The Trihelix transcription factor GT2-like 1 (GTL1) promotes salicylic acid metabolism, and regulates bacterial-triggered immunity

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Abstract

The Trihelix Transcription factor GT2-like 1 (GTL1) was previously shown to be a key regulator of ploidy-dependent trichome growth and drought tolerance. Here, we report that GTL1 plays an important role in coordinating plant immunity. We show that gtl1 mutants are compromised in the regulation of basal immunity, microbial pattern-triggered immunity (PTI) and effector-triggered RIN4-mediated immunity. Transcriptome analysis revealed that GTL1 positively regulates defense genes and inhibits factors that mediate growth and development. By performing hormonal measurements and chromatin-immunoprecipitation studies, we found GTL1 to coordinate genes involved in salicylic acid metabolism, transport and response. Interaction studies and comparative transcriptomics to known data sets revealed that GTL1 is part of the MPK4 pathway and regulates oppositely the expression of differentially expressed genes in mpk4 plants. We introduced the gtl1 mutation in the mpk4 mutant and thereby partially suppressed its dwarfism and the high resistance against a bacterial invader. Our data show that GTL1 is part of the MPK4 pathway and acts as a positive regulator of bacterial-triggered immunity and SA homeostasis.

Author summary

The trihelix-transcription factor GT-2-like 1 (GTL1) belongs to the seven genes containing GT-2 family of the plant-specific trihelix transcription factors. Previously, GTL1 was shown to be a key regulator of ploidy-dependent trichome growth and drought tolerance. In this report, we show that GTL1 is part of the MPK4-signaling cascade that coordinates immunity to virulent and avirulent Pseudomonas syringae strains. gtl1 mutants are compromised in basal immunity, PTI and ETI. Comparative transcriptomics revealed a common set of differentially regulated genes in gtl1 and mpk4. In this context, GTL1 positively regulates defense genes and inhibits factors that mediate growth and development.
Salicylic acid measurements and Chromatin-Immunoprecipitation assays indicate that GTL1 directly binds and regulates genes involved in SA-biosynthesis, transport and response. The mpk4/gtl1 double mutant is compromised in the resistance to Pst AvrRPM1 and partially restored in the growth inhibition compared to mpk4 single mutant. In summary, the reduced resistance of the double mutant indicates MPK4 as a negative regulator of GTL1-mediated AvrRPM1-triggered immunity.

Introduction

Plants are faced with a constant threat of potential infections by a multiplicity of pathogenic microorganisms in their habitat. Pathogen-independent preformed physical borders like the cuticle, cell walls and wax coating represents the first line of plant defense to prevent pathogen invasion. Once the first boundary is breached, plants rely on their innate immune system to cope with different sorts of invaders and to initiate an adequate pathogen-counteracting defense response.

Plant innate immunity can be subdivided in two different recognition and response systems that relies either on the perception of pathogen-associated molecular patterns (PAMP) by plasma-membrane localized receptors (PRR) or on bacterial effectors injected in the plant cell and their recognition by intracellular receptors encoded by nucleotide binding domain leucine-rich repeat proteins (NLR-proteins) [1]. The perception of PAMPs, like FLAGELLIN22 (flg22), a 22 amino acid bacterial flagellum peptide, by the plasma membrane-localised receptor FLAGELLIN-INSENSITIVE2 (FLS2) activates the mitogen-activated protein kinase (MAPK) cascades as part of the pattern-triggered immunity (PTI) [2]. Effector-triggered immunity (ETI) is activated by pathogen-derived “avirulence” (avr) effectors injected into the plant cell by the bacterial type III-secretion system in Pseudomonas syringae cv tomato (Pst). On susceptible (r) hosts, type III effectors can contribute to virulence, by interfering with plant immunity at the level of NPR1-dependent SA signaling [3] or the activation of the MAPKs MPK4 and 11 [4]. Some effectors are recognised by specific disease resistance (R) gene products leading to ETI. R and Avr proteins often co-localize within the plant cell [5]. The most common and widely distributed class of R proteins has a central nucleotide binding (NB) domain and C-terminal Leu-rich repeats (LRRs) [6]. The activation of NB-LRR proteins triggers a multitude of robust defense responses comprising biochemical and cellular events, like localized programmed cell death (hypersensitive response) and massive transcriptional reprogramming to restrict pathogen propagation [7]. Resistance to Pseudomonas syringae strains expressing either AvrB and AvrRpm1 [8, 9] is conferred by Pseudomonas syringae pv maculicola 1 (RPM1), a CC-NB-LRR R protein, that is peripherally associated with the plasma membrane [10]. In this context, RPM1-Interacting Protein 4 (RIN4) acts as a vital defense regulator [11] and is targeted by several pathogen effectors, such as AvrRPM1, AvrRpt2, AvrPto and AvrPtoB [12]. AvrRPM1 triggers RIN4 phosphorylation [5] by RIN4-INTERACTING RECEPTOR-LIKE PROTEIN KINASE [13] to promote the defense repression mediated by RIN4. However, plants producing RPM1 R-protein detect RIN4 phosphorylation and initiate ETI [14]. AvrRpt2 is a Cys-protease that passes through self-activation and cleavage in order to cleave RIN4 at the plasma membrane [15]. RIN4 degradation imposed by AvrRpt2 is considered as a bacterial strategy to bypass AvrRpm1 induced ETI in the presence of RPM1 [16]. Recent studies have shown that SA signaling is an integral part of ETI and PTI [17, 18]. Plant innate immunity activates MAP kinase cascades typically involved in early and late immune responses. MAPK cascades consist of three sequentially activated kinase modules composed of
a MAPK kinase kinase, a MAPK kinase and eventually a MAPK, thereby linking upstream signals to downstream targets. In Arabidopsis as well as throughout the plant kingdom, the MAPK orthologues of MPK3, MPK4 and MPK6 represent the final step in the transmission of PAMP signals to respective target proteins by phosphorylation [1, 19]. Although at least six PAMP-activated MAPKs have been reported to date [20, 21], but so far clear evidence for a role in defense only exists for MPK3, MPK4 and MPK6, all three of which are required for complete activation of defense genes [22]. MPK3 and MPK6 are both activated by MKK4 and MKK5, but their upstream MAP3K(s) have not yet been unambiguously identified [23]. In contrast, there is clear evidence that the MAPKKK MEKK1 activates the MAPKs MKK1 and MKK2, which converge to activate MPK4 [24–27].

