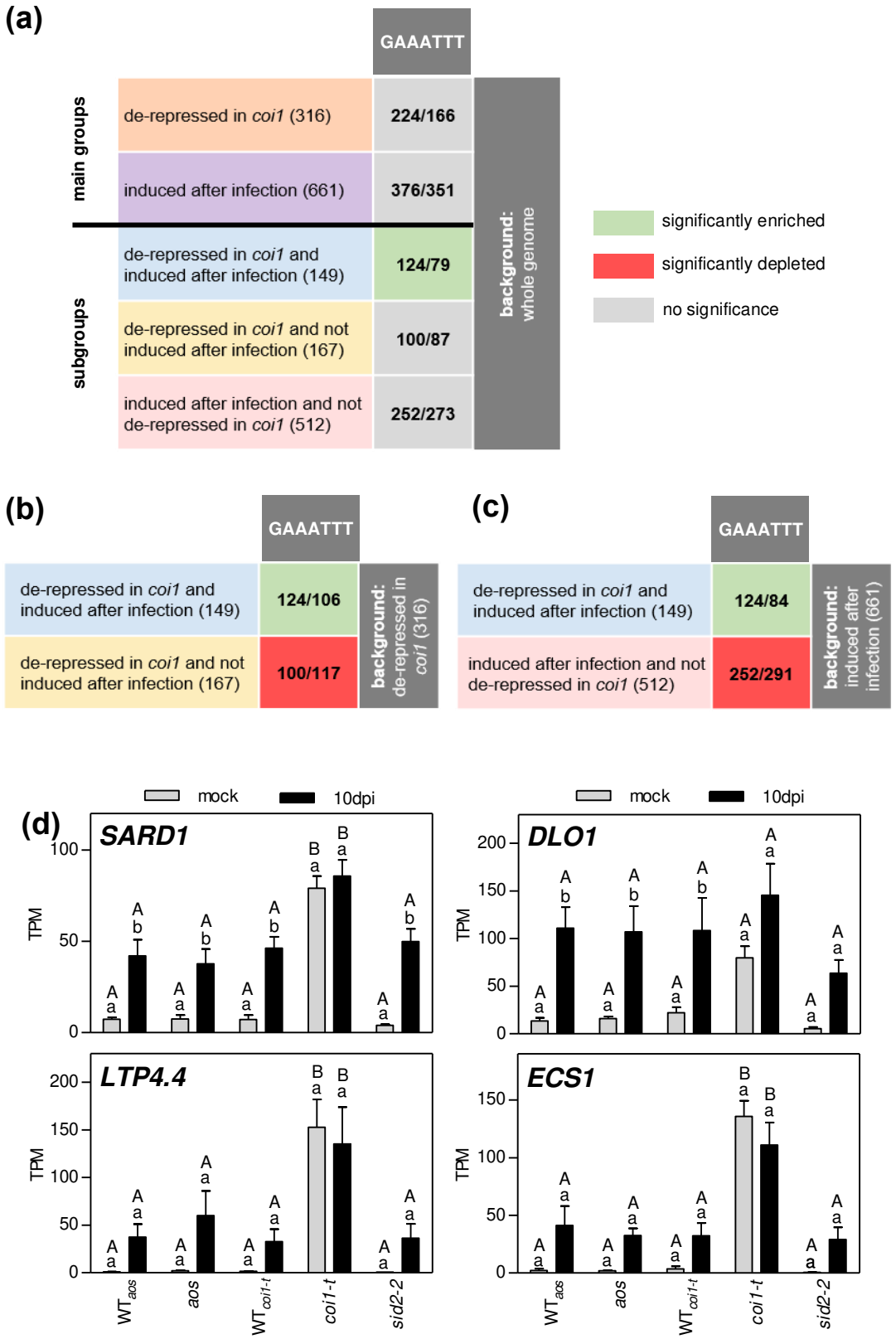

Figure 3. The binding motif for SARD1 and CBP60g 'GAAATTT' is significantly enriched in the 149 defence genes of interest.

Full legend on next page.

Figure 3. The binding motif for SARD1 and CBP60g 'GAAATTT' is significantly enriched in the 149 defence genes of interest.

Motif Mapper cis-element analysis of **(a)** the 316 genes de-repressed in *coi1-t* roots (*coi1-t* mock > *aos*, WT_{aos}, WT_{coi1-t}, *sid2-2* mock; 2-fold; $p < 0.05$), the 661 genes induced after infection (*aos*, WT_{aos}, WT_{coi1-t}, *sid2-2* 10 dpi > *aos,* WT_{aos}, WT_{coi1-t}, *sid2-2* mock; 2-fold; $p < 0.05$), the 149 genes de-repressed in *coi1-t* and induced after infection, the 167 genes de-repressed in *coi1-t* and not induced after infection and the 512 genes induced after infection but not de-repressed in *coi1-t* **(b)** the 149 genes de-repressed in *coi1-t* and induced after infection and the 167 genes de-repressed in *coi1-t* and not induced after infection against the background of the set of all 316 genes de-repressed in *coi1-t* roots **(c)** the 149 genes de-repressed in *coi1-t* and induced after infection and the 512 genes induced after infection and not de-repressed in *coi1-t* roots against the background of the set of all 661 genes induced after infection. Numbers before slashes show the total number of detected motifs in the set of interest. Numbers behind the slashes show the number of expected motif counts in a set of randomly chosen promoters within the indicated background set. Significant enrichment/depletion is defined at $p < 0.05$. **(d)** Relative expression of *SARD1, DLO1, LTP4.4* and *ECS1* transcript levels as quantified by RNA-seq analysis 10 days after mock treatment or inoculation with $1x10^6$ spores/mL sGFP-expressing *V. longisporum*. Bars are means of Transcripts Per Million (TPM) ± SEM of three to four biological replicates of each genotype, with each replicate representing twelve roots from one independent experiment. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). WT_{aos} and WT_{coi1-t} are the two wild-types lines obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds.

When testing the groups of the 149 inducible and 167 non-inducible genes under COI1-control against the total 316 genes de-repressed in *coi1* rather than the whole genome, the 149 inducible genes were enriched with the 'GAAATTT' motif (Figure 3b). The other 167 genes, however, were actually depleted of the motif.

Similarly, we analysed all 661 genes inducible after infection split into two groups of those 149 under negative control of COI1 and those 512 not suppressed by COI1. Only the group of the 149 genes showed enrichment of the 'GAAATTT' motif. The 512 genes not under COI1 control were significantly depleted of the motif (Figure 3c).

Overall, this analysis shows that out of all groups analysed, the 149 genes induced after infection and de-repressed in *coi1* are explicitly enriched with the SARD1/CBP60g binding motif.

Indeed, SARD1 might be a promising candidate for regulating this set of genes as it is found within the group of the 149 genes. Figure 3d shows expression patterns of *SARD1* and three representative marker genes chosen from the 149 genes in this group: *LIPID TRANSFER PROTEIN 4.4 (LTP4.4)* (AT5G55450), a gene involved in SA catabolism *DMR6-LIKE OXYGENASE 1 (DLO1)* (AT4G10500) and the cell wall protein *ECS1* (formerly *CXC750*) (AT1G31580).

COI1 does not suppress target gene expression in shoots

Seeing that COI1 represses basal expression of 316 genes in roots, we analysed gene expression in whole shoots from the same plants whose roots underwent the RNA-seq analysis (Figure 4). In contrast to roots, our marker genes were not de-repressed in *coi1* shoots. Hence, the observed suppressive action of COI1 on gene expression is exclusive to roots.

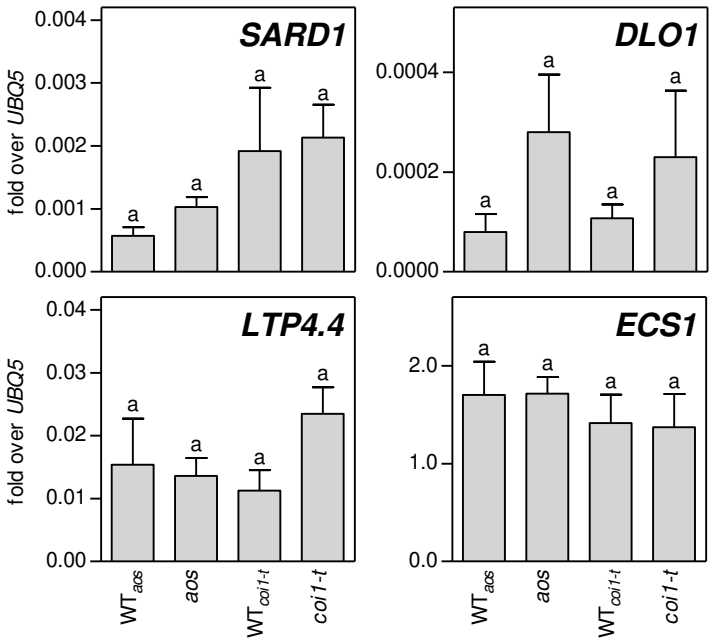

Figure 4. Unlike in roots, COI1 is not a constitutive repressor of target genes in shoots.
SARD1, LTP4.4, DLO1 and *ECS1* transcript levels, measured by qRT-PCR. RNA was extracted from shoots 10 days after mock treatment from the same plants whose roots were subjected to the RNA-seq. Bars are means ± SEM of four replicates, each made up twelve shoots per genotype. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$). WT_{aos} and $WT_{coi1\text{-}t}$ are the two wild-types lines obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds.

Gene induction after *V. longisporum* infection does not require increased SA levels

GO term analysis linked the genes under negative control of COI1 and induced after infection to defence responses, especially SA-mediated defences. In contrast to this, we see our genes of interest induced after infection in *sid2* roots in the RNA-seq data. We repeated experiments not only in *sid2* roots but also included *npr1* mutants to see if the observed gene induction requires the main hub of SA-mediated transcriptional reprogramming. Again, induction of *SARD1, LTP4.4* and *DLO1* was similar in WT and *sid2* roots, confirming observations from the RNA-seq (Figure 5a). *ECS1* showed slightly lower expression in infected *sid2* mutants compared to WT but was still almost 6-fold induced over mock plants. Induction of all genes was still observed in infected *npr1* mutants, albeit to slightly smaller extent in some cases. We measured SA levels in WT and *sid2* mutants and could not detect an increase in SA levels in either of the two genotypes after infection (Figure S4).

This seemingly contradictory fact that SA-defence related genes are upregulated without increased SA levels is not exclusive to *V. longisporum*-infected roots. Local infiltration of leaves with nlp14, a 14 amino acid-long immunogenic peptide found in NLPs, also induced expression of *SARD1* and *LTP4.4* after 24 hours in *sid2* and *npr1* mutant leaves (Figure 5b). *DLO1* and *ECS1* expression, however, was ICS1- and NPR1-dependent under these conditions.

In *coi1* roots, *ICS1* is de-repressed and SA levels elevated (Figures S5a and S5b). Still, basal upregulation of *SARD1*, *LTP4.4* and *ECS1* was still elevated in *coi1 sid2* double mutant roots, which have WT-like SA levels (Figures S5b and S5c). Elevated *DLO1* expression was dependent on upregulated SA levels though.

Our genes of interest can be regulated independently from increased SA levels after *V. longisporum* infection, and mostly so in locally nlp14-triggered leaves and *coi1* roots. Nevertheless, we could establish a connection to SA signalling. Treatment of roots with SA showed that our marker genes - with the exception of *LTP4.4* - are responsive to SA accumulation (Figure S6). Induction after 24 hours of SA treatment requires NPR1 and hence

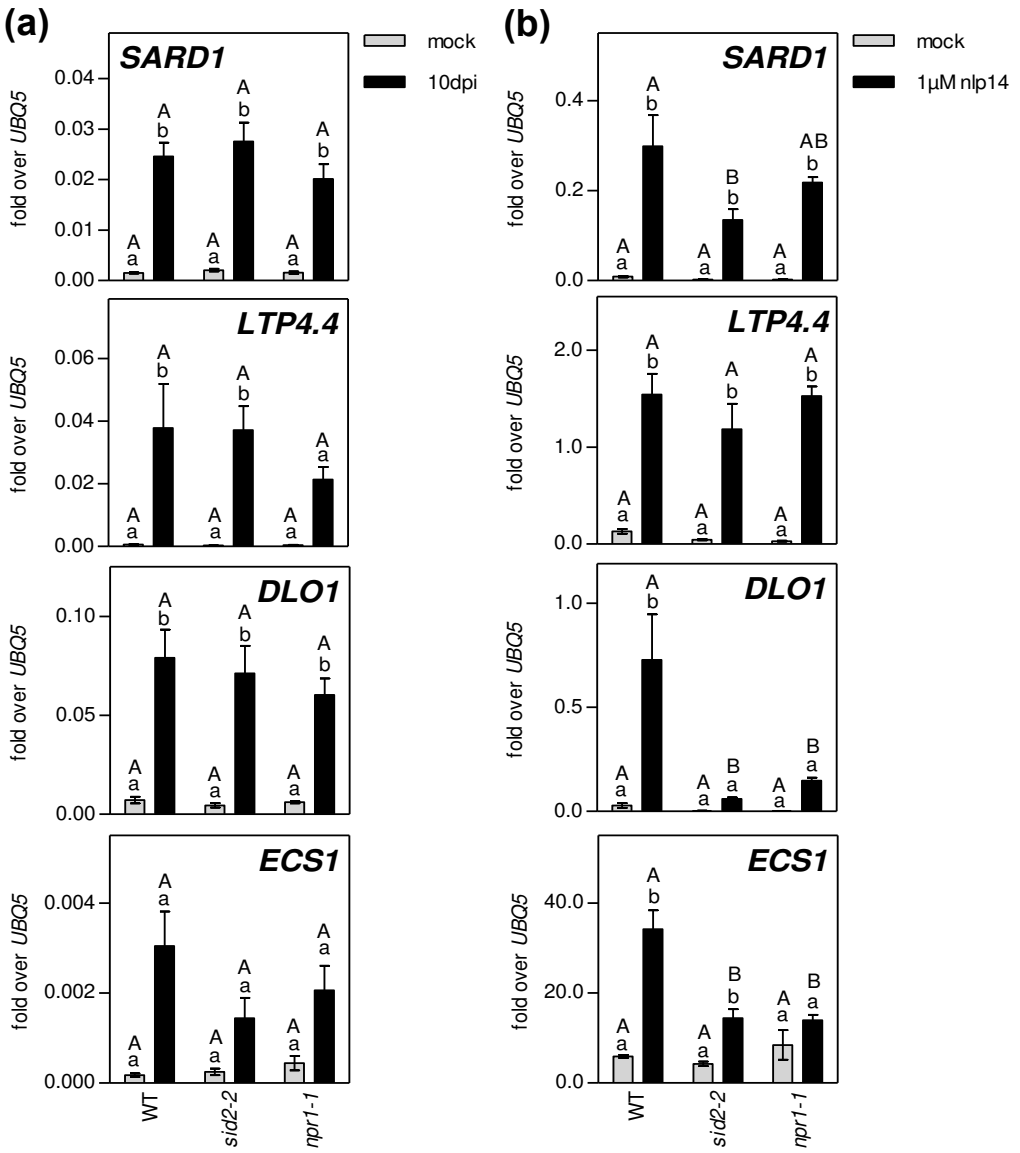

Figure 5. Genes of interest are induced independently of increased SA levels and NPR1 in roots.

(a) *SARD1, LTP4.4, DLO1* and *ECS1* transcript levels, measured by qRT-PCR. RNA was extracted from roots 10 days after mock treatment or infection with 1x10^6 spores/mL *V. longisporum*. Bars are means ± SEM of thirteen to sixteen roots per genotype. For *ECS1* transcript levels in *npr1-1* only six replicates are shown. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). **(b)** *SARD1, LTP4.4, DLO1* and *ECS1* transcript levels, measured by qRT-PCR. RNA was extracted from leaves 24 hours after infiltration with 1µM nlp14. Bars are means ± SEM of four to six replicates per genotype. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 1µM nlp14 ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$).

classical SA defence signalling. Exogenous treatment with SA even hyperinduced gene expression levels compared to the expression level in *coi1* roots for *DLO1* and *ECS1*.

In summary, our genes of interest are connected to classical SA signalling but under the conditions we investigate here, they are regulated independently of increased SA levels.

SARD1/CBP60g are required for gene induction after *V. longisporum* infection and for basal gene upregulation in *coi1* roots

We next turned our attention to the role of SARD1 in regulating our genes of interest, as motif mapping analysis had shown significant enrichment of the SARD1/CBP60g binding motif in their promoters (Figure 3a, 3b and 3c). SARD1 and CBP60g are close homologues that bind to the same motif. Both transcription factors are important for local and systemic pathogen defence responses by regulating key players in SA and N-hydroxy-pipecolic acid synthesis like *ICS1*, *avrPphB SUSCEPTIBLE3 (PBS3)*, *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1)* and *AGD2-LIKE DEFENSE RESPONSE PROTEIN1 (ALD1)* (Wang *et al.*, 2009, 2011; Zhang *et al.*, 2010a; Sun *et al.*, 2015). Moreover, they bind to promoters of a plethora of other defence response genes, such as key players in ETI and PTI, e.g. *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1)*, *PHYTOALEXIN DEFICIENT4* (*PAD4)*, *BRASSINOSTEROID INSENSITIVE 1* (*BAK1)* and *BOTRYTIS-INDUCED KINASE1* (*BIK1)* (Sun *et al.*, 2015). Even though CBP60g is not induced after infection (Figure S7), we decided to work with *sard1 cbp60g* double mutants to exclude any possible compensatory effects in gene activation by CBP60g in the absence of SARD1.

To determine if SARD1/CBP60g are required for gene induction after infection with *V. longisporum*, we infected *sard1 cbp60g* mutants and determined marker gene expression at 10 dpi. As a well-established target gene of SARD1 and CBP60g we also included *ICS1* in our analysis (Zhang *et al.*, 2010b; Sun *et al.*, 2015). As shown above (Figure S5a), *ICS1* is under negative control of COI1 and even though it was not significantly induced after infection in the RNA-seq data, we always observed minor *ICS1* induction in all subsequent infections

experiments. The observed induction of *ICS1* was usually lower than 2-fold, therefore, it is not as strongly responsive to *V. longisporum* infection as our other marker genes (Figure 6a). Overall, in *sard1 cbp60g* roots all four marker genes, *LTP4.4*, *DLO1*, *ECS1* and *ICS1* showed greatly impaired gene induction compared to WT roots at 10 dpi (Figure 6a).

Having established that SARD1/CBP60g are crucial for induction of marker genes in response to infection, we were interested if they are also involved in enhanced gene expression in *coi1* roots. To determine if this was the case, we crossed the *sard1 cbp60g* mutant into the *coi1* background. In the *coi1 sard1 cbp60g* triple mutant expression of our marker genes was reverted back to WT levels (Figure 6b). Hence, SARD1/CBP60g are also responsible for the upregulated expression of *LTP4.4*, *DLO1*, *ECS1* and *ICS1* in *coi1* roots.