MPK3 and MPK6 regulate the expression of a number of pathways, including phytoalexins, indole glucosinolate and ethylene biosynthesis [28–30]. MPK4 positively regulates basal resistance against pathogens [31], and about 50% of flg22-induced genes require MPK4 [22]. On the other hand, mkk1, mkk1 mkk2, and mpk4 mutant plants exhibit extreme dwarfism and autoimmune phenotypes such as spontaneous cell death and constitutive defense gene expression [24, 26, 31]. However, mutations in the NLR protein SUMM2 suppress these phenotypes [32], suggesting that SUMM2 monitors the integrity of the MEKK1-MKK1/2-MPK4 pathway [33, 34]. The Pseudomonas syringae pathogenic effector HopAI1, targets MPK4 to block its kinase activity and activates SUMM2-dependent defense response. In addition to SUMM2, SUMM1 is also required for activation of defense responses in mkk1, mkk1 mkk2, and mpk4 mutant plants and encodes the MAPKKK MEKK2 [35]. MEKK2 functions upstream of SUMM2 as MEKK2 overexpression results in constitutive activation of defense responses in a SUMM2-dependent manner [35]. Recently, CALMODULIN-BINDING RECEPTOR-LIKE CYTOPLASMIC KINASE 3 (CRCK3) was identified as SUMM3. CRCK3 is directly interacting with SUMM2 and is required for the constitutive defense responses of mkk1, mkk1 mkk2, and mpk4 mutant plants and suggested to function as the “guardee” or “decoy” of SUMM2 [32]. However, negative regulation of flg22-induced gene expression occurs through MPK4 phosphorylation of the transcriptional regulator ASR3 (ARABIDOPSIS SH4-RELATED3) [36] and complementation of mpk4 mutants by a constitutively active MPK4 leads to enhanced pathogen susceptibility [37]. ASR3 belongs to a plant-specific trihelix transcription factor family and functions as a negative regulator of PTI. ASR3 suppresses a large subset of PAMP-induced genes via MPK4-mediated phosphorylation.

The trihelix-transcription factor GT-2-like 1 (GTL1) belongs to the seven genes containing GT-2 family of the plant-specific trihelix transcription factor family [38, 39]. Phylogenetic analysis of the GT-2 members shows that GT2, DF1 and GTL1 form a small clade while the other homologues are more distantly related [38–40]. A characteristic for five of the GT-2 members is the highly conserved N- and C-terminal trihelix DNA binding domain that generally binds to GT cis elements (GT1 box, 5’-GGTTAA-3’; GT2 box, 5’-GGTAAT-3’; GT3 box, 5’-GGTAAA-3’) [41–43]. Topological comparisons identified a well-conserved intervening central helix region (alpha-helical coiled-coil domain) of around 70 amino acids of unknown function. Bioinformatic analysis of GTL1 identified a putative 9-amino acid transactivation motif which fully matches to the transactivation domain previously identified for eukaryotic and viral transcription factors [44]. The loss-of-function mutant gtl1 shows large trichomes with increased levels of endoreduplication while the overexpression of GTL1 is sufficient to arrest the endocycling and cell growth in trichome and other leaf epidermal cells [44, 45]. GTL1 actively terminates ploidy-dependent cell growth by the transcriptional repression of CDH1/FZR/CCS52, an activator of the anaphase-promoting complex/cyclosome (APC/C), and is considered as a critical molecular link between developmental programming and cell-size control. In this regard, GTL1 is expressed during the post-branching stage of trichome
development, and the protein is nuclear localised. However, the expression is not restricted to leaf hairs but also found in petals, expanding roots [44], leaves [38], in the abaxial epidermis and stomata [46]. Furthermore, GTL1 is involved in the abiotic stress adaption. In this context, GTL1 was shown to regulate water use efficiency and drought tolerance by the modulation of stomatal density via the trans-repression of STOMATAL DENSITY AND DISTRIBUTION1 (SDD1) expression [46].

In this report, we show that GTL1 is part of the MPK4-signaling cascade that coordinates PTI and ETI. Comparative transcriptomics revealed a common set of differentially regulated genes by GTL1 and MPK4. GTL1 positively regulates defense genes and inhibits factors that mediate growth and development. Hormone measurements and Chromatin-Immunoprecipitation assays indicate that GTL1 directly binds and regulates genes involved in SA biosynthesis and response. The analysis of the mpk4/gtl1 double mutant suggests a genetic linkage of both factors, indicated by its compromised resistance to Pst AvrRPM1 and the increased growth compared to mpk4 single mutant, respectively.

Results

GTL1 associates with the MAPK MPK4 in vitro and in vivo

To identify unknown interaction partners of the immune MAP kinases MPK3, 4 and 6 that potentially contribute to immunity-associated processes in Arabidopsis, we analysed a collection of transcription factors (TF) following two stringent criteria. Firstly, these TFs were shown previously to function in abiotic stress adaptation, and secondly, in silico analysis by using the Eukaryotic Linear Motif resource [47] revealed putative MAP kinase docking sites. One of the transcription factors that emerged from this study was GTL1. The interactions of MAP kinases MPK3, 4 and 6 with GTL1 were assessed via in vitro pull-down assays by the use of MBP-His tagged GTL1 and GST-tagged MPK3, 4 and 6 (Fig 1A). Notably, we observed the predominate interaction of GTL1 with MPK4. However, we could not detect an association of GTL1 with MPK3 and MPK6, suggesting an exclusive biological function of GTL1 in the interplay with MPK4.

To evaluate the in vitro binding data, we applied two in vivo protein-protein interaction studies. Firstly, bimolecular-fluorescence complementation (BiFC) in Nicotiana benthamiana was performed by the use of GTL1-YFPn and MPK4-YFPc constructs. The interaction analysis showed a nuclear signal in tobacco epidermal cells demonstrating the interaction of MPK4 with GTL1 (Fig 1B). The negative control by using MPK3 and the empty-YFPc vector did not display a fluorescence signal.

To further evaluate the binding of MPK4 with GTL1, we performed a co-immuno-precipitation study coupled to mass spectrometry analysis. In this experiment, 18 day-old Arabidopsis plants were used that express an MPK4-Tandem Affinity Purification (TAP)-tagged genomic locus. This method bears the advantage to evaluate the interaction of 2 proteins at native protein levels and thereby minimising the risk to detect false-positive results imposed by the ectopic overexpression of the transgenes. We analysed three biological replicates in which GTL1 was identified and reproducibly quantified via the GTL1-specific peptide EETLALLR (amino acids 66 to 73) (Fig 1C and S1 Table), indicating that GTL1 interacts with MPK4 in vivo. In addition, the binding of MPK4 to GTL1 was evaluated in three biological replicates 15 min after applying flg22. GTL1 was similarly reproducibly identified and the PAMP-treatment did not compromise the interaction of MPK4 with GTL1 (Fig 1C). In the LC-MS/MS analysis, we detected the GTL1-specific peptide EETLALLR with a significant Mascot score of 23.9 (S1 Table). By using the Eukaryotic Linear Motif resource, we performed a protein motif analysis of GTL1 which assigned the peptide EETLALLR to a MAP kinase docking domain at
the N-terminus of the trihelix-domain that comprises amino acids 62–71 (S1A Fig). The putative MAPkinase docking site RWPREETLAL in GTL1 follows the general MAPkinase docking pattern [KR][0,2][KR],[0,2][KR],[2,4][ILVM],[ILVF] with a valid probability of 4.324e⁻⁰³ [47].
As part of the flg22-triggered signaling cascade, MPK4 commonly regulates target protein activity by phosphorylation on SP/TP sites in a PAMP-dependent manner. In several independent phosphoproteomic studies [48–52] SAAFEIAQS\(^{\text{\textbullet}}\)PANR of GTL1 was found to be phosphorylated in a stress-dependent manner. Therefore, to discover motifs in GTL1 which are targeted for phosphorylation by MPK4, in vitro kinase assays were carried out by the use of the constitutively active version of MPK4. Surprisingly, despite the availability of 5 SP and 4 TP sites in GTL1 predominantly targeted by MPK4 [53], MPK4 did not phosphorylate GTL1 at any of the sites (S1B Fig). However, the results of the positive control Target of Myb protein 1 (TOM1) were recently published by Rayapuram, et al. 2017 [54] which confirmed the functionality of the experimental setup. Taken together, we could not detect phosphorylation of GTL1 by MPK4 on SP or TP sites suggesting a regulation mechanism that relies on protein-protein interaction but not on phosphorylation.