Motif mapping had shown that the 'GAAATTT' motif was depleted in the groups of the 167 genes de-repressed in *coi1* and not significantly induced after infection as well as in the 512 genes induced after infection but not under control of COI1. We randomly selected a few genes from each of these two groups and assessed if they are regulated independently of SARD1/CBP60g.

Testing expression of genes found in the 167 COI1-repressed but non-inducible genes, we saw that a RmlC-like cupins superfamily protein (*AT5G39120*) was still de-repressed in *coi1 sard1 cbp60g* roots. *WRKY54* and *WRKY46* expression was fully and largely SARD1/CBP60g-dependent, respectively (Figure S8a). However, upregulation of *WRKY54* in *coi1* is ICS1-dependent (Figure S8b), hence the downregulation in *coi1 sard1 cbp60g* is likely due to the reduction of ICS1-derived SA levels (Figure 6b). *WRKY46* is still upregulated in *coi1 sid2* mutants confirming that it is mostly SARD1/CBP60g-dependent (Figures S8a and S8b). Two genes picked from the group of the 561 genes induced after infection but not suppressed by COI1, *ANAC076* and *ERF54*, were still inducible in *sard1 cbp60g* mutants after infection (Figure S8c).

Taken together, these observations show that SARD1/CBP60g are necessary for activation of our defence genes of interest in *V. longisporum*-infected and *coi1* roots

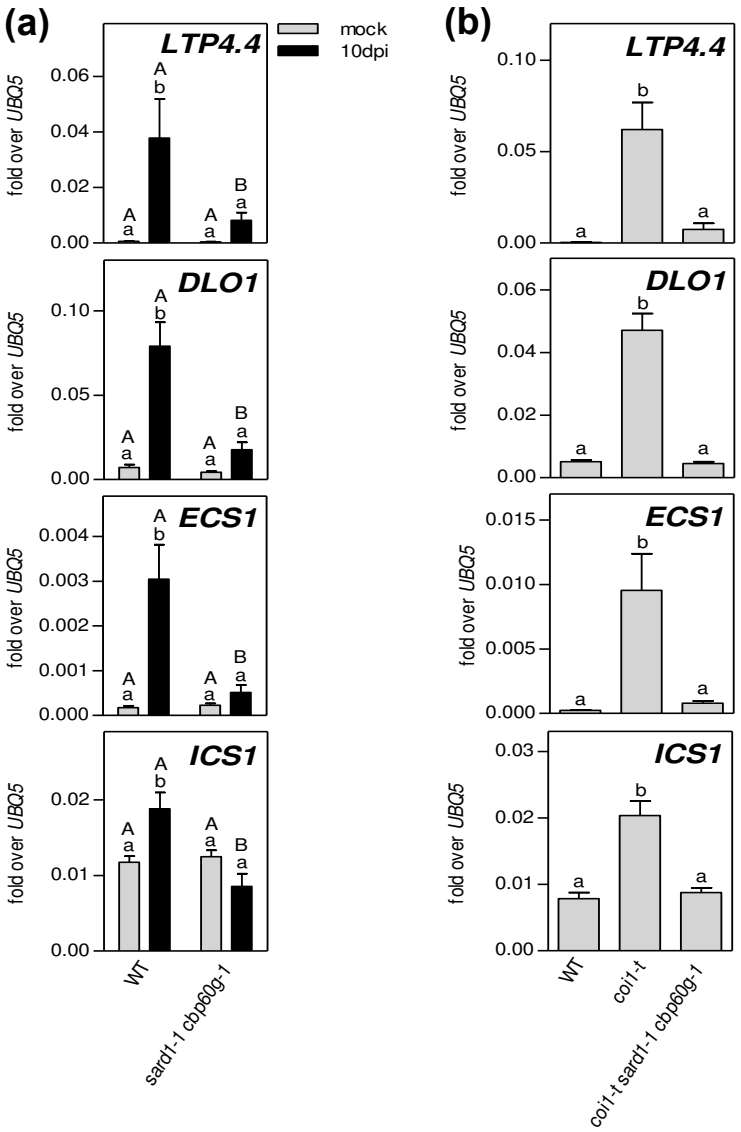

Figure 6. SARD1/CBP60g are responsible for induction of genes of interest after infection with *V. longisporum* and for basally upregulated gene expression in *coi1*.

(a) *LTP4.4, DLO1, ECS1* and *ICS1* transcript levels, measured by qRT-PCR. RNA was extracted from roots 10 days after mock treatment or infection with $1x10^6$ spores/mL *V. longisporum*. Bars are means ± SEM of thirteen to sixteen roots per genotype. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). **(b)** *LTP4.4, DLO1, ECS1* and *ICS1* transcript levels, measured by qRT-PCR. RNA was extracted from roots 10 days after mock treatment. Bars are means ± SEM of eight roots per genotype. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$).

SARD1 is required but not sufficient for induction of genes of interest

Having identified SARD1/CBP60g as main regulators of our genes of interest, we aimed to create SARD1 overexpression lines to see if we could induce expression of our target genes in such plants. Plants overexpressing SARD1 in roots should mimic enhanced gene expression levels that we see after infection with *V. longisporum* and basally in *coi1*. To this end, we constructed a *SARD1* clone using the genomic sequence from the transcriptional start site with a C-terminal three times HA and Strep-II tag under control of the *UBIQUITIN10* (*UBQ10*) promoter. Zhang *et al.* (2010) reported increased SA levels and a dwarfed phenotype of their *SARD1* overexpression lines (*SARD1 OXs)* under control of the endogenous *SARD1* promoter. The underlying reason for this is likely the activation of *ICS1* by SARD1, activating SA defence signalling. As we wanted to avoid growth defects, we not only transformed our *SARD1* construct into Col-0 plants but also into *sid2* mutants. Overexpressing *SARD1* in the *sid2* background avoids triggering ICS1-derived SA production and its possible negative effects on plant growth. *SARD1* overexpression in Col-0 lead to about 2-fold higher induction of *SARD1* compared to *V. longisporum*-infected WT plants (Figure S9a) and thus is approximately comparable to *SARD1* transcript levels in *coi1* roots (Figure 3d). In the end, our overexpression line in the Col-0 background only showed mild defects in rosette size compared to the empty vector control (Figure S9b). As expected, overexpression of *SARD1* had no effect on rosette size in the *sid2* background.

To our surprise, we found that overexpressing *SARD1* in roots in either Col-0 or *sid2* led to weak to no induction of target genes (Figure 7a). *LTP4.4* showed no induction in Col-0 or *sid2* background. *DLO1* and *ECS1* showed approximately 2-fold induction in *SARD1 OXs* in Col-0 but no induction in the lines in *sid2* background. *ICS1* was not at all induced in the *SARD1* overexpression line in Col-0.

In contrast, expression of our target genes in shoots of the exact same plants showed much stronger gene induction (Figure 7b). *LTP4.4* was 25-fold and 11-fold induced in Col-0 and *sid2*,

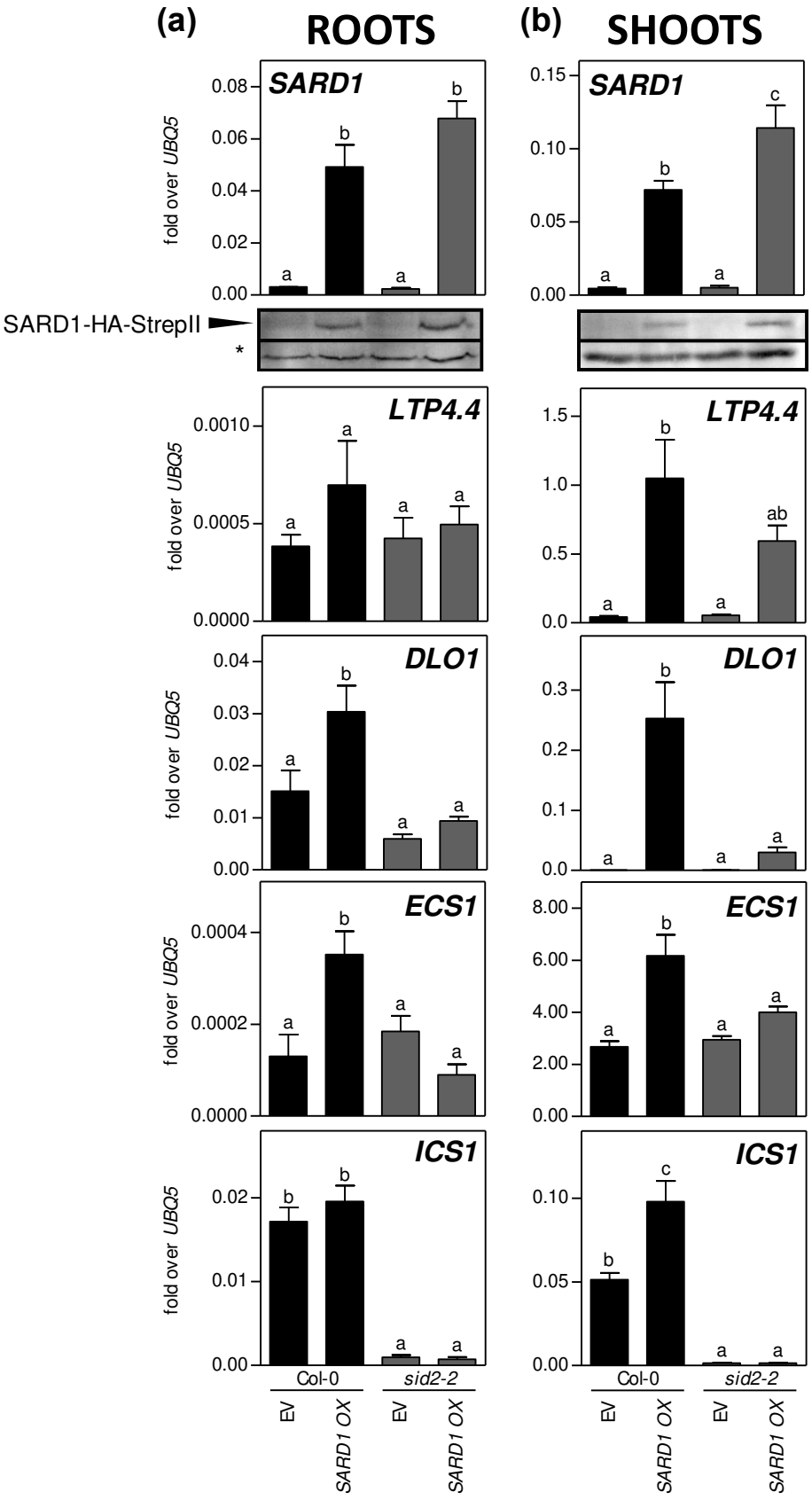

Figure 7. Overexpression of SARD1 in roots does not lead to strong target gene activation.

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Figure 7. Overexpression of SARD1 in roots does not lead to strong target gene activation.
SARD1, LTP4.4, DLO1, ECS1 and *ICS1* transcript levels, measured by qRT-PCR. RNA was extracted from **(a)** roots or **(b)** shoots 10 days after mock treatment of *SARD1* overexpression lines *(SARD1 OX)* and empty vector (EV) controls in both Col-0 (black bars) and *sid2-2* (gray bars) background. Bars are means ± SEM of three to six roots or shoots per line. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$).
Insert: Western blot of protein extracts obtained from roots and shoots of *SARD1* overexpression lines *(SARD1 OX)* and empty vector (EV) controls in Col-0 and *sid2-2* background. Per lane, six roots or three shoots were pooled from each line. C-terminally 3xHA-StrepII tagged SARD1 protein levels were detected using an anti-HA antibody. * depicts an unspecific band shown as loading control.

respectively. *DLO1* was 372-fold induced in Col-0 plants, when it was only 2-fold induced in roots in the same genotype. In *sid2*, *DLO1* was 33-fold induced, albeit non-significantly in statistical analysis. *ECS1* showed no stronger induction in shoots as compared to roots, only stronger expression overall. In shoots, *ICS1* was also induced twofold.

We also tested expression of *WRKY46* and *WRKY54* in the *SARD1 OX* lines (Figures S10a and S10b). *WRKY46* showed a similar induction pattern to *LTP4.4*, with *SARD1 OX* lines only inducing gene expression in shoots. For *WRKY54,* lower expression in *sid2* compared to Col-0 can be seen in both EV and *SARD1 OX* plants. *WRKY54* shows weak induction in the Col-0 *SARD1 OX* line in roots and slightly stronger induction in shoots, again with strong differences in Col-0 and *sid2*.

Overall, in roots, SARD1 alone is not sufficient to induce gene expression to a scale that was observed in infected roots or in *coi1* roots. In contrast to roots, SARD1 can induce expression of target genes strongly in shoots, where COI1 does not act as a repressor of these genes (Figure 4). The only exception is *ECS1* which is similarly expressed in *SARD1 OXs* in roots and shoots. Possibly, *SARD1 OXs* do not show strong target gene induction in roots because SARD1 is unable to overcome the COI1-mediated repression of these genes. This evidence is in favour of our hypothesis, that COI1 mediated repression is overcome by infection with *V. longisporum*. Only after the negative effect of COI1 on these promoters is lifted, SARD1 is able to activate gene expression.

Basal upregulation of SARD1-controlled defence gene expression in roots is not responsible for the tolerance of *coi1* plants against *V. longisporum*

Our main interest is the regulation of the 149 genes de-repressed in *coi1* roots and induced after infection with *V. longisporum*. We anticipated that understanding their regulation may provide insights into the mechanism through which COI1-mediated repression works and how it may be inactivated. Nevertheless, having identified SARD1/CBP60g as the master regulators of this set of genes, we were now also able to address the question whether the upregulation of these genes in *coi1* roots is the underlying reason for the tolerance of *coi1* plants against *V. longisporum*.

Grafting experiments had shown that plants with *coi1* root stock and WT shoots were tolerant against fungal infection (Ralhan *et al.*, 2012). Hence, *coi1* roots and not shoots are important for tolerance. This seemingly contradicts the fact that fungal entry into *coi1* roots is not hindered and that infection is only contained by reduced proliferation of *V. longisporum* in shoots of *coi1* plants (Ralhan *et al.*, 2012). The genes we are investigating here are induced after infection and GO term analysis identified them to be defence-related (Figure 2). Some of these genes, like *LTP4.4*, are secreted to the apoplast (McLaughlin *et al.*, 2015), others have a mobile mRNA, like the call wall protein *ECS1* (Thieme *et al.*, 2015). It is possible that products of these genes, constitutively made in the root, are transported up into the shoot where they accumulate. Once the fungus reaches the shoot, its proliferation is impaired by the defence compounds that are deposited there.

With this idea in mind, we again used mutations in *SARD1* and *CBP60g* to examine the phenotype of infected plants unable to activate our genes of interest. Initially, we compared the phenotype of *sard1 cbp60g* plants against WT plants to see if the mutants would be hypersusceptible to infection with *V. longisporum*. Even at 21 dpi we could not determine any difference between *sard1 cbp60g* and WT plants (Figure S11). To better address the question if the constant activation of these genes in *coi1* roots is the reason for the tolerance, we infected *coi1 sard1 cbp60g* plants. We have already shown that in these roots target gene activity is reduced to WT levels again in the *coi1* background (Figure 6b). *coi1* plants only showed 11%

loss of leaf area at 15 dpi, whereas WT plants showed much greater susceptibility to *V. longisporum* with 46% loss of leaf area (Figure 8). With 21%, the *coi1 sard1 cbp60g* plants only showed a slightly increased and non-significant loss of leaf area compared to *coi1* plants. Hence, the upregulation of the group of 149 SARD1/CBP60g–regulated defence genes in *coi1* roots has only a very slight effect towards tolerance and is not the overall reason for the tolerance against *V. longisporum*.

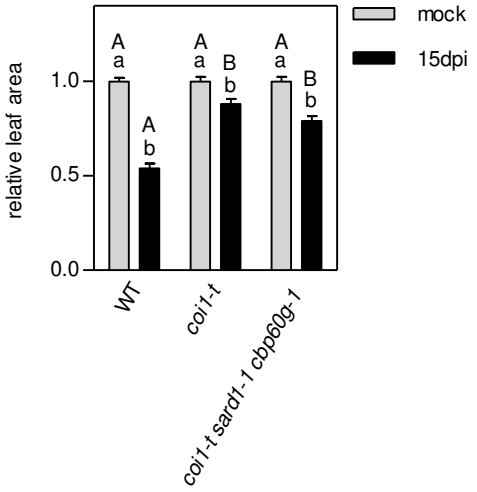

Figure 8. The SARD1/CBP60g-controlled genes upregulated in *coi1* roots are not responsible for the tolerance of *coi1* plants against infection with *V. longisporum*.
Relative leaf area of plants 15 days after mock treatment or infection with $5x10^5$ spores/mL sGFP-expressing *V. longisporum*. Bars are means ± SEM of a total of 47-48 plants per treatment from three independent experiments with 15-16 plants per treatment each. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 15 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$).

Discussion

In this manuscript, we report the identification of genes that are differentially expressed in roots of *V. longisporum*-infected Arabidopsis plants at 10 dpi. Induction of the large majority of genes did not require biosynthesis of the major defence hormones SA and JA. Apart from genes associated with cell wall remodelling, a set of genes associated with the SA-dependent shoot

defence response systemic acquired resistance were induced even in the SA biosynthesis mutant *sid2*. These genes were already up-regulated in mock-treated *coi1* plants, suggesting that inactivation of COI1 by a yet unknown signal might be responsible for their SA-independent induction.