GTL1 and innate immunity

MPK4 and the associated signaling cascades are considered as key elements in Arabidopsis innate immunity. Thus, the interaction of GTL1 with MPK4 suggests that GTL1 might play a role in the defense in Arabidopsis. To test this hypothesis, pathogen assays were performed with different Pseudomonas syringae DC3000 strains by the use of the allelic GTL1 mutants gtl1-2 (SALK_005965) [44] and gtl1-5 (Salk_044308) [46], previously described as knock-out lines. In addition, we evaluated 2 independent GTL1-GFP lines (GTL1ox1, GTL1ox2) driven by the UBIQUITIN10 promoter. The phenotype of gtl1 is very similar to WT plants (S2A Fig) underpinned by a comparable leaf morphology and area, trichome number per leaf [44] and shoot dry weight [46]. However, the trichome and in particular the trichome-branch length is enlarged, and the stomatal density is reduced which in turn is accompanied by physiological characteristics like increased drought tolerance and increased water deficit tolerance [46]. The phenotype of the GTL1ox lines is indistinguishable from WT (S2A Fig) showing comparable biomass. For the pathogen application, we decided to apply spray inoculation of different Pseudomonas strains because this treatment reflects most closely the natural course of infection. To analyse the biological function of GTL1 in basal immunity, the allelic gtl1 mutants and the two GTL1ox lines were treated by the use of the virulent hemibiotrophic pathogen Pst DC3000 and Pst DC3000 \(\Delta\)avrPto/avrPtoB. Two hours after spray infection, the infection levels in the different transgenic lines corresponded to those in WT plants indicating that stomatal immunity was not affected (Fig 1D and 1E). By contrast, after 72 hours, the allelic gtl1 mutants showed a higher proliferation level of both Pst DC3000 strains of approximately one log\(_{10}\) value than WT (Fig 1D and 1E). However, the bacterial titer in the GTL1ox lines was significantly reduced after PstDC3000 infection compared to WT. This finding shows that gtl1 mutants are compromised in basal resistance to Pst infection whereas the overexpression of GTL1 leads to a reduced susceptibility. We evaluated these results and leaf-infiltrated Pst DC3000 in WT plants and gtl1 mutants. In accordance to the spray infection, the proliferation level of the bacteria was increased in the mutant background compared to WT (S2B Fig). Based on our study, we conclude that GTL1 functions as a positive regulator of basal immunity. We also tested the susceptibility of gtl1 mutants to spray infection by the non-virulent PTI marker strain Pst DC3000 hrcC- which is mutated in the type-III secretion system and hence unable to deliver effector proteins. gtl1 mutants showed higher proliferation levels of Pst hrcC- while the GTL1ox lines exhibit a WT-like resistance (Fig 1F, S2C Fig). These results indicate that GTL1 is a positive regulator of basal immunity and PTI.
Basal hydrogen-peroxide level is affected in gtl1 and GTL1ox lines

To characterise the enhanced susceptibility of gtl1 mutants and the increased resistance of GTL1ox lines in more detail, the levels of the reactive oxygen species (ROS) H$_2$O$_2$ was assessed by 3,3’-diaminobenzidine (DAB) staining in untreated WT plants, gtl1-2 mutant and GTL1ox lines. We observed that the H$_2$O$_2$ level in gtl1 is reduced in comparison to WT as depicted by a weaker staining intensity (Fig 2A–2C). In contrast, two independent GTL1ox lines displayed intense staining after the DAB exposure which demonstrates higher H$_2$O$_2$ levels than WT (Fig 2D). These findings show that the basal H$_2$O$_2$ level depends on the GTL1 function. Furthermore, ROS production and release are among the first defense reactions in response to pathogen perception. The ROS burst after flg22 treatment was significantly reduced in gtl1 mutants to approximately 50%, 15 min after application (Fig 2E, S2D Fig). However, the GTL1ox lines showed elevated ROS release after flg22 treatment (Fig 2F). The differences in the ROS efflux after flg22 application in gtl1 and GTL1ox lines might be a direct consequence of the affected basal H$_2$O$_2$ levels. The activation of the flg22-triggered signaling cascade was evaluated by pTpY antibody-based immunoprecipitation that targets the phosphorylated MAPK3, 4 and 6 versions. The highest activation of the three MAPK was achieved 15 min after flg22 treatment.
in both gtl1-2 and WT plants (Fig 2G). The comparable activation of MPK3, 4 and 6 suggests a function of GTL1 downstream of the flg22-induced MAP kinase signaling cascades.

**Comparative transcriptome composition**

Firstly, to identify biological processes and genes that are governed by GTL1, the transcriptome of 14 day-old plants of gtl1-2 mutant and WT was analysed by performing RNA-seq. At a stringency of \( p \leq 0.01 \), 1448 genes differently regulated genes (S1 Table) could be identified that show a log2 fold change from -5.29 to -0.53 of negatively regulated genes and from 0.52 to 5.00 of positively regulated genes. Among these 1448 genes, 678 genes are up-regulated, and 770 genes are down-regulated (Fig 3A). The GO term analysis of down-regulated genes revealed gene functions for Innate Immune Response, Systemic Acquired Resistance and Response to biotic stimulus and suggests a reduced ability of gtl1-2 in these processes (Fig 3B). Furthermore, we found genes being down-regulated in gtl1-2 that contribute to hydrogen peroxide metabolic process (S1 Table). For example, ATRBOHC/RHD2 [55] and a substantial number of peroxidases (S1 Table) that contribute to \( \text{H}_2\text{O}_2 \) generation [56], such as PRXCB, PER4, PRX37 and PRX25, are compromised in their expression. The GO terms in the set of up-regulated genes emphasised gene functions in Nucleotide Biosynthesis Process, Ribosome Biogenesis and Response to Sucrose Stimulus and can be summarized in support of plant growth and development (Fig 3C, S1 Table). All in all, the GO analysis indicates GTL1 as a positive regulator of immunity-related processes and a suppressor of biological functions related to plant growth.

Secondly, we sought to investigate whether the transcriptome composition of gtl1 and mpk4 is compromised in the same set of downstream targets. Therefore, deregulated genes in the mpk mutants [57] and gtl1 were analysed by hierarchical clustering with mutants of MAPKs 3, 4 and 6 (Fig 3D). Interestingly, mpk3 and mpk6 mutants showed only a small overlap in gene expression with gtl1. However, a large number of genes in the gtl1 mutant showed an opposite pattern of gene expression in the mpk4 mutant. Among the two main clusters that were identified in the comparison between gtl1 and mpk4, the 123 genes in cluster I are up-regulated in gtl1-2 and down-regulated in mpk4 (Fig 3E, S2 Table). The GO term analysis highlights gene functions in Response to Light, RNA Metabolism and Lipid Biosynthesis Process. Cluster II, comprising 319 genes, which are down-regulated in gtl1-2 and up-regulated in mpk4, displays assigned GO terms for Innate Immunity and Response to bacterium (Fig 3F, S2 Table). The consensus matrix (Fig 3G, S2 Table) illustrates the dissimilarity of the gene sets in the gtl1 and mpk4 transcriptomes and also shows the difference to the transcriptomes of mpk3 and mpk6. These findings indicate a genetic interaction of GTL1 and MPK4 in the regulation of distinct biological processes. To evaluate the RNAseq-based transcriptome comparison, the expression of three representative genes that contribute to SA-biosynthesis and response (S2E Fig) was analysed in the gtl1 and mpk4 mutant by qPCR. Firstly, CAM-BINDING PROTEIN 60-LIKE G (CBP60g) works cooperatively with SARD1 [58, 59] to regulate the expression of ICS1 to induce SA-metabolism; secondly, PHYTOALEXIN DEFICIENT 3 (PAD3), that catalyses the conversion of dihydrocamalexin acid to camalexin [60, 61] and lastly, ELICITOR-ACTIVATED GENE 3 (ELI3-2/CAD8) acting as alcohol:NADP+ oxidoreductase [62]. The expression of CBP60g, PAD3 and CAD8 is diminished in gtl1 when compared to WT, but enhanced in the mpk4 mutant (S3A–S3C Fig).

**Salicylic acid metabolism and homeostasis are affected in gtl1 and GTL1ox**

In addition to CBP60g, the RNAseq-based transcriptome analysis also revealed that a number of genes are affected in gtl1 mutants that contribute to the regulation of SA biosynthesis or its
GTL1 promotes salicylic acid metabolism and regulates bacterial-triggered immunity

A. GO-term down-regulated genes
- MAPKKK cascade
- response to bacterium
- systemic acquired resistance
- innate immune response
- response to biotic stimulus

B. GO-term up-regulated genes
- response to sucrose stimulus
- galactolipid metabolic process
- ribosome biogenesis
- nucleotide biosynthetic process
- RNA modification

D. Cluster I
- response to light stimulus
- RNA metabolic process
- response to nutrient levels
- cellular response to starvation
- lipid biosynthetic process

F. Cluster II
- innate immune response
- response to oxidative stress
- response to bacterium
- response to biotic stimulus
- defense response

G. Consensus Matrix
- mpk3
- mpk4
- mpk6
- gtl1-2

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signaling events (Fig 4A). Compared to WT, the transcriptome analysis of gtl1 mutants before and after PAMP application highlights in down-regulated genes GO categories for SA Biosynthesis, Systemic Acquired Resistance and Response to SA (Fig 4B and 4C). In untreated plants, the genes involved in SA biosynthesis, such as CBP60g, PBS3 and WRKY46 (Fig 4A) as well as SA signaling and PAMP-response targets, such as WRKY72, PR1 and FRK1 (Fig 4A and 4E) are down-regulated. To assess PAMP-triggered SA-metabolism and signaling in gtl1-2, the transcriptome composition was analysed 1 hour after flg22-treatment (S3 Table). In this regard, the expression of the key-SA biosynthesis gene ICS1, as well as its transcriptional activator CBP60g, is diminished (Fig 4D and 4F). Furthermore, the expression of the genes considered as central factors in SA signaling NPR1 and NIMIN1 is reduced (Fig 4D) as well as those of FRK1 (S3D Fig), WAK2 and PAD3 contributing to SA-mediated response (Fig 4D and 4E). If GTL1 acts as an activator of genes involved in SA metabolism and signaling, then the expression of these genes is expected to be predominantly elevated in GTL1ox lines compared to the gtl1 mutant and WT, respectively. Indeed, the expression FRK1, CBP60g and PAD3 (Figs 4E, 5C and 5I) are significantly increased in the GTL1ox lines. These results indicate that GTL1 functions as a positive regulator of SA-mediated processes.