Induction of the majority of *Verticillium longisporum*-induced genes is independent of *de novo* synthesis of jasmonic acid or salicylic acid

Transcriptome analysis of *Verticillium longisporum*-infected Arabidopsis roots has been performed before with axenically grown seedlings (Iven *et al.*, 2012; Ulrich *et al.*, 2021). In our hands, *V. longisporum* only entered the xylem when seedlings were cultured for one day without any nutrients. Under these conditions, we did not observe any changes at the transcriptional level at 4 dpi. In contrast, *Verticillium*-induced gene expression was observed when seedlings continued to be cultivated on MS plates (Iven *et al.*, 2012). Under these conditions, the fungus was able to colonize the cortex, but entry into the xylem was not documented. Here, we harvested tissue from plants that had been cultivated on soil for 10 dpi. At this stage, microscopical data is hampered by strong autofluorescence of the root system. We detected GFP-tagged fungal hyphae only occasionally in a few roots (Ralhan *et al.*, 2012). In contrast to the infection system on MS plates, the root surface was not covered with fungal mycelium. In WT plants segregating from the sterile *aos* and *coi1* plants, we observed that 881 and 948 genes were induced, respectively. Although both wild-types should react in the same manner to *V. longisporum*, only 772 genes were induced in both lines. Most of the genes that do not fall into this group barely missed the threshold in one of the wild-types. GO term enrichment analysis unravelled preferential up-regulation of cell wall remodelling genes, with *VND7* coding for a master transcriptional regulator of *de novo* xylem formation being strongly induced. In contrast, Iven *et al.* (2012) had observed enrichment of genes involved in tryptophane biosynthesis and tryptophane-derived secondary metabolism. Marker genes of this pathway like *CYP79b2* and *CYP79b3* were not induced under our conditions. Since these genes were induced even at 8 dpi in the axenic infection system (Iven *et al.*, 2012), we do not

think that the difference in the time point of analysis is responsible for distinct gene expression patterns. We rather imagine that growth conditions and/or localisation of the fungus within or around the root affect the outcome of the transcriptional response. The response of *aos* and *sid2* were to the same degree different as the two wild-types indicating that increased levels of SA and JA do not play a major role for the induction. This notion was already evident from the principal component analysis and is supported by the fact, that key biosynthesis enzymes of the pathways like *ICS1*, *AOS* or *OPDA REDUCTASE 3* (*OPR3)* were not induced.

A portion of *Verticillium*-inducible genes is constitutively de-repressed in *coi1*

Principal component analysis revealed that the *coi1* transcriptome showed the largest difference to the transcriptomes of the other genotypes, both in the mock-treated and the fungal-infected samples. This difference is mainly due to the constitutive up-regulation of 316 genes in *coi1* roots. This repressive effect of COI1, which is only observed in roots and not in shoots, has been detected before under axenic growth conditions (Ulrich *et al.*, 2021).

Roughly half of these genes are induced upon infection of soil-grown plants with *V. longisporum*. Notably, constitutive expression of these genes in *coi1* is not further enhanced by *V. longisporum*. This raises the hypothesis that – with regard to transcriptional activation of these genes – infection cannot be sensed without COI1. A possible scenario is that the repressive function of COI1 is inactivated upon infection. This postulated inactivation is almost as efficient as the genetic inactivation, suggesting that it occurs systemically and not only in a few locally infected cells. We currently have no information on the spatial distribution of root gene activation after *V. longisporum* infection, but it is unlikely that all COI1-expressing cells are in direct contact with the fungus. Therefore, we favour the idea that *V. longisporum* infection leads to systemic inactivation of at least those genes that are suppressed by COI1.

To explain systemic effects in the root system, we have to postulate a signal generated in locally infected roots that travels to the shoot, where a second signal moving back to the root system is generated (Figure 9). Primary signals travelling from the root to the shoot might either

be synthesized by the fungus or the plant. It is known that plant-derived small peptides can be transported to the shoot where they can diffuse into the phloem. Their perception leads to the generation of other signalling molecules that are transported down to all parts of the root via the phloem. Examples for this mechanism have been described before in the context of nitrogen starvation, where a small peptide (CEP) is generated in N-starved roots (Tabata *et al.*, 2014). This peptide is recognized by a receptor (CEPR) in the shoot, leading to the synthesis of a glutaredoxin-like small protein (CEPD) which promotes gene expression leading to increased nitrate uptake (Tabata *et al.*, 2014; Ohkubo *et al.*, 2017). Indeed, expression of the 79 amino acid-long peptide *CLE1* is highly induced upon infection in all five genotypes. Alternatively, root volatiles or small RNAs might serve as mobile signals (Godard *et al.*, 2008; Tsikou *et al.*, 2018; Okuma *et al.*, 2020).

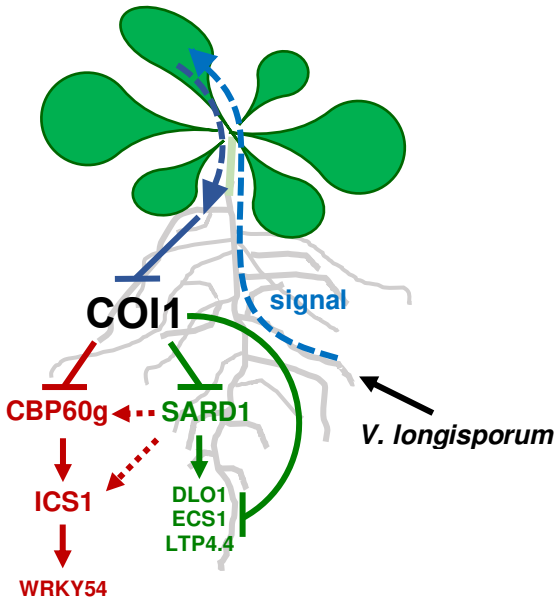

Figure 9. After *V. longisporum* infection, inactivation of COI1 leads to activation of the SARD1-branch but not the CBP60g-branch of downstream defence gene activation.
Model where *V. longisporum* infection generates a root to shoot signal (light blue) that leads to the systemic inactivation of COI1's repressive function in roots (dark blue). Inactivation of COI1 leads to activation of SARD1 and its downstream targets (green pathway) but inactivation of COI1 after *V. longisporum* infection is not sufficient to induce the CBP60g-regulated *ICS1*-branch (red pathway). COI1 also has a direct repressive effect on SARD1-target genes like *DLO1*, *ECS1* and *LTP4.4* which is lifted after infection (green pathway).

SARD1/CBP60g are the master regulators of COI1-supressed genes

Motif mapper analysis identified the 'GAAATTT' motif as being enriched particularly in the group of promoters that is repressed by COI1 and induced by *V. longisporum*. The 'GAAATTT' motif is recognised by the partially redundant transcription factors CBP60g and SARD1. In shoots, these proteins are required for activation of SA production after pathogen infection, for the activation of SA-dependent signalling and for the activation of SA-independent immune responses (Wang *et al.*, 2009, 2011; Zhang *et al.*, 2010; Sun *et al.*, 2015). Consistent with the GO term-based findings that our COI1-repressed genes are functionally connected to SA-related defence responses and consistent with repression of *SARD1/CBP60g* transcription by COI1, we hypothesise that SARD1/CBP60g might play an important role for the expression of COI1-repressed genes. By analysis of the *coi1 sard1 cbp60g* triple mutant, we found that genes upregulated in *coi1* (*LTP4.4*, *ECS1*, *DLO1*, *ICS1*, *WRKY46* and *WRKY54*) indeed require SARD1 (Figures 6a and S8a). At least in shoots, direct *in vivo* binding of SARD1 to the promoter regions of *LTP4.4*, *ECS1* and *ICS1* has been demonstrated by chromatin immunoprecipitation experiments (Sun *et al.*, 2015). *LTP4.4* and *ECS1* are de-repressed in the *coi1 sid2* mutant, supporting the idea that they are directly regulated by SARD1 and not indirectly through SARD1-mediated activation of SA synthesis (Figure S5c). The expression pattern of *WRKY46* was similar to *LTP4.4* and *ECS1* in *coi1 sid2* and *coi1 sard1 cbp60g*, even though it was not in the group of 149 genes but in the 167 genes not induced after infection (Figures S8a and S8b). Expression in *coi1 sard1 cbp60g* roots shows that it is not as strongly dependent on SARD1 as the others. As the group of the 167 genes contains all those that are not significantly induced at 10 dpi under control of COI1, it might also contain more SARD1-dependent genes that have missed the set threshold for significant induction in our data analysis. The upstream regions of *DLO1* and *WRKY54* are not bound by SARD1 in shoots. The expression of these genes is reduced in *coi1 sid2*, indicating that they are predominantly regulated by SARD1-activated SA synthesis. However, there is also at least one exception, namely *AT5G39120*, which is activated through a different mechanism.

Our loss of function analysis suggests that enhanced expression of *SARD1* in *coi1* might be the reason for enhanced expression of downstream genes. However, constitutive expression of SARD1 in Col-0 background did not activate downstream genes to the same extent as enhanced expression of *SARD1* in *coi1*. This is different from the situation in shoots, where SARD1 can have a clear activating effect even in the absence of ICS1-derived SA. It can be envisioned that COI1 interferes with SARD1 activity at downstream genes or that – in roots – SARD1 action requires additional proteins that are de-repressed in *coi1*.

ICS1 is not efficiently induced after *Verticillium longisporum* infection

In shoots, SARD1 binds to the promoters of the two SA biosynthesis enzymes *ICS1* and *PBS3*, resulting in their transcriptional activation and subsequent SA synthesis (Sun *et al.*, 2015). In *coi1* roots, *ICS1* and *PBS3* expression is also enhanced in a manner that requires SARD1 (shown for *ICS1*) leading to the activation of SA-inducible genes like *WKRY54*. However, upon *V. longisporum* infection, only *PBS3*, but not *ICS1,* is induced (Table 1 and subtables). Our first idea was that *V. longisporum* might encode a specific effector that interferes with *ICS1* expression. However, since we consider systemic induction as being likely, we propose that other mechanisms might be responsible for the divergent regulation of *ICS1* and *PBS3*. In shoots, *ICS1*, *PBS3*, *SARD1* and *CBP60g* are highly co-expressed. In roots, we observe a divergent expression pattern with *PBS3* and *SARD1* being up-regulated in *coi1* and being induced by *V. longisporum.* In contrast, *CBP60g* and *ICS1* are up-regulated in *coi1* but not induced by *V. longisporum*. Transcription of *CBP60g* is less than 2-fold enhanced in *coi1*. Assuming a more prominent role for CBP60g in inducing *ICS1*, it can be envisioned that inactivation of COI1 by *V. longisporum* might not yield sufficient amounts of CBP60g to support expression of *ICS1* (Figure 9). Another possible scenario would be that COI1 is only inactivated at specific promoters or that is universally inactivated but other repressors take over on specific promoters as a second line of regulation.

When first reporting the repressive function of COI1 on SA-related genes in roots, we speculated that inappropriate up-regulation of this pathway might interfere with the composition

of the microbiome in the rhizosphere, making an extra layer of repression necessary. Indeed, it has been shown that beneficials colonise roots faster and better when root SA-defences are blocked or impaired (Herrera Medina *et al.*, 2003; Martínez-Medina *et al.*, 2017). Likewise, elevated SA levels delay and reduce colonisation by beneficials (Martínez-Abarca *et al.*, 1998; Herrera Medina *et al.*, 2003; Martínez-Medina *et al.*, 2017). COI1-mediated gene repression might serve as a safety stop to allow the onset of a strong defence response only when necessary. To lift this repression after colonisation of the root with a pathogen, a novel induction mechanism had to be developed. Our findings that this is not relevant for the interaction with *V. longisporum* does not rule out the option that this programme might be efficient against other biotrophic root pathogens.

Materials and Methods

Plant Material

All plants used in this article are *Arabidopsis thaliana* Col-0 background. Genotypes used in the study, corresponding references and sources are: *aos* (SALK_017756) from Nottingham Arabidopsis Stock Centre (NASC); *coi1-1* (Xie *et al.*, 1998) from John Turner (University of East Anglia, Norwich, UK); *coi1-t* (SALK_035548) (Mosblech *et al.*, 2011) from Ingo Heilmann (Martin-Luther-University, Halle, Germany); *coi1-1 sid2-2* (Ulrich *et al.*, 2021); *sard1-1 cbp60g-1* (Zhang *et al.*, 2010) from Yuelin Zhang (UBC Vancouver, Canada); *sid2-2* (Wildermuth *et al.*, 2001) from Frederick M. Ausubel (Harvard University, Boston, USA). The c*oi1-t sard1-1 cbp60g-1* triple mutant was generated through crossing of the respective above-mentioned genotypes. Primers for genotyping are listed in Table S1.

Plant Growth Conditions and Treatments

Surface sterilised seeds were sown onto Murashige-Skoog-medium (MS) supplemented with 2% Sucrose and kept at 4°C for 24-72 hours in darkness. Plates were placed horizontally into

growth chambers with short day conditions (8-h-day/16-h-night cycle, 22°C/22°C, 60% humidity) with a photon flux density of 80-100 µmol m^{-2} s^{-1}. After 14 days plants were carefully transferred onto a 1:1 mix of sand (white, 1-2 mm grain size, Rosnerski, Königslutter, Germany) and twice steamed soil (Fruhstorfer Erde, Spezial Substrat, Typ T, Str. 1 fein, HAWITA, Vechta, Germany) on a thin layer of Seramis (Westland Deutschland, Mogendorf, Germany). The mixture was initially watered with 0.1% Wuxal Super (Manna, Ammerbuch-Pfäffingen, Germany) in dH_2O. Plants were grown on the sand-soil mixture for another 14 days under above-mentioned short-day conditions with increased photon flux density of 120-140 µmol m^{-2} s^{-1}. For the first seven days plants were covered with a transparent hood, on day seven the hood was opened and on day nine it was fully removed. If genotyping was required, a single leaf was clipped from each plant during the first week of growth on the sand-soil mixture. Subsequently, plants were carefully uprooted from the sand-soil mixture and their roots washed in tap water. Roots were then dipped in tap water as mock treatment or *V. longisporum* spore suspension for 45 minutes. Afterwards plants were planted into individual pots containing twice steamed soil (Fruhstorfer Erde, Spezial Substrat, Typ T, Str. 1 fein, HAWITA, Vechta, Germany) soaked with 0.2% Wuxal Super, where plants were kept for a final 10 to 21 days in short day conditions at 120-140 µmol photons m^{-2} s^{-1}. During the first two days on soil pots, plants were kept under transparent hoods. A rootstock or shoot of one single plant was harvested for one biological replicate if not otherwise specified.

For nlp14 infiltration in Figure 5b plants were grown in single pots for four and a half weeks in 12-h-day/12-h-night cycle, 22°C/22°C, 60% humidity. nlp14 (GVYAIMYSWYFPKD; GenScript, Leiden, Netherlands) was solved in 100% DMSO and aliquots were stored at 10mM in -70°C. Using a needleless syringe, three leaves of similar age from each plant were infiltrated with 1µM nlp14 in Millipore H_20 with 0.01% DMSO or just with Millipore H_20 with 0.01% DMSO as mock treatment and harvested after 24h.

For root treatment with 1mM SA (Figure S6), plants were grown as specified above. After growth on sand-soil mixture, plants were carefully uprooted and their roots dipped into tap

water for mock treatment or 1mM sodium salicylate solution (Sigma-Aldrich/Merck KGaA, Darmstadt, Germany) for 24 hours.

For phenotypic analysis of plants in Figure 8, heterozygous seed batches of c*oi1-t* and c*oi1-t sard1-1 cbp60g-1* (homozygous for *sard1-1 cbp60g-1* but heterozygous for c*oi1-t*) were initially placed on MS medium supplemented with 2% Sucrose and 50µM methyl jasmonate to identify plants homozygous for the *coi1-t* mutation (Feys *et al.*, 1994). Consecutive treatment was the same as described above.

Fugal culture and inoculation

Verticillium longisporum isolate *Vl43* (Zeise and Von Tiedemann, 2002) provided by Daguang Cai (Christian-Albrechts-University, Kiel, Germany) and *Verticillium longisporum Vl43* sGFP (Eynck *et al.*, 2007) provided by Andreas von Tiedemann (Georg-August University, Goettingen, Germany) conidia stocks were stored in 21.5% glycerol at -70°C. For preparation of conidia batches for plant inoculation, stock conidia from glycerol were cultivated in liquid simulated xylem medium (SXM) (Hollensteiner *et al.*, 2017), supplemented with 275mg/L Cefotaxim, for 7 days in a rotary shaker at 23°C and 90rpm. Conidia were harvested by filtering through a fluted filter (Nucleo Bond folded filters, Macherey-Nagel, Düren, Germany), washed in sterile tap water and their concentration determined with a hemocytometer. Glycerol was added to a final concentration of 21.5%. The conidia infection stocks were initially stored in -20°C for 5 days and subsequently stored at -70°C until the day of inoculation. On inoculation day conidia stocks were thawed, centrifuged for 8 mins at 8000rpm and resuspended in tap water to a final concentration of 5×10^5 or 1×10^6 spores/mL for plant inoculation.

Leaf Area Measurement

For disease phenotype analysis, photographs of individual plants were taken at 15 or 21 dpi. The surface area of the whole rosette was determined with the 'BlattFlaeche' Software (Datinf GmbH, Tübingen, Germany) (Ralhan *et al.*, 2012).

RNA-seq Analysis

coi1-t plants are male sterile whereas fertility of the *aos* mutant can be rescued by methyl jasmonate (MeJA) treatment. As we wanted to avoid differences in the history of the seed batches by rescuing *aos* mutants with MeJA treatment, we again used heterozygous populations of *coi1-t* and *aos* plants that were genotyped with primers specified in Table S1 (Ulrich *et al.*, 2021). For RNA-Seq analysis, twelve single homozygous roots of either *aos,* WT_{aos}, *coi1-t,* $WT_{coi1\text{-}t}$ or *sid2-2* were combined for one replicate; replicates per genotype and treatment were obtained from four independent infection experiments. RNA was extracted using the Trizol method (Chomczynski and Mackey, 1995) and RNA quality was controlled with an AGILENT BIOANALYZER 2100. Single-end 50-bp raw reads from mRNA sequencing were generated with the Illumina HiSeq 2000 platform and sequence images were transformed with the Illumina BaseCaller software to BCL files, which were subsequently demultiplexed to FASTQ files with CASAVA (v1.8.2). Using a Galaxy platform (Afgan et al., 2018), mapping of reads to the *Arabidopsis thaliana* genome reference sequence (TAIR10 release-39, ftp://ftp.ensemblgenomes.org/pub/plants/release-39) was carried out with RNA STAR (Galaxy version 2.5.2b-2 (Afgan *et al.*, 2018) and aligned reads were quantified using HTSeq-count (Galaxy version 0.9.1 (Afgan *et al.*, 2018)). Normalization and differential expression analysis was performed with DESeq2 (Galaxy version 2.11.40.6+galaxy1 (Love *et al.*, 2014)) to obtain log2-fold changes and adjusted *p* values (Benjamini-Hochberg-corrected).

For Gene Ontology (GO) term enrichment analysis, the agriGO v2.0 program was used (Tian *et al.*, 2017). Categories > 5-fold enrichment against the Arabidopsis genome are shown in Figures 2, S2 and S3.

Quantitative Reverse Transcription (qRT)-PCR

RNA extraction, cDNA synthesis and qRT-PCR were performed as previously described (Ulrich *et al.*, 2021). Calculations were done according to the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001) using the *UBQ5* (AT3G62250) or *PP2*A (At1G13320) transcripts as a reference. Primers used for qRT-PCR are listed in Table S2.

Statistical Analysis

GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) was used to conduct statistical analysis.