Consequently, we determined the levels of free SA in WT, mpk4-2 and gtl1-2 mutant, and GTL1ox1 line in at least three biological replicates. Peterson et al, 2000 [31] showed that the SA accumulation in mpk4 mutants is up to 10 fold higher than WT and our measurement are in accordance with these results (Fig 4G). After analysing 6 biological replicates, we determined a concentration of 16.56 ng SA/mg dry weight in mpk4 compared to 0.47 ng/mg in WT. The high SA values in mpk4 indicate MPK4 as a repressor of SA accumulation. Remarkably, the basal SA amount in the gtl1-2 mutant is consistently lower than WT levels (Fig 4H), while the basal SA concentration in the GTL1ox line is significantly increased compared to WT (Fig 4I). Taken together, these results indicate that GTL1 is a positive regulator of genes involved in SA biosynthesis and promotes basal SA accumulation.

**GTL1 regulates genes involved in SA-metabolism and signaling**

In genome-wide binding studies (ChIP-chip) [45], the association of GTL1 to regulatory sequences upstream and downstream of a large set of genes was revealed. A consensus binding motif for GTL1 was identified and described as GT3 box [5'-GGTAAA-3']. In a previous invitro study, it was shown that the N-terminal DNA binding domain of GTL1 associates both to GT1 and GT2 boxes [46]. The ChIP-chip approach was carried out by the usage of the whole aerial part of 12 day-old gtl1-1 plants that were complemented by pGTL1::GTL1::GFP. Among the total number of 2398 target genes, GTL1 was found to bind to the promoter regions of CBP60g, EDS5 which codes for an SA-transporter [63] and PAD3 (Fig 5A, 5D and 5G) as indicated in the Integrated Genome Browser diagram. To evaluate the association of GTL1 to these direct target genes, we performed Chromatin-Immunoprecipitation (ChIP) followed by quantitative PCR (qPCR) using gene-specific primer sets (P1, P1, G1). 14 day-old Arabidopsis seedlings expressing pUB110::GTL1::GFP (S4A Fig) were generated and 3 independent transgenic lines were selected and used to confirm the binding of GTL1 to selected chromatin
Fig 4. The expression of SA-metabolism/signaling genes and the levels of salicylic acid are compromised in gt1-2 mutant. A, D) Depiction of down-regulated genes (A) in gt1-2 contributing to SA-biosynthesis and signaling without treatment (S1 Table) and (D) after flg22 treatment (S3 Table) compared to the respective expression in WT. B, C) Significantly down-regulated genes (p < 0.01, 769 genes) in gt1-2 before and after flg22 treatment (p < 0.0001, 715 genes) can be grouped in GO terms describing gene functions for SA-signaling and metabolism (S2 and S3 Tables). E-F) Expression of SA/PTI-response gene FRK1 (E, S3D Fig) is reduced in gt1-2 before and after flg22 treatment but shows elevated expression in the GT1 overexpression lines in untreated conditions. The expression of the SA-biosynthesis gene ICS1 (F) is diminished in gt1-2 after flg22 treatment. Statistical significance was analyzed by Student’s test. Error bars, mean ± SEM, letters above bars represent significance groups, p < 0.01. G-I) Quantitative analysis of free salicylic acid by using LC-MS/MS. Box plots are depicted for (G) mpk4-2, (H) gt1-2 and (I) GT1ox-1 compared to WT. Boxes showing the interquartile range (IQR) 25th to 75th percentiles, inner square representing the median, whiskers show the SEM, outliers are
regions. To discriminate against false-positive binding caused by GFP, a negative control expressing GFP under the UBI10 promoter was employed (S4A Fig). By ChIP-qPCR, the binding preference of GTL1 to the promoter region of CBP60g close to the transcriptional start sequence (TSS) could be confirmed (Fig 5B). Intriguingly, in our ChIP-qPCR study, GTL1 binds predominantly to the region-790 bp to 707 bp upstream of the TSS that contains one GT2 motif previously described as the binding motif of GT transcription factors [43]. Accordingly, the binding of GTL1 to the region upstream of the TSS of EDS5 could be confirmed to the 5’region P1 and P2 (Fig 5D and 5E) [45]. Upstream of the TSS of EDS5 several GT boxes can be found facilitating the binding of GTL1. Moreover, GTL1 binds to the promoter region of PAD3 upstream of the TSS and as well in the 5’-ORF (Fig 5G and 5H). Notably, several GT-boxes (GT1) dedicated to GTL1 binding can be found indicating specific binding of GTL1 to the PAD3 genomic region. Taken together, in accordance with the genome-wide binding studies of Breuer et al, 2012 and the presented ChIP evaluations, CBP60g, EDS5 and PAD3 could be confirmed as direct downstream targets of GTL1. To find out whether GTL1 exerts transcriptional control on CBP60g, EDS5 and PAD3, we analysed their expression in the gtl1 mutant and the GTL1ox lines. On the one hand, the expression of CBP60g and PAD3 is reduced in the gtl1 mutant and elevated in the GTL1ox lines (Fig 5C and 5I) under untreated conditions which suggest GTL1 as a transcriptional activator of these genes under non-stress conditions. After flg22-treatment, the expression of CBP60g and PAD3 is also reduced in the gtl1 mutant, but in the GTL1ox lines, the expression is ambiguous for CBP60g and WT-like in the case of PAD3 (S4B and S4D Fig). On the other hand, the expression of EDS5 is elevated in gtl1 and reduced in the GTL1ox lines (Fig 5F) which implies GTL1 as a repressor of EDS5 expression. After flg22-treatment, the expression of EDS5 is not broadly perturbed from WT (S4C Fig). Our results suggest GTL1 as a transcriptional regulator of these genes involved in SA-biosynthesis, transport and response.