Motif Mapper Analysis

Analysis of *cis* element enrichment was done using the Cluster Analysis Real Randomization algorithm incorporated into the Motif Mapper Version 5.2.4.0 (Berendzen *et al.*, 2012). By comparison to 1000 randomly composed, equally sized, reference promoter datasets, significant distribution alterations were defined as described in (Zander *et al.*, 2014).

Generation of Transgenic Plants and Western Blot Analysis

Recombinant SARD1 plasmids were created via GATEWAY cloning (Invitrogen, Karlsruhe, Germany). The genomic sequence of *SARD1* was amplified from the annotated transcriptional start site to the last amino acid of the coding region, using primers SARD1GWfwd and SARD1noStopGWrev (Table S3). The primers added GATEWAY recombination sites to the PCR product, which were used to introduce it into pDONR207. From there, *SARD1* was further introduced into pUBQ10GW3HAstrepII7 (Budimir *et al.*, 2021) adding a three times HA and StrepII C-terminal tag. The final plasmid pUBQ10-SARD1-3HAstrepII7 was introduced into Col-0 and *sid2-2* plants via *Agrobacterium tumefaciens*-mediated gene transfer (Clough and Bent, 1998). As empty vector (EV) controls the original plasmid pUBQ10GW3HAstrepII7 was

transformed into plants. Transgenic plants were characterised via BASTA (Bayer CropScience AG, Monheim, Germany) selection and Western Blot analysis was used to assess SARD1-3xHA-StrepII protein levels in homozygous plants.

For Western Blot analysis, 250µL extraction buffer (4M urea, 16.6% glycerol, 5% SDS, 5% β-mercaptoethanol) was added per 100mg ground root or shoot material. The Pierce 660nm assay kit (Thermo Scientific, Rockford, IL USA) was used to determine protein concentrations of extracts. 50µg of root or shoot protein were loaded and separated on a 10% SDS gel. Transfer of proteins to a polyvinylidene difluoride membrane was done via semi-dry electroblotting. Proteins were detected using an αHA-antibody (Abcam, Cambridge, United Kingdom) and Super SignalTM West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL 1606, USA).

SA Measurements

Measurements of salicylic acid in root material were kindly carried out by Krzysztof Zienkiewicz at the Department of Biochemistry, Albrecht-von-Haller Institute for Plant Sciences, Georg-August University, Göttingen, Germany. Nanoelectrospray (nanoESI) analysis was carried out as previously described (Kusch *et al.*, 2019). After reversed phase separation of constituents by an ACQUITY UPLC® system (Waters Corp., Milford, MA, USA) equipped with an ACQUITY UPLC® HSS T3 column (100mm x 1mm, 1.8µm; Waters Corp., Milford, MA, USA), phytohormones were ionized in a negative mode and determined in a scheduled multiple reaction monitoring mode with an AB Sciex 4000 QTRAP® tandem mass spectrometer (AB Sciex, Framingham, MA, USA). Mass transitions were as described previously (Iven *et al.*, 2012).

Accession numbers

Sequence data from this article can be found in The Arabidopsis Information Resource (http://www.arabidopsis.org/) under the following accession numbers: *ANAC076* (AT4G36160), *AOS* (AT5G42650), *CBP60G* (AT5G26920), *COI1* (AT2G39940), *DLO1* (AT4G10500), *ECS1* (AT1G31580), *ERF54* (AT4G28140), *ICS1* (AT1G74710), *LTP4.4* (AT5G55450), *PP2A* (*At1g13320*), RmlC-like cupins superfamily protein (AT5G39120), *SARD1* (AT1G73805), *UBQ5* (AT3G62250), *UBQ10* (AT4G05320), *WRKY46* (AT2G46400), *WRKY54* (AT2G40750).

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Author contributions

L.U. and C.G. wrote the manuscript; L.U. designed the experiments, acquired and analysed most of the data; C.G. designed and supervised the research; J.S. performed experiments for the RNA-seq; C.T. designed and supervised the research and analysed the RNA-seq data.

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Supplementary Information

Table S1. Primers for Genotyping

Table S2. Primers for qRT-PCR

Table S3. Primers for Cloning

Figure S1. Gene induction patterns are largely overlapping in *aos,* WT_{aos}, $WT_{coi1\text{-}t}$, and *sid2-2* at 10 dpi.

Figure S2. Gene Ontology (GO) term enrichment analysis of the 772 genes significantly induced in WT_{aos} and $WT_{coi1\text{-}t}$ at 10 dpi (> 2-fold; $p < 0.05$).

Figure S3. Gene Ontology (GO) term enrichment analysis of (a) the 167 genes upregulated in mock-treated *coi1-t* compared to mock-treated *aos,* WT_{aos}, $WT_{coi1\text{-}t}$ *and sid2-2* (> 2-fold; $p < 0.05$) and not significantly induced in *aos*, WT_{aos}, $WT_{coi1\text{-}t}$ *and sid2-2* at 10 dpi and (b) the 512 genes induced in *aos*, WT_{aos}, $WT_{coi1\text{-}t}$ *and sid2-2* at 10 dpi (> 2-fold; $p < 0.05$) but not upregulated in mock-treated *coi1-t* compared to mock-treated *aos,* WT_{aos}, $WT_{coi1\text{-}t}$ *and sid2-2*.

Figure S4. Infection with *V. longisporum* does not lead to accumulation of SA in roots.

Figure S5. In *coi1* roots, basally elevated marker gene expression is independent of elevated SA levels in most cases.

Figure S6. Genes of interest can mostly be induced by SA in roots.

Figure S7. *CBP60g* is weakly de-repressed in *coi1* roots and not induced after infection in WT_{aos}, $WT_{coi1\text{-}t}$ and *aos*.

Figure S8. SARD1/CBP60g-dependency of gene expression in the two groups of COI1-suppressed but not significantly induced genes at 10 dpi and the genes induced after infection but not under control of COI1.

Figure S9. SARD1 overexpression leads to mild growth defects in Col-0 plants.

Figure S10. *WRKY54* and *WRKY46* induction is stronger in shoots overexpressing SARD1 compared to roots.

Figure S11. Mutations in SARD1 and CBP60g do not affect the disease phenotype after *V. longisporum* infection.

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Figure Legends

Table 1. RNA-seq gene expression data from *coi1-t*, *aos*, WT_{aos}, WT_{coi1-t} and *sid2-2* roots 10 days after mock treatment or incoculation with $1x10^6$ spores/mL sGFP-expressing *V. longisporum.*

Table 1 will be desposited at the department of Plant Molecular Biology and Physiology, Georg-August University Göttingen, until the manuscript is published. For access, please contact Prof. C. Gatz (cgatz@gwdg.de).

Figure 1. A group of 149 genes is de-repressed in *coi1-t* roots and induced in *aos,* WT_{aos}, WT_{coi1-t}, and *sid2-2* at 10 dpi.

(a) Principal component analysis of the normalised root transcriptome data acquired by RNA-seq analysis 10 days after mock treatment or inoculation with $1x10^6$ spores/mL sGFP-expressing *V. longisporum*. Biological replicates from four independent experiments are symbolised by circles (mock) or plus signs (10 dpi). For WT_{coi1-t} only three replicates were analysed for both mock and 10 dpi treatments. WT_{aos} and WT_{coi1-t} are the wild-types obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds. **(b)** Venn diagram showing the overlap between 316 genes constitutively upregulated in mock-treated coi1-t roots vs mock-treated aos, WT_{aos}, WT_{coi1-t} and *sid2-2* (> 2-fold, $p < 0.05$) and 661 genes induced in *aos*, WT_{aos}, WT_{coi1-t} and *sid2-2* at 10 dpi (> 2-fold, $p < 0.05$). Expression data was obtained by RNA-seq analysis from root material 10 days after mock treatment or inoculation with $1x10^6$ spores/mL sGFP-expressing *V. longisporum*. Circles are drawn to scale with respect to the number of genes represented in each group.

Figure 2. The 149 genes de-repressed in *coi1* and induced after infection are related to defence responses.

Gene Ontology (GO) term enrichment analysis of the 149 genes basally upregulated in *coi1-t* compared to *aos,* WT_{aos}, WT_{coi1-t} *and sid2-2* (> 2-fold; $p < 0.05$) and induced in *aos*, WT_{aos},

$WT_{coi1\text{-}t}$ *and sid2-2* at 10 dpi (> 2-fold, $p < 0.05$). Bars represent the fold enrichment of the number of genes found per GO term in the group of 149 genes against the number of genes found within the Arabidopsis genome associated with that GO term. Only GO terms with > 5-fold enrichment against the genome are shown. SA defence related GO terms are underlined in red.

Figure 3. The binding motif for SARD1 and CBP60g 'GAAATTT' is significantly enriched in the 149 defence genes of interest.

Motif Mapper cis-element analysis of **(a)** the 316 genes de-repressed in *coi1-t* roots (*coi1-t* mock > *aos*, WT_{aos}, $WT_{coi1\text{-}t}$, *sid2-2* mock; 2-fold; $p < 0.05$), the 661 genes induced after infection (*aos*, WT_{aos}, $WT_{coi1\text{-}t}$, *sid2-2* 10 dpi > *aos,* WT_{aos}, $WT_{coi1\text{-}t}$, *sid2-2* mock; 2-fold; $p < 0.05$), the 149 genes de-repressed in *coi1-t* and induced after infection, the 167 genes de-repressed in *coi1-t* and not induced after infection and the 512 genes induced after infection but not de-repressed in *coi1-t* **(b)** the 149 genes de-repressed in *coi1-t* and induced after infection and the 167 genes de-repressed in *coi1-t* and not induced after infection against the background of the set of all 316 genes de-repressed in *coi1-t* roots **(c)** the 149 genes de-repressed in *coi1-t* and induced after infection and the 512 genes induced after infection and not de-repressed in *coi1-t* roots against the background of the set of all 661 genes induced after infection. Numbers before slashes show the total number of detected motifs in the set of interest. Numbers behind the slashes show the number of expected motif counts in a set of randomly chosen promoters within the indicated background set. Significant enrichment/depletion is defined at $p < 0.05$. **(d)** Relative expression of *SARD1, DLO1, LTP4.4* and *ECS1* transcript levels as quantified by RNA-seq analysis 10 days after mock treatment or inoculation with $1x10^6$ spores/mL sGFP-expressing *V. longisporum*. Bars are means of Transcripts Per Million (TPM) ± SEM of three to four biological replicates of each genotype, with each replicate representing twelve roots from one independent experiment. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10

dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). WT_{aos} and $WT_{coi1\text{-}t}$ are the two wild-types lines obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds.

Figure 4. Unlike in roots, COI1 is not a constitutive repressor of target genes in shoots.

SARD1, LTP4.4, DLO1 and *ECS1* transcript levels, measured by qRT-PCR. RNA was extracted from shoots 10 days after mock treatment from the same plants whose roots were subjected to the RNA-seq. Bars are means ± SEM of four replicates, each made up twelve shoots per genotype. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$). WT_{aos} and $WT_{coi1\text{-}t}$ are the two wild-types lines obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds.

Figure 5. Genes of interest are induced independently of increased SA levels and NPR1 in roots.

(a) *SARD1, LTP4.4, DLO1* and *ECS1* transcript levels, measured by qRT-PCR. RNA was extracted from roots 10 days after mock treatment or infection with $1x10^6$ spores/mL *V. longisporum*. Bars are means ± SEM of thirteen to sixteen roots per genotype. For *ECS1* transcript levels in *npr1-1* only six replicates are shown. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). **(b)** *SARD1, LTP4.4, DLO1* and *ECS1* transcript levels, measured by qRT-PCR. RNA was extracted from leaves 24 hours after infiltration with 1µM nlp14. Bars are means ± SEM of four to six replicates per genotype. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 1µM nlp14 ($p < 0.05$),

uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$).

Figure 6. SARD1/CBP60g are responsible for induction of genes of interest after infection with *V. longisporum* and for basally upregulated gene expression in *coi1*.

(a) *LTP4.4, DLO1, ECS1* and *ICS1* transcript levels, measured by qRT-PCR. RNA was extracted from roots 10 days after mock treatment or infection with $1x10^6$ spores/mL *V. longisporum*. Bars are means ± SEM of thirteen to sixteen roots per genotype. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). **(b)** *LTP4.4, DLO1, ECS1* and *ICS1* transcript levels, measured by qRT-PCR. RNA was extracted from roots 10 days after mock treatment. Bars are means ± SEM of eight roots per genotype. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$).

Figure 7. Overexpression of SARD1 in roots does not lead to strong target gene activation.

SARD1, LTP4.4, DLO1, ECS1 and *ICS1* transcript levels, measured by qRT-PCR. RNA was extracted from **(a)** roots or **(b)** shoots 10 days after mock treatment of *SARD1* overexpression lines *(SARD1 OX)* and empty vector (EV) controls in both Col-0 (black bars) and *sid2-2* (gray bars) background. Bars are means ± SEM of three to six roots or shoots per line. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$).

Insert: Western blot of protein extracts obtained from roots and shoots of *SARD1* overexpression lines *(SARD1 OX)* and empty vector (EV) controls in Col-0 and *sid2-2* background. Per lane, six roots or three shoots were pooled from each line. C-terminally 3xHA-

StrepII tagged SARD1 protein levels were detected using an anti-HA antibody. * depicts an unspecific band shown as loading control.

Figure 8. The SARD1/CBP60g-controlled genes upregulated in *coi1* roots are not responsible for the tolerance of *coi1* plants against infection with *V. longisporum*.

Relative leaf area of plants 15 days after mock treatment or infection with $5x10^5$ spores/mL sGFP-expressing *V. longisporum*. Bars are means ± SEM of a total of 47-48 plants per treatment from three independent experiments with 15-16 plants per treatment each. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 15 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$).

Figure 9. After *V. longisporum* infection, inactivation of COI1 leads to activation of the SARD1-branch but not the CBP60g-branch of downstream defence gene activation.

Model where *V. longisporum* infection generates a root to shoot signal (light blue) that leads to the systemic inactivation of COI1's repressive function in roots (dark blue). Inactivation of COI1 leads to activation of SARD1 and its downstream targets (green pathway) but inactivation of COI1 after *V. longisporum* infection is not sufficient to induce the CBP60g-regulated *ICS1*-branch (red pathway). COI1 also has a direct repressive effect on SARD1-target genes like *DLO1*, *ECS1* and *LTP4.4* which is lifted after infection (green pathway).

Table S1. Primers for Genotyping.

	Primer ID	Sequence 5'-3'
aos	aos-fwd	AATCGTAGGACCAATCAAAGACCG
	aos-rev	CAGATCCTTCTTCGCTCTACCGTA
cbp60g-1	SALK_023199_LP	TGGTTACAGTGTCTTTAGAGCTCG
	SALK_023199_RP	ATTCTCCTCGTTGGTCTCTACATC
	LBb1.3	ATTTTGCCGATTTCGGAAC
coi1-1	coi1-1 up	GTAATCGGAGATAGGGGTCTAGAGG
	coi1-1 low	TGTACCCACAAGTATCTCAGTGAAGG
		Subsequent digestion with *Mva*1296I
coi1-t	COI1gen-1936fwd	CATCTTCTGGCTTTTCTGAAACAGCTG
	COI1gen1115rev	CACCAATTTCATTAAGGACAAAAAGTATCCAC
	LBb1	GCGTGGACCGCTTGCTGCAACT
sard1-1	SALK_138476.15.35.x_LP	GAGCATTGATCTCAGAAAACACC
	SALK_138476.15.35.x_RP	ACACTTACTTCTCCGGCAAGTAAC
	LBb1.3	ATTTTGCCGATTTCGGAAC
sid2-2	sid2-2 fwd1	TTCTTCATGCAGGGGAGGAG
	sid2-2 fwd2	CAACCACCTGGTGCACCAGC
	sid2-2 rev	AAGCAAAATGTTTGAGTCAGCA

Table S2. Primers for qRT-PCR.

Primer ID	Sequence 5'-3'
ANAC076	QuantiTect QT00727076 (Qiagen)
ERF54	QuantiTect QT00816893 (Qiagen)
DLO1 RT fwd	AATATCGGCGACCAAATGC
DLO1 RT rev	CGCTCGTTCTCGGTGTTTAC
ECS1	QuantiTect QT00871619 (Qiagen)
ICS1	QuantiTect QT00893473 (Qiagen)
LTP4.4	QuantiTect QT00842660 (Qiagen)
PP2A RT fwd	AAGCAGCGTAATCGGTAGG
PP2A RT fev	GCACAGCAATCGGGTATAAAG
SARD1 RT fwd	TCAAGGCGTTGTGGTTTGTG
SARD1 RT rev	CGTCAACGACGGATAGTTTC
UBQ5 fwd RT	GACGCTTCATCTCGTCC
UBQ5 rev RT	GTAAACGTAGGTGAGTCCA
WRKY46 RT fwd	ACCTGCTGCTGTTGAGAATTCCG
WRKY46 RT rev	ACGACCACAACCAATCCTGTCC
WRKY54	QuantiTect QT00720846 (Qiagen)
AT5G39120	QuantiTect QT00734748 (Qiagen)

Table S3. Primers for Cloning.

Primer ID	Sequence 5'-3'
SARD1GWfwd	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAACAATGGCA GGGAAGAGGTTATTTCAAG
SARD1noStopGWrev	GGGGACCACTTTGTACAAGAAAGCTGGGTCGAAAGGGTTT ATATGATTTTGAGACGAAG

Figure S1

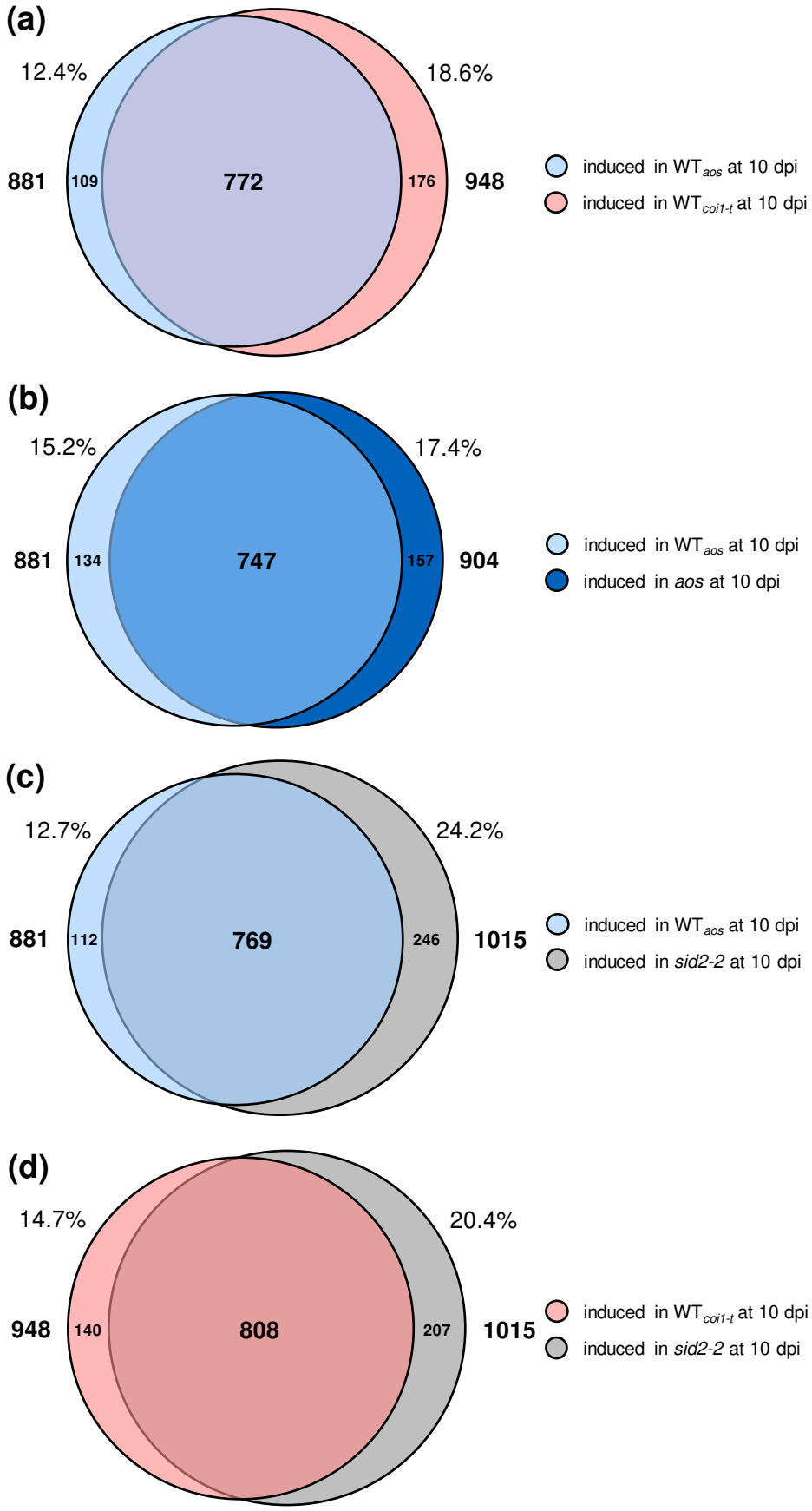

Figure S1. Gene induction patterns are largely overlapping in *aos*, WT_{aos}, $WT_{coi1\text{-}t}$, and *sid2-2* at 10dpi.

Full legend on next page.

Figure S1. Gene induction patterns are largely overlapping in *aos*, WT_{aos}, $WT_{coi1\text{-}t}$, and *sid2-2* at 10 dpi.

Venn diagrams showing **(a)** the overlap between genes induced in WT_{aos} and $WT_{coi1\text{-}t}$ at 10 dpi (> 2-fold, $p < 0.05$), **(b)** the overlap between genes induced in WT_{aos} and *aos* at 10 dpi (> 2-fold, $p < 0.05$), **(c)** the overlap between genes induced in WT_{aos} and *sid2-2* at 10 dpi (> 2-fold, $p < 0.05$), **(d)** the overlap between genes induced in $WT_{coi1\text{-}t}$ and *sid2-2* at 10 dpi (> 2-fold, $p < 0.05$). Expression data was obtained by RNA-seq analysis from root material 10 days after mock treatment or inoculation with $1x10^6$ spores/mL sGFP-expressing *V. longisporum*. Circles are drawn to scale with respect to the number of genes represented in each group. WT_{aos} and $W_{coi1\text{-}t}$ are the wild-types obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds.

Figure S2

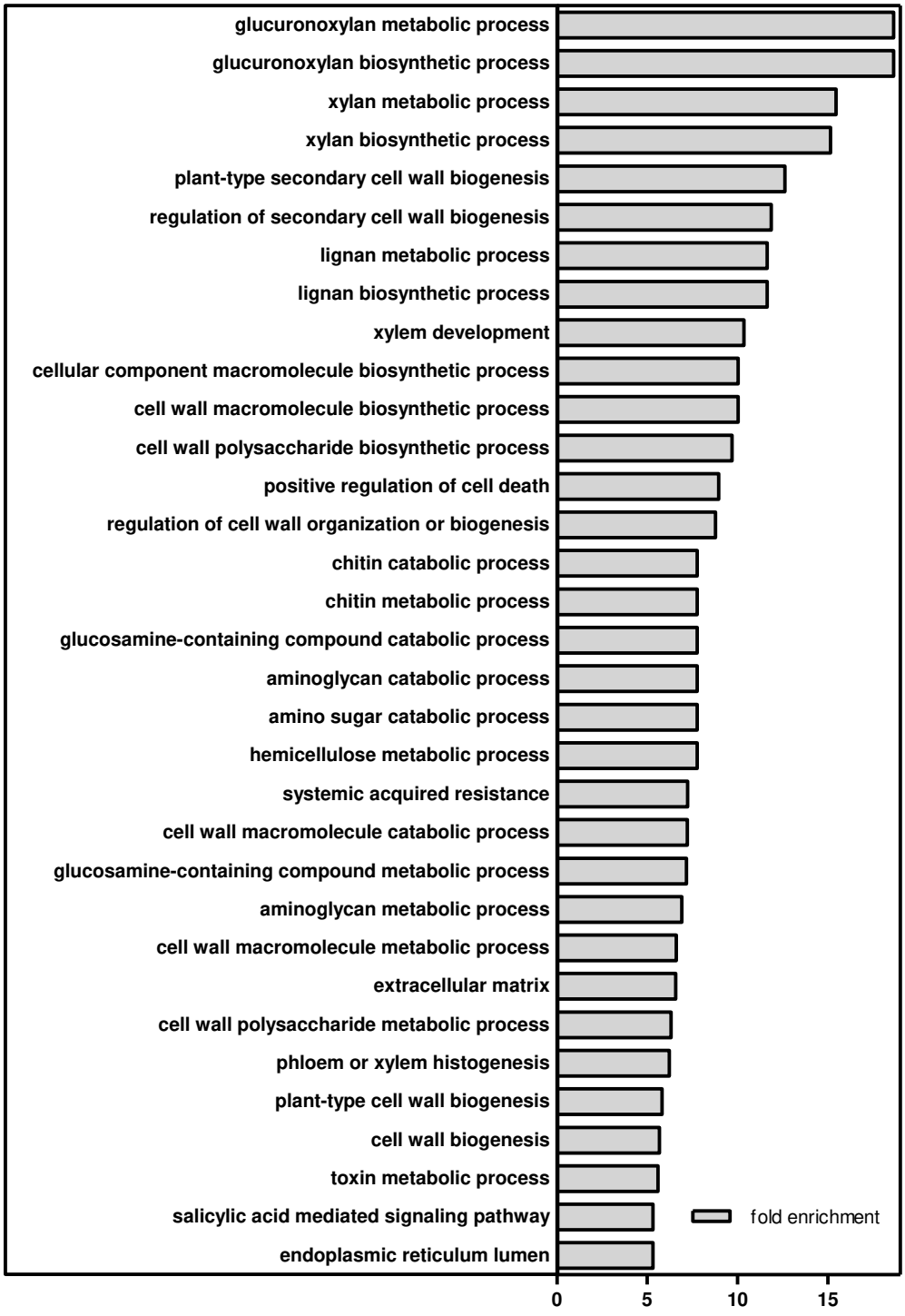

Figure S2. Gene Ontology (GO) term enrichment analysis of the 772 genes significantly induced in WT_{aos} and $WT_{coi1\text{-}t}$ at 10 dpi (> 2-fold; $p < 0.05$).

Bars represent fold enrichment of number of genes found per GO term in the group of 772 genes against the number of genes found within the Arabidopsis genome associated with that GO term. Only GO terms with > 5-fold enrichment against the genome are shown.

Figure S3

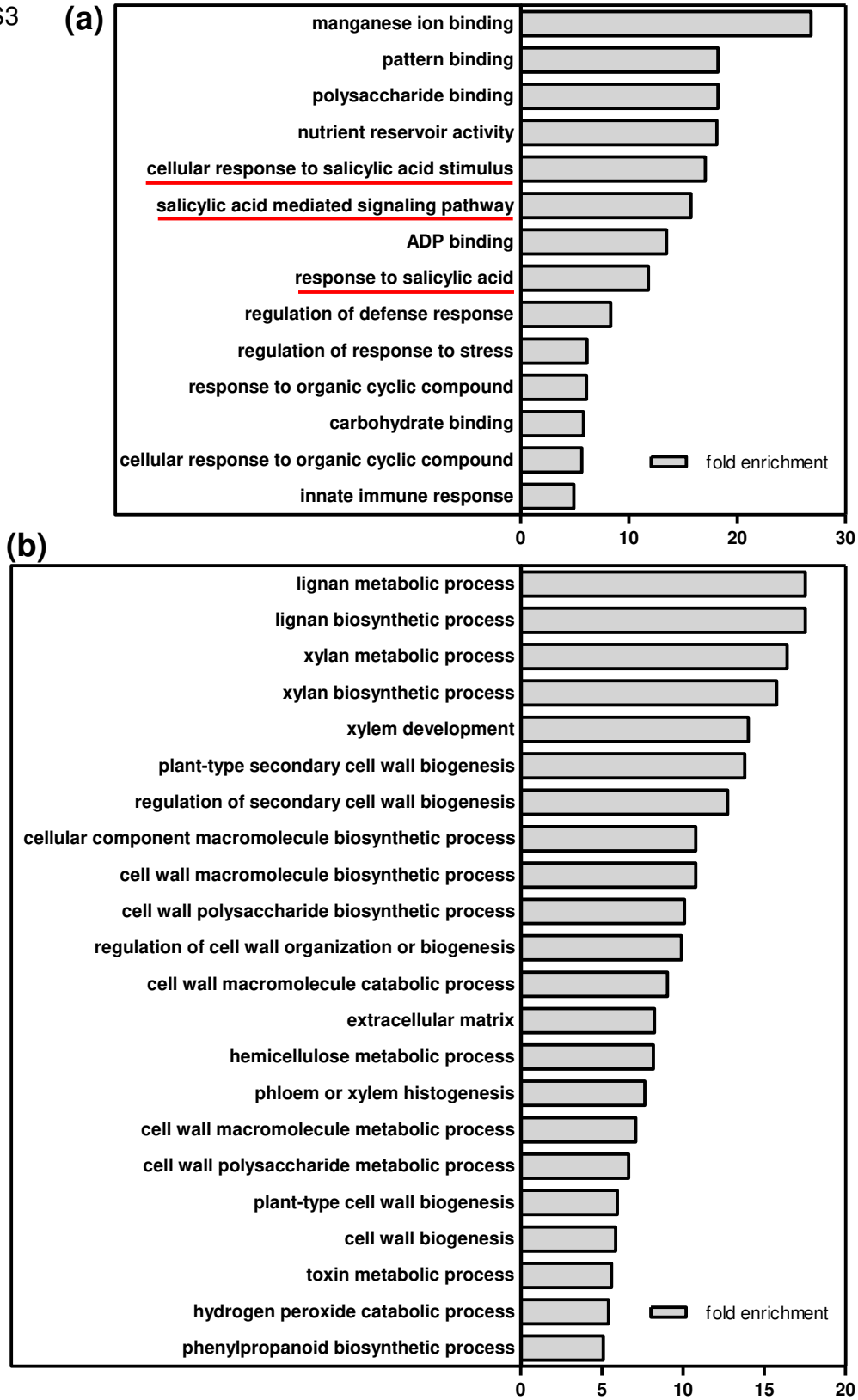

Figure S3. Gene Ontology (GO) term enrichment analysis of (a) the 167 genes basally upregulated in *coi1-t* compared to *aos*, WT_{aos}, WT_{coi1-t} *and sid2-2* (> 2-fold; $p < 0.05$) and not significantly induced in in *aos*, WT_{aos}, WT_{coi1-t} *and sid2-2* at 10 dpi and (b) the 512 genes induced in *aos*, WT_{aos}, WT_{coi1-t} *and sid2-2* at 10 dpi (> 2-fold; $p < 0.05$) but not basally upregulated in *coi1-t* compared to *aos*, WT_{aos}, WT_{coi1-t} *and sid2-2* (> 2-fold; $p < 0.05$).

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Figure S3. Gene Ontology (GO) term enrichment analysis of (a) the 167 genes upregulated in mock-treated *coi1-t* compared to mock-treated *aos,* WT_{aos}, WT_{coi1-t} *and sid2-2* (> 2-fold; $p < 0.05$) and not significantly induced in *aos*, WT_{aos}, WT_{coi1-t} *and sid2-2* at 10 dpi and (b) the 512 genes induced in *aos*, WT_{aos}, WT_{coi1-t} *and sid2-2* at 10 dpi (> 2-fold; $p < 0.05$) but not upregulated in mock-treated *coi1-t* compared to mock-treated *aos,* WT_{aos}, WT_{coi1-t} *and sid2-2*.
Bars represent fold enrichment of the number of genes found per GO term in the group of 167 or 512 genes against the number of genes found within the Arabidopsis genome associated with that GO term. Only GO terms with > 5-fold enrichment against the genome are shown. SA defence related GO term are underlined in red.

Figure S4

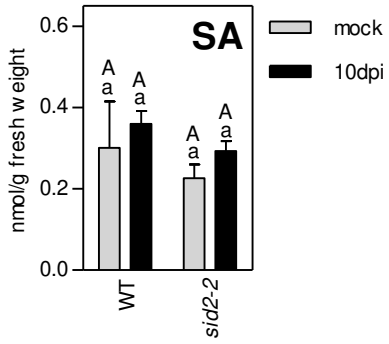

Figure S4. Infection with *V. longisporum* does not lead to accumulation of SA in roots.

SA levels in roots at 10 days after mock treatment or infection with $1x10^6$ spores/mL *V. longisporum*. Per sample eight to ten roots were pooled. Bars are means ± SEM of two samples per genotype. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$).

Figure S5

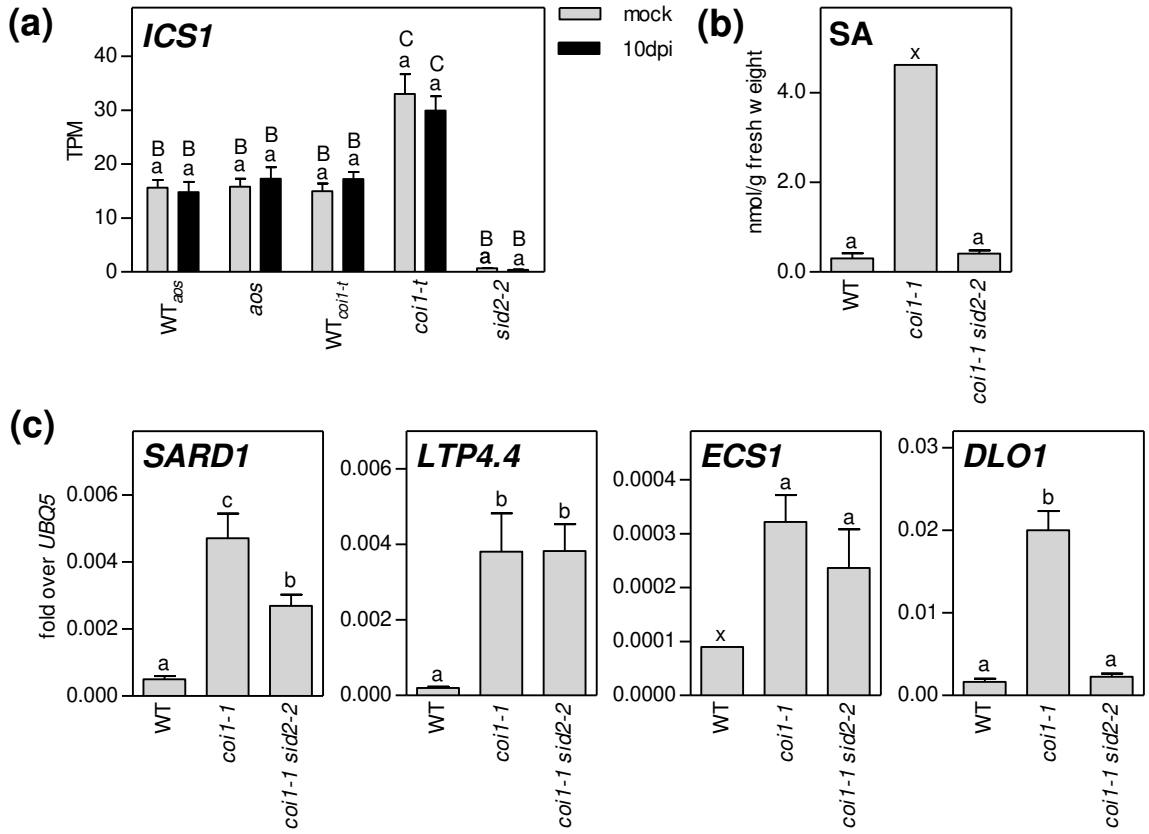

Figure S5. In *coi1* roots, basally elevated marker gene expression is independent of elevated SA levels in most cases.

Full legend on next page.

Figure S5. In *coi1* roots, basally elevated marker gene expression is independent of elevated SA levels in most cases.

(a) Relative expression of *ICS1* transcript levels as quantified by RNA-seq analysis 10 days after mock treatment or inoculation with $1x10^6$ spores/mL sGFP-expressing *V. longisporum*. Bars are means of Transcripts Per Million (TPM) ± SEM of three to four biological replicates of each genotype, with each replicate representing twelve roots from one independent experiment. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). WT_{aos} and $WT_{coi1\text{-}t}$ are the two wild-types lines obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds. **(b)** SA levels in roots at 10 days after mock treatment. Per sample eight to ten roots were pooled. Bars are means ± SEM of two samples per genotype. For *coi1-1* only one sample made up of eight pooled roots is shown. For statistical analysis, an unpaired Student's t-test (two-tailed) was performed between WT and *coi1-1 sid2-2*; lowercase letters denote significant differences between samples ($p < 0.05$). x denotes that *coi1-1* was excluded from statistical analysis as only one replicate is shown. (c) *SARD1, LTP4.4, ECS1* and *DLO1* transcript levels, measured by qRT-PCR. RNA was extracted from roots 10 days after mock treatment. Bars are means ± SEM of five to six roots per genotype. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$). x denotes that for *ECS1* levels in WT only one sample is shown as the others fell below the detection threshold in our analysis. Therefore, WT *ECS1* levels had to be excluded from statistical analysis and an unpaired Student's t-test (two-tailed) was performed between WT and *coi1-1 sid2-2*; lowercase letters denote significant differences between samples ($p < 0.05$).