GTL1 regulates immunity against the bacterial effectors AvrRpm1 and AvrRpt2

Since MPK4 is also involved in effector-triggered immunity [37], we tested the bacterial strains Pst DC3000_AvrRpm1 and PstDC3000_AvrRpt2 which upon injection of the bacterial effectors trigger RIN4-dependent ETI [5]. Two hours after spray infection, the growth level of the bacteria in the transgenic lines was indistinguishable from WT (Fig 6A and 6B). However 72 hours after infection, we observed enhanced bacterial growth of about one fold change in either Pst DC3000 strains in the allelic gtl1 mutants (Fig 6A and 6B), while the GTL1ox lines are not broadly perturbed in their immunity compared to WT, respectively (S4E and S4F Fig). These results show that gtl1 mutants are compromised in RIN4-AvrRpm1/AvrRpt2 induced immunity which indicates a function of GTL1 in the effector-triggered immunity. To pinpoint whether GTL1 contribute to SA accumulation after bacterial infection, we determined the free SA levels by LC-MS/MS analysis 24 hours after Pst DC3000_AvrRpm1 infection in the gtl1 mutant. We found that the SA accumulation is significantly reduced to 3.5 ng/mg compared to WT showing an SA level of about 4.7 ng/mg in the average of 4 biological replicates, respectively (Fig 6C). Our results show that GTL1 is necessary for the SA accumulation as part of the ETI.
Fig 5. The SA-biosynthesis activator CBP60g, the SA-transporter EDS5 and the SA-responsive gene PAD3 are bound and regulated by GTL1. A, D, G) Microarray data, provided by Breuer et. al 2012[45] show significant enrichment of GTL1 binding in
gtl1 partially restores susceptibility of mpk4 and growth defects

Previously, it was reported that the strong autoimmune phenotype in mpk4-3 largely depends on SUMM2 [34]. However, the accumulation of H$_2$O$_2$ and PR gene expression are only to some extent diminished in the mpk4/summ2 double mutant and show still significant enhancement compared to WT [34]. Furthermore, the severe dwarfism of the mpk4 mutant is not fully restored by the introduction of different allelic summ2 mutations [34]. Eventually, these previous results indicate that MPK4 is involved in immune and growth regulation independently of SUMM2 [34]. To test whether defense response to bacterial attack in GTL1 depends on MPK4, we generated and analysed the mpk4-2/gtl1-2 double mutant. As depicted in Fig 7A and 7B, the fresh weight of the double mutant is increased by about 14% of 4 week-old plants and 34% of 7 week-old plants (S4G Fig) compared to mpk4-2 single mutants, respectively. Furthermore, the trichome branch length is extended in the double mutant compared to mpk4 single mutant suggesting a partial suppression of developmental defects in mpk4 plants by the gtl1 mutation (Fig 7C and 7D). To evaluate the genetic interaction of
MPK4 with GTL1 in the effector-triggered immunity, the double mutant was treated by \textit{Pst DC3000 AvrRPM1}. At two hours after infection, the bacterial titer was indistinguishable from WT in \textit{gtl1}, \textit{mpk4} single mutants, and \textit{mpk4/gtl1} double mutant thereby suggesting that stomatal immunity is not perturbed (Fig 7E). However, after 72 hours, the proliferation level in the \textit{mpk4} single mutant was significantly reduced compared to WT, while the bacterial titer was elevated in \textit{mpk4/gtl1} compared to \textit{mpk4} single mutants (Fig 7E). Based on our findings, we postulate that MPK4 functions as a negative regulator of GTL1 in \textit{AvrRPM1}-triggered RIN4-mediated immunity.