Figure S6

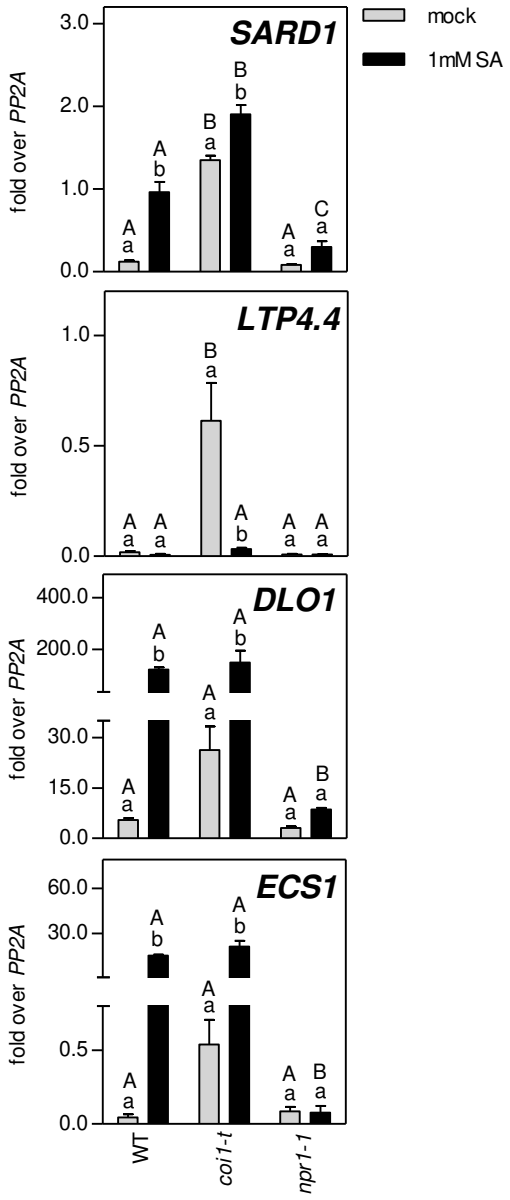

Figure S6. Genes of interest can mostly be induced by SA in roots.

SARD1, LTP4.4, DLO1 and *ECS1* transcript levels, measured by qRT-PCR. RNA was extracted from roots treated with 1mM SA for 24 hours. Per sample five to six roots were pooled. Bars are means ± SEM of three to five replicates per genotype. For *LTP4.4* transcript levels in WT roots treated with 1mM SA only two replicates are shown. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 1mM SA treatment ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$).

Figure S7

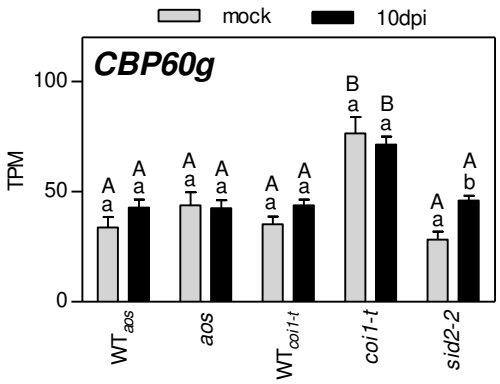

Figure S7. *CBP60g* is weakly de-repressed in *coi1* roots and not induced after infection in WT_{aos}, $WT_{coi1\text{-}t}$ and *aos*.

Relative expression of CBP60g transcript levels as quantified by RNA-seq analysis 10 days after mock treatment or inoculation with $1x10^6$ spores/mL sGFP-expressing *V. longisporum*. Bars are means of Transcripts Per Million (TPM) ± SEM of three to four biological replicates of each genotype, with each replicate representing twelve roots from one independent experiment. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). WT_{aos} and $WT_{coi1\text{-}t}$ are the two wild-types lines obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds.

Figure S8

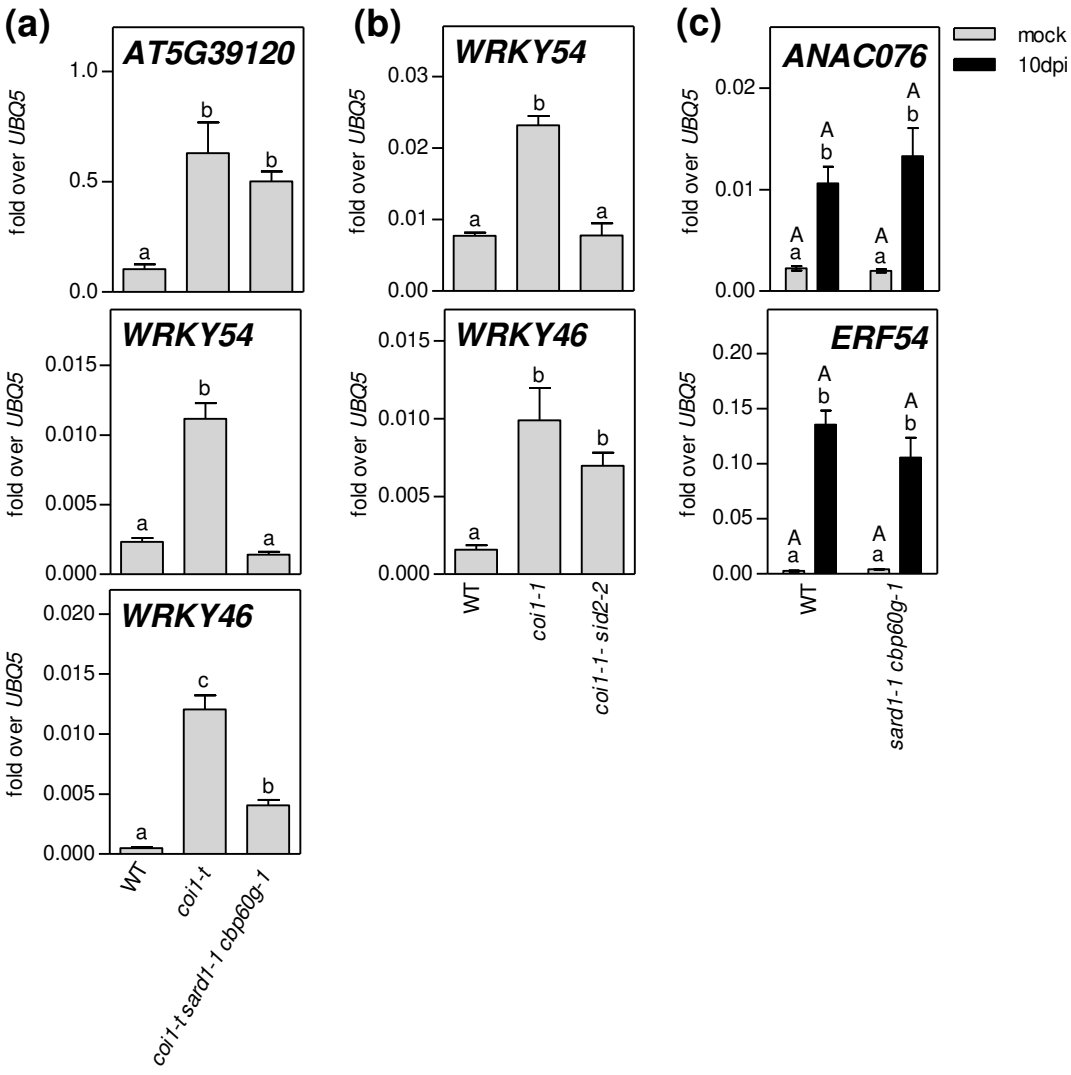

Figure S8. SARD1/CBP60g-dependency of gene expression in the two groups of COI1-suppressed but not significantly induced genes at 10 dpi and the genes induced after infection but not under control of COI1.

(a) *AT5G39120* (a RmlC-like cupins superfamily protein), *WRKY54* and *WRKY46* transcript levels measured by qRT-PCR. RNA was extracted from roots 10 days after mock treatment. Bars are means ± SEM of six to eight roots per genotype. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$). **(b)** *WRKY54* and *WRKY46* transcript levels measured by qRT-PCR. RNA was extracted from roots 10 days after mock treatment. Bars are means ± SEM of six roots per genotype. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$). **(c)** *ANAC076* and *ERF54* transcript levels, measured by qRT-PCR. RNA was extracted from roots 10 days after mock treatment or infection with $1x10^6$ spores/mL *V. longisporum*. Bars are means ± SEM of ten to eleven roots per genotype. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$).

Figure S9

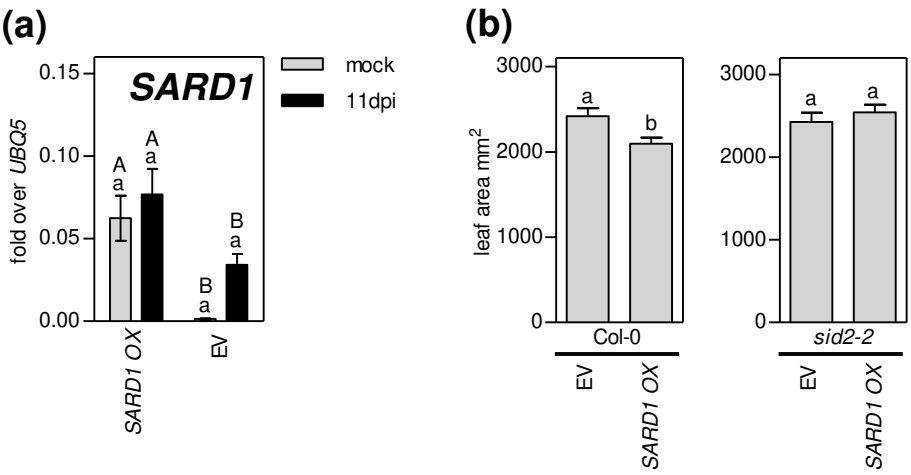

Figure S9. SARD1 overexpression leads to mild growth defects in Col-0 plants.

(a) SARD1 transcript levels, measured by qRT-PCR. RNA was extracted from roots 10 days after mock treatment or infection with 1x10^6 spores/mL *V. longisporum* of the *SARD1* overexpression line *(SARD1 OX)* and empty vector (EV) control line in Col-0 background. The primers used do not differentiate between endogenous and transgenic transcript in the *SARD1 OX* line. Bars are means ± SEM of five to six roots per genotype. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). **(b)** Leaf area of SARD1 overexpression *(SARD1 OX)* and empty vector (EV) lines in Col-0 and *sid2-2* background 15 days after mock treatment. Bars are means ± SEM of 16 plants. For statistical analysis, an unpaired Student's t-test (two-tailed) was performed between *SARD1 OX* and EV; lowercase letters denote significant differences between samples ($p < 0.05$).

Figure S10

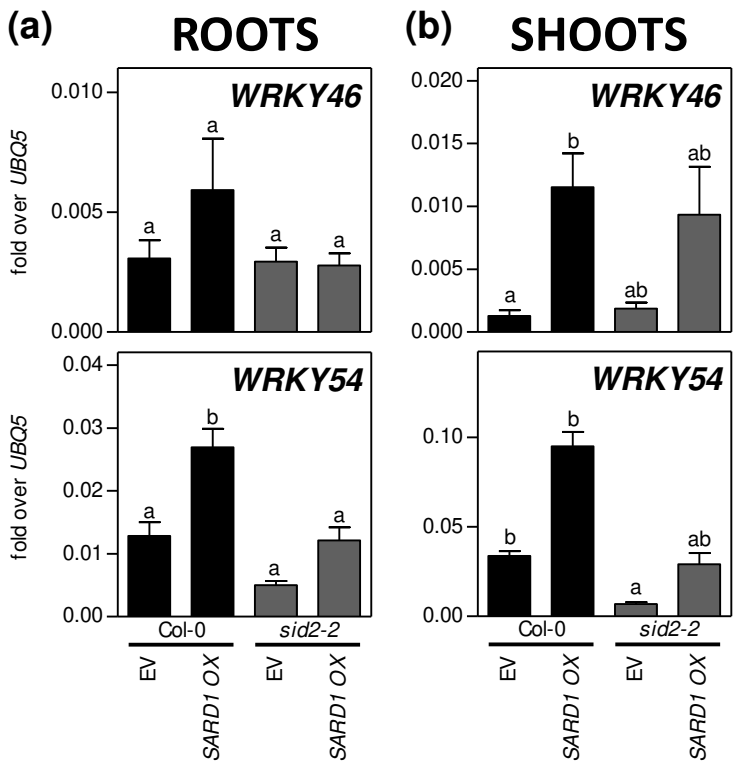

Figure S10. *WRKY54* and *WRKY46* induction is stronger in shoots overexpressing SARD1 compared to roots.

WRKY54 and *WRKY46* transcript levels, measured by qRT-PCR. RNA was extracted from **(a)** roots or **(b)** shoots 10 days after mock treatment of *SARD1* overexpression lines *(SARD1 OX)* and empty vector (EV) controls in both Col-0 (black bars) and *sid2-2* (gray bars) background. Bars are means ± SEM of five to six roots or shoots per line. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test; lowercase letters denote significant differences between samples ($p < 0.05$).

Figure S11

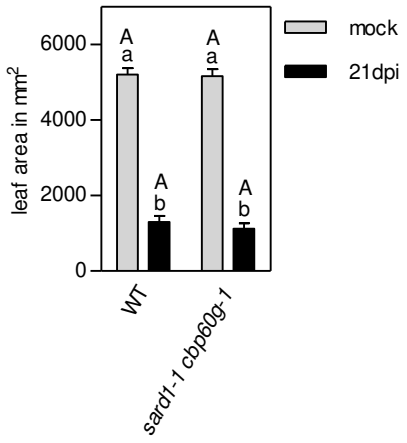

Figure S11. Mutations in SARD1 and CBP60g do not affect the disease phenotype after *V. longisporum* infection.

Leaf area of WT and *sard1-1 cbp60g-1* mutants 21 days after mock treatment or infection with $1x10^6$ spores/mL *V. longisporum*. Bars are means ± SEM of 16 plants. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 21 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$).

V. Additional Data to Articles

1. Additional Data

1.1 The role of *AT3G05770* in facilitating susceptibility to *V. longisporum* could not be determined

In the sand-soil root transcriptome analysis described in Article 2, we found 11 genes that are lower expressed in *coi1-t* after infection compared to *aos*, $WT_{aos,}$ $WT_{coi1\text{-}t}$ and *sid2-2* roots (< 2-fold, $p > 0.05$) (Article 2, Table 1 and subtables). Especially genes induced after infection in susceptible plants but not induced in the tolerant *coi1-t* plants would be candidates for factors that confer susceptibility to *V. longisporum*. Three genes were highly induced after infection in susceptible plants but not in *coi1-t* (Article 2, Table 1 and subtables). One of them is *AT3G05770*, a hypothetical protein (Figure AD1). Even though it showed good induction in the

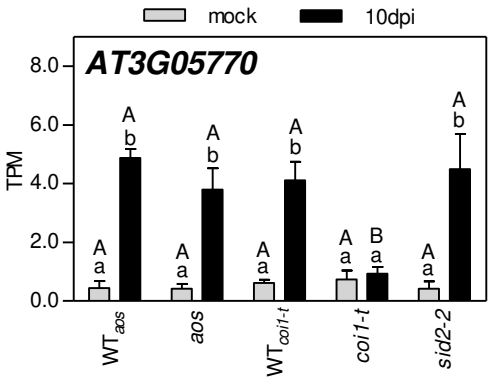

Figure AD1. *AT3G05770* is induced in susceptible genotypes after infection with *V. longisporum* but not in the tolerant *coi1-t* plants.

Relative expression of *AT3G05770* transcript levels as quantified by RNA-seq analysis 10 days after mock treatment or inoculation with $1x10^6$ spores/mL sGFP-expressing *V. longisporum* (RNA-seq described in Article 2). Bars represent the average of Transcripts Per Million (TPM) ± SEM of three to four biological replicates of each genotype, with each replicate representing twelve roots from one independent experiment. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). WT_{aos} and $WT_{coi1\text{-}t}$ are the two wild-types lines obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds.

transcriptome analysis, we could not re-created induction at 10 dpi in roots via qRT-PCR. Nevertheless, we tried to obtain knockout mutants for this gene but did not get viable homozygous offspring from heterozygous mutants we obtained from the Nottingham Arabidopsis Stock Centre (GABIseq_064G08.2, containing an insertion in the coding region). Hence, we were unable to examine whether mutants of this gene would be tolerant to infection with *V. longisporum*.

1.2 *WRKY49* is not a susceptibility gene enabling effective infection by *V. longisporum*

Another gene that we found intriguing was *WRKY49*. It is not induced after infection, so it is not a classical susceptibility candidate gene, however, it is lower expressed in *coi1-t* after infection compared to the susceptible genotypes (Figure AD2a). Possibly some basal levels of WRKY49 need to be present in roots to facilitate favourable conditions for *V. longisporum.* We obtained two different *wrky49* mutant lines; GABIseq_428F12.2 and SALK_091556C (both containing a T-DNA insertion in the coding region) and assessed their susceptibility to *V. longisporum*. Both *wrky49* mutant lines showed similar leaf area loss compared to WT plants (Figure AD2b).

1.3 Neither HDA6 nor SARD1 interacts with COI1 in yeast

COI1 has been shown to interact with HISTONE DE-ACETYLASE 6 (HDA6) in a yeast two hybrid assay, in transiently transformed *A. thaliana* cell cultures but not *in planta* (Devoto *et al.*, 2002). De-acetylation of histones leads to a more closed chromatin structure and reduced gene expression. We hypothesise that recruiting HDA6 to de-acetylate histones could be the mechanism by which COI1 achieves repression of genes in roots. To address this idea, we first of all aimed to reproduce the interaction between COI1 and HDA6 in the exact same yeast two hybrid system as reported by Devoto *et al.* (2002). Interaction experiments were performed in *Saccharomyces cerevisiae* using the LexA system in which COI1 was fused to the DNA

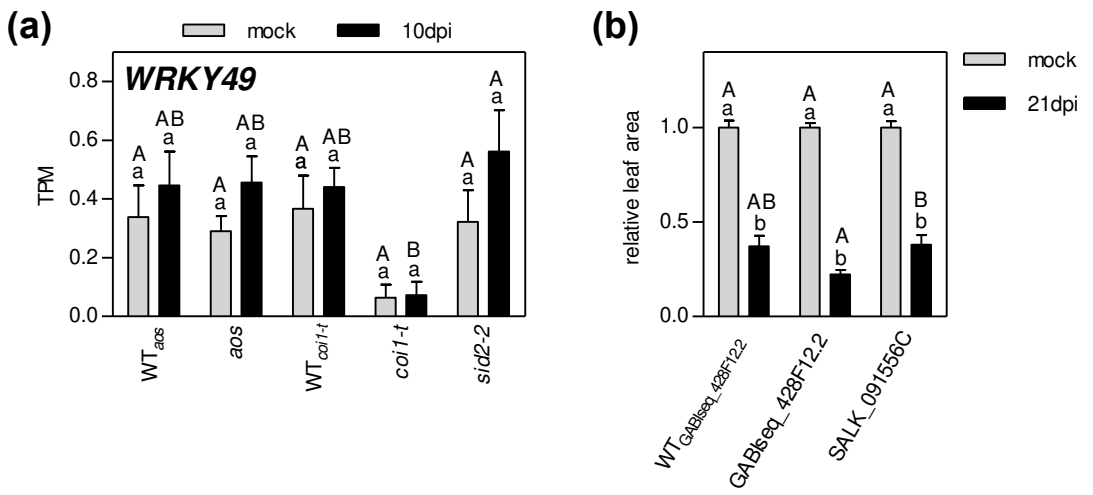

Figure AD2. *WRKY49* is not a susceptibility gene facilitating effective infection by *V. longisporum*.

(a) Relative expression of *WRKY49* transcript levels as quantified by RNA-seq analysis 10 d after mock treatment or inoculation with $1x10^6$ spores/mL sGFP-expressing *V. longisporum*. Bars represent the average of Transcripts Per Million (TPM) ± SEM of three to four biological replicates of each genotype, with each replicate representing four roots from one independent experiment. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). WT_{aos} and $WT_{coi1\text{-}t}$ are the two wild-types lines obtained from the segregating offspring of heterozygous *aos* and *coi1-t* seeds. **(b)** Leaf area of WT and two *wrky49* mutant lines (GABIseq_428F12.2 and SALK_091556C both containing a T-DNA insertion in the coding region) 21 days after mock treatment or infection with $1x10^6$ spores/mL *V. longisporum*. Bars are means ± SEM of 16 plants. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$). $WT_{GABIseq\_428F12.2}$ is the wild-type line obtained from the segregating offspring of heterozygous GABIseq_428F12.2 seeds.

binding domain (BD) and HDA6 to the activation domain (AD) (Brent and Ptashne, 1985; Van Criekinge and Beyaert, 1999). If the proteins interact, close contact between BD and AD drives expression of the *LacZ* gene, coding for ß-galactosidase. ß-Galactosidase hydrolyses the X-Gal added to the yeast growth medium, leading to blue staining of the yeast colonies. No interaction was observed between COI1 and HDA6 (Figure AD3a). However, as we are investigating COI1 as a repressor protein, we wondered if COI1 and HDA6 were interacting

but were forming a repressor complex that overcame the intended activation of *LacZ*. To test this idea, we generated a COI1 construct that contained a DNA binding domain together with an activation domain (VP). This construct should alone be sufficient to drive activation of *Lac*. If COI1 and HDA6 interacted and indeed acted as a repressor together, co-transformation of COI1 in the VP construct with HDA6 should abolish activation of *LacZ*. However, no such repression of the active *LacZ* promoter was observed (Figure AD3b).

Nevertheless, as the interaction had been reported before (Devoto *et al.*, 2002), we tested if *hda6* mutant plants would show the same tolerant phenotype as *coi1* plants after infection with

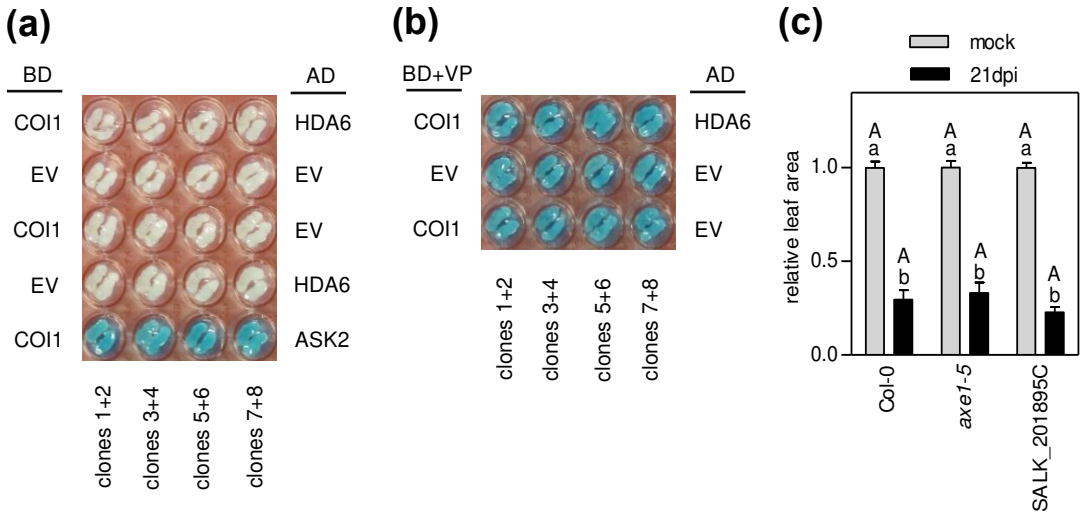

Figure AD3. HDA6 is most likely not involved in COI1-mediated repression.

(a) Interaction analysis of COI1 fused to the LexA binding domain (BD) and HDA6 fused to the B42 activation domain (AD) two days after streaking yeast colonies onto X-Gal supplemented medium. As a positive control the interaction between COI1 fused to the LexA binding domain and ASK2 fused to the B42 activation domain is shown. Blue colour indicates protein interaction. EV denotes empty vector controls. The experiment displayed was performed by Natalie Leutert as part of her Bachelor Thesis supervised by Louisa Ulrich. **(b)** COI1 fused to the LexA binding domain (BD) and the VP16 activation domain (VP) constitutes a functional unit to drive expression of *LacZ* (indicated by blue colour) two days after streaking colonies onto X-Gal supplemented medium. HDA6 fused to the B42 activation domain (AD) was added to assess the formation of a functional repressor unit. EV denotes empty vector controls. The experiment displayed was performed by Natalie Leutert as part of her Bachelor Thesis supervised by Louisa Ulrich. **(c)** Leaf area of WT and two *hda6* mutant lines (*axe1-5* and SALK_201895C (containing a T-DNA insertion in the coding region)) 21 days after mock treatment or infection with $1x10^6$ spores/mL *V. longisporum*. Bars are means ± SEM of 16 plants. For statistical analysis, a two-way ANOVA was performed followed by Bonferroni's multiple comparison test; lowercase letters denote significant differences within each genotype between mock and 10 dpi ($p < 0.05$), uppercase letters denote significant differences between genotypes subjected to the same treatment ($p < 0.05$).

V. longisporum. If HDA6 is part of the mechanism though which COI1 achieves gene repression, then *hda6* plants should show the same basally upregulated gene expression in roots. In turn, if basally upregulated gene expression in *coi1* roots is the underlying reason for the tolerance, *hda6* mutants should show the same tolerant pathophenotype as *coi1* plants. We infected two different *hda6* mutants; *axe1-5* (Murfett *et al*., 2001) and SALK_201895C (T-DNA insertion in the coding region). Both *hda6* mutants were as susceptible to the fungus as WT plants (Figure AD3c).

As we show in Article 2, SARD1 is involved in the activation of genes like *LTP4.4*, *DLO1* and *ECS1*. Hence, we hypothesised that SARD1 might recruit COI1 directly to promoters of genes with the GAAATTT SARD1 binding site for repression. To address this hypothesis, we again performed interaction experiments between SARD1 and COI1 in *Saccharomyces cerevisiae.* No interaction between COI1 and SARD1 was observed (Figure AD4).

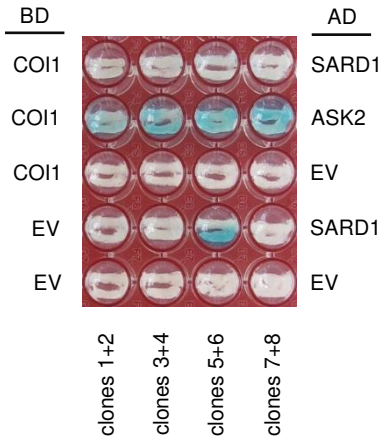

Figure AD4. SARD1 is most likely not involved in COI1-mediated repression.
Interaction analysis of COI1 fused to the LexA binding domain (BD) and SARD1 fused to the B42 activation domain (AD) two days after streaking yeast colonies onto X-Gal supplemented medium. As a positive control the interaction between COI1 fused to the LexA binding domain and ASK2 fused to the B42 activation domain is shown. Blue colour indicates protein interaction. EV denotes empty vector controls.

2. Methods for Additional Data to Articles

Relative expression of *AT3G05770* and *WRKY49* is data from the RNA-seq analysis described in Article 2. Fungal infections and leaf area measurements were performed as described in Article 2. GABIseq_428F12.2, SALK_091556C and SALK_201895C and *axe1-5* were obtained from Nottingham Arabidopsis Stock Centre (NASC). The primers for genotyping of T-DNA insertion lines GABIseq_428F12.2, SALK_091556C and SALK_201895C are given in Table AD1 in Section VIII. Appendix. *axe1-5* mutants were confirmed by sequencing (Murfett *et al.*, 2001).

For interaction analyses in the LexA yeast two-hybrid system, *COI1*, *ASK2*, *HDA6* and *SARD1* were introduced into the GATWAY-compatible vectors pGILDA-GW and pB42AD-GW, described in Li *et al.* (2019). COI1 was also introduced into pGILDA-VP16-GW, additionally containing the VP16 activation domain. The sequence of pGILDA-VP16-COI1 is given in Section VIII. Appendix. Yeast two hybrid assays were performed as described in Article 1 (Ulrich *et al.*, 2021).

VI. General Discussion

Prompted by the tolerance-mediating effect of *coi1* roots against *V. longisporum*, RNA-seq analysis of *coi1*, *aos* and WT roots was conducted. We have observed de-repression of SA-related defence genes in *coi1* but not *aos* roots. In Article 1 we showed that this repressor function of COI1 operates independently from JA-Ile and most likely also independently from JAZ proteins. Moreover, experiments in shoot material have shown that COI1 only acts as a repressor of gene expression in roots. In Article 2 we describe that about 50% of the genes under negative influence of COI1 are induced at 10 dpi. We postulate that this part of the *V. longisporum*-induced defence programme is only initiated after a fungal or plant-derived signal leads to inactivation of COI1 in roots. In addition to our findings on COI1 and its role in root gene expression regulation, we could show that at 10 dpi the *V. longisporum*-induced processes in Arabidopsis roots run mostly independently of JA and SA and are in large part cell wall biogenesis-related, including xylem differentiation.

1. Responses to *V. longisporum* infection differ at early and late time points

GO term analysis of genes upregulated in WT plants at 10 dpi show upregulation of cell wall biogenesis proteins (Article 2, Figure S2). Especially when excluding the COI1-repressed portion of these inducible genes, which are mainly SA-defence-related genes, there are almost exclusively cell wall biosynthesis processes left (Article 2, Figure 2 and S3b). Induction of cell wall biogenesis-related genes and metabolites has previously been reported for infected *A. thaliana* shoots at 18 dpi and 25 dpi (Tappe, 2008; Floerl *et al*., 2012).

Tappe (2008) observed induction of cell wall-related genes at 18 dpi in whole *A. thaliana* rosettes. Floerl *et al.* (2012) analysed the metabolome of apoplastic fluid and examined cell wall properties at 25 dpi in *A. thaliana* leaf material. They found increased production of cell wall carbohydrates with reduced esterification and increased lignification in infected plants.

The shared secretome of *V. longisporum* cultivated in simulated xylem medium and filtered *B. napus* xylem sap contains a large number of carbohydrate degrading enzymes (Leonard *et al.*, 2020). This indicates that at infection stages when the fungus has entered the xylem, carbohydrate degrading enzymes are secreted. The fitness gain in producing cell wall reinforcements has been shown in a *V. longisporum*-resistant *B. napus* cultivar (Eynck *et al.*, 2009). In comparison to a susceptible cultivar, the resistant *B. napus* cultivar SEM 05-500256 showed greater reinforcement of tracheary elements and build-up of vascular occlusions in the hypocotyl at 21dpi (Eynck *et al.*, 2009). The upregulation of cell wall material production genes that we observe at 10dpi in roots might indicate the reinforcement of structural barriers. However, Reusche *et al.* (2012) observed *VASCULAR-RELATED NAC DOMAIN 7* (*VND7)*-dependent *de novo* xylem formation in *A. thaliana* in leaves, hypocotyl and roots, starting between 7 and 14 dpi. In our RNA-seq data from 10dpi, we found *VND7* and *VND6*, both master regulators of xylem differentiation, in the group of non-COI1 dependent genes induced after infection (Article 2, Table 1 and subtables). Moreover, we see upregulation of *MYB46* and *MYB83* which coordinate biosynthesis of cellulose, hemicellulose and lignin downstream of *VND6* and *VND7* (Ko *et al.*, 2014; Růžička *et al.*, 2015). Since we do not have microscopic data from this time point, we cannot differentiate if *de novo* xylem formation is the sole process leading to upregulation of cell wall biogenesis genes or if the plant also reinforces barriers to restrict fungal spreading.

The induction of cell wall biogenesis programmes in *V. longisporum* infected plants at these later time points, when the fungus resides in the xylem or has reached the shoot, contrasts the responses observed at earlier time points after *V. longisporum* infection.

Studies analysing the transcriptome or translatome at 1, 2 or 3 dpi in roots show no cell wall biosynthesis-related gene expression patterns (Iven *et al.*, 2012; Fröschel *et al.*, 2021). Instead, they report induction of genes involved in the production of secondary metabolites, like *CYP81F2* and *PENETRATION 2 (PEN2)* which are important for the production of indole glucosinolates (IGs) and *CYP71A12*, *CYP71A13*, *PHYTOALEXIN DEFICIENT 3* (*PAD3)* and

GLUTATHIONE S-TRANSFERASE 6 (*GST6)* which are part of the camalexin biosynthesis pathway (Iven *et al.*, 2012; Fröschel *et al.*, 2021).

In our transcriptome data we only see induction of *CYP71A13* but not any of the other genes discussed above. Moreover, GO term analysis did not hint at any IG or camalexin biosynthesis-related processes. In accordance, neither Tappe (2008) reports induction of these pathways at 18 dpi, nor were IGs or camalexin found to be secreted at 25dpi (Floerl *et al.*, 2012). Hence, it seems that tryptophan-derived secondary metabolite synthesis is an early response to *V. longisporum* infection.

However, Iven *et al.* (2012) also saw production of camalexin and indole-3-carboxylic acid at 8 dpi. This might be an artefact of media-dependent saprophytic growth of the fungus outside the root on MS medium. Possibly, IG and camalexin production is enhanced by detection of MAMPs on the outermost root layers. The studies reporting on early transcriptome/translatome responses derived their data from infection systems on MS medium (Iven *et al.*, 2012; Fröschel *et al.*, 2021). In contrast, the ones describing later responses to *V. longisporum* infection, including us, used a soil-based system. In our hands, infections on MS-medium provide an environment for saprophytic growth of the fungus outside the root and did not force the fungus to enter the xylem. We observed xylem entry of *V. longisporum* in plate-based infections only when transferring plantlets to nutrient-poor agarose plates before infection. Since roots are capable of perceiving the presence of MAMPs (Millet *et al.*, 2010; Zhou *et al.*, 2020), extended fungal growth on the outside of roots caused by MS infection systems might lead to distorted responses. Hence, studying responses in MS plate-based systems at later timepoints, when the fungus would usually not be growing on the root surface anymore, might not accurately reflect root responses.

The combined data hints at IGs and camalexin synthesis being an early response against *V. longisporum* infection, whereas cell wall remodelling is initiated after the fungus has reached the xylem.

2. Regulation of COI1-mediated gene repression differs from COI1's mode of action in canonical JA signalling

Both our RNA-seq analyses have clearly shown that COI1 is a repressor of defence gene expression in roots. As the *aos* mutant does not show this de-repression of gene expression, the novel COI1 function must be JA-Ile independent. To describe this repressor function of COI1, we selected marker genes identified in the RNA-seq analysis, represented by *PGM, PRLIP2* and *SARD1*.

The interaction with JAZ proteins for a COI1 repressor function that requires no JA-Ile would need to involve JA-Ile-independent turnover of JAZs by COI1. The first idea of how COI1 might lead to gene suppression that comes to mind is similar to COI1's role in canonical JA signalling. Under basal conditions SCF^{COI1} leads to ligand-independent turnover of JAZs which suppress transcription of a repressor of genes like *PRLIP2*, *PGM* and *SARD1*. Such a repressor would be lower expressed in *coi1* roots where JAZs are not degraded. However, we could exclude the involvement of the only two candidates for such repressors found in the transcriptome. One of the potential repressor candidates did not show lower expression in *coi1* compared to WT in purely soil-grown roots, where de-repression of *PGM* and *SARD1* is still observed (Article 1, Figure S4). The other repressor candidate was so lowly expressed under these conditions that no specific PCR product could be detected.

Another possible way JAZ proteins would be involved in the repressive function of COI1 is if JAZs accumulation interfered with the action of a transcriptional repressor of genes like *PGM*, *PRLIP2* and *SARD1*. Again, ligand-independent turnover of JAZs by SCF^{COI1} in WT and *aos* roots would keep JAZ proteins from over-accumulating. In contrast, in *coi1* (possibly only specific) JAZs might accumulate to higher levels than even in *aos* and might interfere with this repressor of gene repression. However, this explanation was not supported by results from transiently transformed protoplasts, where no basal turnover of any JAZ in the absence of JA-Ile or coronatine was observed (Article 1, Figure S7). Nevertheless, we additionally generated a COI1 protein that is severely impaired in JAZ interaction ($COI1_{AA}$). $COI1_{AA}$ only very weakly

complemented canonical *VSP2* induction after wounding and did not restore fertility. Still, we observed highly suppressed gene expression of *PGM* and *PRLIP2* in *coi1-t/COI1$_{AA}$* roots (Article 1, Figure 5).

Hence, the involvement of JAZs in the newly discovered repressor function of COI1 is unlikely. Therefore, COI1 must act differently in gene repression than it does in canonical JA signalling.

In a previous attempt, an N-terminally tagged COI1 protein could not complement gene suppression of *PGM* and *PRLIP2* in *coi1* while being able to complement the canonical function (Article 1, Figure 4). The N-terminal domain of COI1 is the F-box domain, which is important for the formation of the SCF^{COI1} complex, which acts as an E3 ubiquitin ligase. In this functional context, an N-terminal tag does not seem to interfere with COI1 function. The F-box domain is also required for the recruitment of COI1 to JA-Ile responsive promoters by MED25, thereby establishing proximity between COI1 and JAZ. Apparently, this function is also not compromised by the N-terminal tag. Hence, the novel repressive function of COI1 requires a yet unknown process that is disturbed by an N-terminal tag.

PGM and *SARD1* are lower expressed in untreated roots of *med25* compared to WT plants (Article 1, Figure 6). This suggests that MED25 is present at these promoters to activate basal gene expression. Since MED25 interacts with COI1 (An *et al.*, 2017) and since it might sit at these promoters, it is a candidate for recruiting COI1.

Additionally, we investigated if COI1 might interact with HDA6 or SARD1 to carry out its repressive function. HDA6 is involved in gene repression by de-acetylation of histone H3 and H4 and has been shown to interact with COI1 (Devoto *et al.*, 2002; Yu *et al.*, 2011; Wang *et al.*, 2013). We were unable to reproduce the interaction using exactly the same protocol (Additional data to articles, Figures AD3a and AD3b). Moreover, *hda6* mutants did not show tolerance against infection with *V. longisporum* (Additional data to articles, Figure AD3c). Similarly, as we have discovered that SARD1 controls a group of COI1-repressed genes (Article 2), we hypothesised that SARD1 might recruit COI1 to these promoters. However, we also did not observe interaction of COI1 and SARD1 in yeast (Additional data to articles, Figure

AD4). Hence, we have no evidence that suggests the involvement of either HDA6 or SARD1 in COI1-mediated gene repression.

We have shown that infection with *V. longisporum* leads to induction of COI1-suppressed genes such as *LTP4.4* in a SARD1/CBP60g-dependent manner. *SARD1* is itself under negative control of COI1 (Article 2, Figure 3d). Moreover, increased levels of SARD1 can only induce expression of downstream target genes in unstressed *coi1* roots but not in WT roots. Therefore, COI1 must have an additional repressive effect on these downstream genes (Article 2, Figures 6 and 7). Thus, we postulate that COI1 is inactivated after infection.

Only a fraction of COI1 repressed genes is hyper-induced in *coi1* after infection (Article 2, Table 1 and subtables). Hardly any genes inducible in both WTs, *aos* and *sid2* roots are lower expressed in these genotypes compared to *coi1* at 10 dpi (Article 2, Table 1 and subtables). Hence, we hypothesise systemic inactivation of COI1 in roots after infection. The fact that the JA defence response is not induced might be evidence for the fact that COI1 is generally inactivated, not just in its role as a repressor of gene expression.

Systemic inactivation would require a systemic signal after infection. A systemic signal traveling only in the root vasculature is implausible as there is no source to sink gradient creating directional flow within the root. Instead, systemic root to shoot signals have been described where peptides, microRNAs and hormones are transported shootward with the transpirational stream and down via the source to sink gradient (Tabata *et al.*, 2014; Ohkubo *et al.*, 2017; Tsikou *et al.*, 2018; Wang *et al.*, 2019; Okuma *et al.*, 2020). We can only speculate about the nature of such a signal so far. In our transcriptome we see induction of *CLE1*, coding for a small peptide hormone of the CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION-related (CLE) family. *CLE1, 3, 4* and *7* have been shown to be induced in roots in response to low nitrogen conditions and have been proposed to travel through the phloem to systemically regulate root system architecture (Araya *et al.*, 2014). Hence, CLE1 would be a potential candidate for inactivation of COI1 upon *V. longisporum* infection. Alternatively, the signal could be a volatile spreading through the root. Volatiles leading to changes in root behaviour have

been described in the context of root-fungus interactions before (Schenkel *et al.*, 2018; Dreher *et al.*, 2019; Moisan *et al.*, 2021).

If the infected plant generates the proposed signal, we assume it would be to activate the COI1-repressed root defence. On the other hand, it is possible that the mobile signal is fungus-derived. Possibly, *V. longisporum* secretes a signal that that inactivates COI1 to avoid the launch of the JA pathway, which leads to activation of COI1-suppressed root defences unintendedly. This signal might travel up to the shoot and back down to inactivate COI1 systemically. Alternatively, a *V. longisporum*-derived molecule travels to the shoot where it is perceived by plant receptors that pass down a signal for COI1 inactivation. Tappe (2008) showed that *V. longisporum*-responsive genes are induced in shoots before fungal DNA can be detected there. Similarly, Reusche *et al.* (2012) report trans-differentiation in leaves in the absence of fungal hyphae. Although it is possible that the findings of Tappe (2008) are limited by PCR sensitivity, this suggests that *V. longisporum*-derived molecules can be transported to the shoot via the transpiration stream, inducing responses ahead of fungal colonisation. However, it is also possible that this signal is a DAMP generated by *V. longisporum* degrading the plant's cell walls.

The idea that COI1 repression can be overcome by pathogen infection of the root tempts to speculate that COI1 is a safety check for root responses. How plant roots manage and regulate perception of pathogens in an environment continuously providing exposure to MAMPs, has been a long-standing question in plant-microbe interactions. Zhou *et al.* (2020) have shown that differentiated roots employ a switch-like system in which the presence of MAMPs or DAMPS alone does not lead to root defence responses, instead responses are only launched when damage and perception of MAMPs coincide. Possibly, COI1 might be a similar root switch that prevents excessive triggering of root defences in response to trivial stimuli, to avoid excessive energy expenses and determent of beneficial root microbes. In line with this idea, it has been shown that elevated SA levels interfere with colonisation of roots by beneficials (Martínez-Abarca *et al.*, 1998; Herrera Medina *et al.*, 2003; Martínez-Medina *et al.*, 2017). Here

it would be interesting to see if other root pathogens can also cause inactivation of COI1 and induction of SA-related defences.

3. Basal upregulation SARD1/CBP60g-dependent defence genes in roots is not the reason for tolerance of *coi1*

As *coi1* roots confer tolerance to *V. longisporum* that only later takes effect when the fungus already resides in the shoot, we hypothesised that products of de-repressed defence genes in *coi1* roots travel with the transpiration stream and accumulate in the shoot. At later stages of infection when the fungus has reached the shoot its proliferation is impaired, leading to lower fungal biomass in *coi1* shoots as compared to *aos* and WT and fewer visible infection symptoms.

By mutating SARD1 and CBP60g in the *coi1* background, we could reverse pre-induction of their downstream targets (Article 2, Figure 6). However, *coi1 sard1 cbp60g* triple mutants were not as susceptible as WT plants (Article 2, Figure 8). With 21% leaf area loss compared to *coi1* plants with 11% loss, only a small, reproducible, albeit non statistically significant effect on leaf area loss was observed. Thus, this SARD1/CBP60g-coordinated defence programme alone is not the reason for the observed tolerance in *coi1*. Nevertheless, it adds a fraction to the overall tolerance observed. We initially assumed a major role of COI1 on one single type of response that would explain the tolerance. However, it is possible that a multitude of effects combined render *coi1* plants tolerant against infection by *V. longisporum.*

As described earlier, cell wall reinforcements and production of IGs and camalexin are somewhat effective defences against *V. longisporum* infection (Eynck *et al.*, 2009; Iven *et al.*, 2012). We did not see basal upregulation of *PEN2, CYP81F2, GST6, CYP71A12, CYP71A13* or *PAD3* in *coi1,* so exclude that these defences are preinduced in *coi1* which could explain the tolerance.

Germin-like proteins have been shown to inhibit mycelial growth and spore germination of *V. dahliae* and *F. oxysporum* (Pei *et al.*, 2019; Pei *et al.*, 2020). Moreover, Floerl *et al.* (2012) detected enrichment of GLP-3 in *A. thaliana* apoplastic fluid after infection. Hence, GLPs present suitable candidates for root to shoot transported antifungal proteins that hamper *V. longisporum* proliferation in shoots. Indeed, our transcriptome data shows that in *coi1* roots three GLPs are basally upregulated: *GLP2A* (AT5G39190), *AT5G39160*, and *AT5G39120,* all of which have a predicted signal peptide (Article 2, Table 1 and subtables). They are not induced in response to *V. longisporum*, however, their constitutive expression in *coi1* roots and the fact that they are most likely secreted make them candidates for transport to and accumulation in the shoot. For *AT5G39120* we have shown that it is still upregulated in mostly tolerant *coi1 sard1 cbp60g* plants, thus it remains a candidate for conferring tolerance (Article 2, Figure S8a). Hence, it would be interesting to assess if these GLPs are involved in *coi1*-mediated tolerance to *V. longisporum*. As these GLPs are found in close proximity in the genome, a CRISPR/Cas9 approach could prove efficient for knocking out these and other GLPs simultaneously in *coi1*.

Besides COI1, *CRT1a* has been shown to facilitate infection by *V. longisporum* (Pröbsting *et al.*, 2020). Our RNA-seq analysis yielded very few susceptibility gene candidates, that might explain the tolerance-mediating effect of *coi1* roots. Only three genes which are induced in susceptible genotypes at 10dpi are not induced in *coi1* after infection (Article 2, Table 1 and subtables). We aimed to address if the absence of *AT3G05770* induction in *coi1* (Additional data to articles, Figure AD1) might convey tolerance, however, were unable to obtain mutants for analysis. The other two candidates have not been under investigation, yet. Moreover, we tested if lower basal levels of *WRKY49* might affect tolerance in response to *V. longisporum* but did not see differences in susceptibility after infection in two different *wrky49* mutants (Additional data to articles, Figure AD2).

4. The effectiveness of SARD1-regulated defences seems to differ against *V. longisporum* and *V. dahliae*

SARD1 and CBP60g have been shown to be important in defence against *V. dahliae. V. dahliae* possesses an effector, VdSCP41, that interacts with SARD1 and CBP60g in the nucleus (Qin *et al.*, 2018). Arabidopsis plants infected with a mutant *V. dahliae* strain lacking VdSCF41 (VdΔscp41) show increased expression levels of *ICS1* and *FMO1*. Arabidopsis and cotton plants infected with VdΔscp41 show less severe disease symptoms. Qin *et al.* (2018) did not, however, address if *sard1 cbp60g* mutants were more susceptible to infection with *V. dahliae* or whether infection of *sard1 cbp60g* plants with VdΔscp41 had an effect on susceptibility. For CBP60g at least, it has been shown that interaction with VdSCF41 reduces its DNA binding activity; probably the mechanism by which transcriptional activity on target promoters is reduced (Qin *et al.*, 2018).

In *V. longisporum*-infected roots we see induction of *SARD1* and activation of downstream target genes. If *V. longisporum* had an effector that interfered with SARD1 activity it is unlikely that we would see induction of SARD1-dependent targets to similar levels as in *coi1*. Moreover, we have shown *sard1 cbp60g* double mutants were not hyper-susceptible to infection compared to WT plants and mutating SARD1 and CBP60g only had a minor effect on *coi1* tolerance (Article 2, Figures 8 and S11). Hence, it seems that SARD1/CBP60g do not play important roles in effective defence against *V. longisporum*.

During infection, *V. dahliae* has also been shown to secrete an effector acting as an isochorismatase (VdICS1), interfering with SA accumulation (Liu *et al.*, 2014; Zhu *et al.*, 2017). Indeed, we do not see an increase in SA after infection, however, we also only observe weak induction of *ICS1* after infection. It is more likely that the lack of SA results from the minor induction of *ICS1* than an effector hydrolysing isochorismate.

5. Outlook

The findings reported here uncover a novel role of COI1 in suppression of gene expression in roots. To act as a repressor, COI1 does likely not interact with its canonical JA-signalling partners, the JAZ proteins. Pull-down experiments with the tagged COI1-lines described in Article 1 could be used to identify novel interaction partners COI1 associates with in its role as a repressor of gene expression. As pull downs might be impeded by the low endogenous concentration of COI1 in roots, generating C-terminally tagged COI1 overexpression lines might be necessary. Chromatin immunoprecipitation (ChIP)-PCR could help to elucidate if COI1 is directly present at promoters of repressed genes and also if its presence there is diminished after infection.

Based on transcriptome data we postulate that COI1 is systemically inactivated in roots of *V. longisporum*-infected plants. β-glucuronidase (GUS)-reporter lines of COI1-suppressed and highly *V. longisporum*-inducible genes like *SARD1* or *DLO1* in combination with a split root infection assay could be conducted to address this hypothesis. Infecting only one part of the root and analysing gene induction in distant non-infected parts of the root would help to shed light on the propagation of root responses to *V. longisporum*. Gene expression analysis of infected *cle1* roots could be conducted to address if *CLE1* is involved in this potential systemic signalling pathway.

To address the idea that COI1 might act as a regulator of root defence onset, infections with other vascular and root pathogens should be conducted in *A. thaliana*. To see if there is any biological significance to keeping these genes suppressed, root growth assays should be considered and colonisation of *coi1* roots by beneficials assessed. Investigating if COI1 also acts as a liftable repressor of root gene expression in other plant species, would enhance our understanding of the broader significance of this newly discovered COI1 function.

VII. References

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VIII. Appendix

Table AD1. Primers for Genotyping.

	Primer ID	Sequence 5'-3'
GABIseq_428F12.2	GK428F12Chr1-LP	GTTCAGTTGCATACAAAGCGCAG
	GK428F12Chr1-RP	CGCTGACCAAGGGACACGAGTAC
	o8409mod	CCATATTGACCATCATACTCATTGC
SALK_091556C	SALK_091556C_LP	TTTCATACATGCCTCGAATCTATCC
	SALK_091556C _RP	TTTTCGGTCACAAGCCTAATGTTAC
	LBb1.3	ATTTTGCCGATTTCGGAAC
SALK_201895C	SALK_201895C_LP	ATATCTATGTAGAGAACCCGCTGC
	SALK_201895C_RP	GTGCGTGTATATATAAGCTGTGCC
	LBb1.3	ATTTTGCCGATTTCGGAAC

Sequence of pGILDA-VP16-COI1

CTTGAATTTTCAAAAATTCTTACTTTTTTTTTGGATGGACGCAAAGAAGTTTAATAATCATATTACAT
GGCATTACCACCATATACATATCCATATACATATCCATATCTAATCTTACTTATATGTTGTGGAAAT
GTAAAGAGCCCCATTATCTTAGCCTAAAAAAACCTTCTCTTTGGAACTTTCAGTAATACGCTTAACT
GCTCATTGCTATATTGAAGTACGGATTAGAAGCCGCCGAGCGGGTGACAGCCCTCCGAAGGAAG
ACTCTCCTCCGTGCGTCCTCGTCTTCACCGGTCGCGTTCCTGAAACGCAGATGTGCCTCGCGCC
GCACTGCTCCGAACAATAAAGATTCTACAATACTAGCTTTTATGGTTATGAAGAGGAAAAATTGGC
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TTATTCAAATGTAATAAAAGTATCAACAAAAAATTGTTAATATACCTCTATACTTTAACGTCAAGGA
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ATTGAAATTGTTTCCGGCGCATCACGCGGGATTCGTCTGTTGCAGGAAGAGGAAGAAGGGTTGC
CGCTGGTAGGTCGTGTGGCTGCCGGTGAACCACTTCTGGCGCAACAGCATATTGAAGGTCATTA
TCAGGTCGATCCTTCCTTATTCAAGCCGAATGCTGATTTCCTGCTGCGCGTCAGCGGGATGTCGA
TGAAAGATATCGGCATTATGGATGGTGACTTGCTGGCAGTGCATAAAACTCAGGATGTACGTAAC
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CATGCCGACGCGCTAGACGATTTCGATCTGGACATGTTGGGGGACGGGGATTCCCCGGGGCCG
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GTCAGTGACTTAGATCTAGATCGTTTAGCTAAAGCTAGAGCAGATGATCTTGAGACTTTGAAGCTA
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ACTTTGTTAATGGAAGAGAGTTCTTTTAGTGAAAAGGATGGTAAGTGGCTTCATGAGCTTGCTCAG
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TCCGATCTCTTTTGATTGGATGCAAGAAACTCAGACGATTTGCATTCTATCTGAGACAAGGCGGCT
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X. Curriculum Vitae

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DATE OF BIRTH 30/01/1993

PLACE OF BIRTH Braunschweig, Germany

NATIONALITY German

EDUCATION

11/2017- 07/2021 **Georg-August Universität - Göttingen, Germany**
PhD student in the department of Plant Molecular Biology and Physiology lead by Prof. Gatz as part of the PhD Programme Biology by the Georg-August University School of Science (GAUSS)

PhD Thesis:
Analysis of the JA-Ile-independent function of COI1 in *Arabidopsis thaliana* upon infection with *Verticillium longisporum*

08/2016 -11/2017 **University of Glasgow – Glasgow, United Kingdom**
1st class Master of Science in Biotechnology

Master dissertation, supervised by Prof. Anna Amtmann, Institute of Molecular Cell and Systems Biology, Molecular Plant Physiology:
Characterisation of *Arabidopsis thaliana* mutants for genes with potentially cell-type specific functions in root pericycle and xylem parenchyma

09/2012 - 06/2016 **University of Stirling – Stirling, United Kingdom**
1st class Bachelor of Science with Honours in Marine Biology

Bachelor dissertation, supervised by Dr. Andrew Desbois, Institute of Aquaculture:
Interaction of *Vibrio anguillarum* with extracellular traps in the innate immune response of *Onchorynchus mykiss*

08/2004 - 07/2011 **High School Gymnasium Martino Katharineum – Braunschweig, Germany**

Abitur: 1,9

WORK EXPERIENCE

14/08/17 – 08/09/17 **University of Glasgow, Institute of Molecular Cell and Systems Biology, Department of Plant Science lead by Prof. Amtmann- Glasgow, United Kingdom**
Student Laboratory Research Assistant

- Assisted PhD projects on salt stress responses in *A. thaliana*
- Continued research from Master's dissertation

07/06/2016 – 10/07/2016 **University of Stirling, Bacteriology Laboratory – Stirling, United Kingdom**
Student Laboratory Research Assistant

- Independently conducted experiments as part of ongoing research of Dr. Andrew Desbois, Experiments aimed to establish optimal timing and dosage of antibiotics against vibriosis in *Salmo salar* using *Galleria mellonella* larvae as surrogates

10/06/2013 – 09/07/2013 **Helmholtz Zentrum für Infektionsforschung – Braunschweig, Germany**
Intern in the Department of Microbial Active Substances

PUBLICATIONS

Ulrich, L.*, Schmitz, J.*, Thurow, C. and Gatz, C. (2021) 'The jasmonoyl-isoleucine Receptor CORONATINE INSENSITIVE1 suppresses defence gene expression in Arabidopsis roots independently of its ligand', *The Plant Journal*. doi: 10.1111/TPJ.15372.

*Authors contributed equally to this work

PRIZES AND AWARDS

July 2016 **Research Based Learning Prize – University of Stirling**

Awarded for the best overall BSc research project grade within the Institute of Aquaculture in 2016

September 2019 **Best Poster Award**

Awarded by the Germany Botanical Society at the German Botanical Congress 2019

Louisa Ulrich Göttingen, 28/7/21