**Discussion**

**Role of GTL1 in basal resistance and PTI**

In this study, we identified the trihelix transcription factor GTL1 as a regulator of immunity. Using pathogen assays with virulent \textit{Pst DC3000}, \textit{Pst DC3000 ΔavrPto/avrPtoB} and non-virulent \textit{Pst DC3000 hrcC}- strains, we showed that GTL1 is a positive regulator of basal defense and PTI, respectively. Transcriptome analysis suggested that GTL1 functions on similar targets as the MPK4 pathway. However, whereas MPK4 negatively regulates the overlapping set of targets contributing to defense and immunity, GTL1 regulates them in a positive manner. In this context, the \textit{mpk4} mutant exhibits enhanced resistance to \textit{Pst DC3000} and elevated expression of defense markers [31]. Since PAMP-triggered MAPK activation is not affected in \textit{gtl1} mutants, it is likely that GTL1 functions downstream of the MEKK1-MKK1/2-MPK4 cascade. This hypothesis is supported by the fact that GTL1 forms part of the MPK4 protein complex. We also found that GTL1 can directly interact with MPK4 and that this interaction is specific as no interaction was detected with the related immune MAPKs MPK3 and MPK6. Interestingly, we could not detect phosphorylation of GTL1 by MPK4, suggesting a regulation mechanism that relies on protein-protein interaction rather than phosphorylation.
GTL1 is a positive regulator of SA biosynthesis and signaling but negatively regulates growth

The transcriptome pattern of gtl1 mutants revealed that GTL1 is a positive factor for defense gene expression but a negative regulator of genes involved in growth, supporting the concept that growth and defense are inversely coupled. In agreement with a role of GTL1 in suppressing growth, gtl1 mutants are also slightly bigger than WT plants under water-deficiency [46], but as shown here, this feature comes with the caveat of being more susceptible to pathogen attack. Conversely, mpk4 mutant plants are dwarfed but are incredibly pathogen resistant. A characteristic feature of mpk4 mutants is the increased SA level that correlates with its enhanced resistance to the virulent strains of Pseudomonas syringae [31]. Interestingly, SA amounts in gtl1-2 mutant and lines expressing a constitutively active MPK4 version [37] were consistently lower than WT suggesting an opposing regulation of SA homeostasis. Since gtl1 has reduced whereas mpk4 massively, enhanced SA levels, one might be tempted to conclude that these different sensitivities could be solely due to SA amounts. However, this assumption is probably too simple as suppression of SA levels in mpk4 mutants could only relieve the dwarf phenotype to some extent [31].

Transcriptome analysis confirmed a role of GTL1 as a positive regulator of SA and defense as well, by showing reduced levels of the SA/PAMP-marker genes, such as PR1 and FRK1. This effect seems to be mediated both at the level of SA signaling genes, exemplified by NPR1 and NIMIN1, as well as at the level of the biosynthesis gene ICS1/SID2 and its regulator CBP60g. The analysis of available ChIP-chip data [45] indicated that GTL1 binds to a number of its target genes via interaction of the GT boxes. By ChIP-qPCR, we could verify that GT elements are involved in the regulation of the CBP60g, EDS5 and PAD3 genes by GTL1. CBP60g binds to and promotes the expression of the SA-biosynthesis gene ICS1 and the SA-signaling NPR1 [59]. Furthermore, WRKY33 and MKS1 are two downstream target proteins of flg22-activated MPK4 that mutually regulate the expression of PAD3 encoding an enzyme required for synthesis of antimicrobial camalexin [61, 64]. Recently, it was shown that the MEKK1-MKK1/2-MPK4 cascade is guarded by the NB-LRR gene SUMM2 and that the guardee of this system is CRCK3 which directly interacts with SUMM2 [32]. The double mutant of mpk4/summ2 is to some extent suppressed in the mpk4 autoimmune phenotype and partially restored in growth. Eventually, these data explain the severe mpk4 mutant phenotypes and suggest that MPK4 acts actually as a positive regulator of defense. However, the only partial suppression indicates that MPK4 is involved in immune and growth regulation independently of SUMM2. The up-regulation of PAD3 is unaffected in the summ2 mutant after flg22-application [34].

Unlike the gtl1-2 mutant, the compromised up-regulation of PAD3 after flg22-treatment refers directly to the cooperation of MPK4 and GTL1 in a SUMM2 independent manner.

However, MPK4 also negatively regulates defense genes as evidenced by the fact that expression of a constitutively active version of MPK4 results in pathogen hypersensitivity [37]. Moreover, a negative role of MPK4 in defense gene expression is also provided by the work on the transcriptional repressor ASR3, whereby PAMP-induced MPK4 phosphorylation of ASR3 was shown to enhance its DNA binding and repression of a considerable number of defense target genes [36]. ASR3 acts as a transcriptional repressor through its EAR motif and displays opposite FRK1 regulation as GTL1. Interestingly, ASR3 is also a member of the plant-specific trihelix transcription factor family but belongs to an SH4 clade. The asr3 mutant shows, unlike gtl1, an enhanced resistance against virulent bacterial strains. By contrast, the susceptibility of asr3 to infection by the avirulent strain PstDC3000 avrRpt2 matches WT plants. Pathogen resistance to PstDC3000 AvrRpt2 and PstDC3000 AvrRpm1 is triggered upon perception by the CC-NB-LRR receptor. The fact that gtl1 exhibits an enhanced susceptibility to either
PstDC3000 strains demonstrates that both trihelix TF family members do not act redundantly and exert distinct and opposite biological functions in immunity.

Interestingly, GTL1 was recently also shown to alter drought tolerance of Arabidopsis [46], and the underlying mechanism was suggested to be due to the altered, reduced number of stomates in gtl1 plants making them more robust under drought conditions. GTL1 is assumed to monitor the water status in plants to determine the most appropriate number of stomates during plant development. This effect was shown to be exerted through the repression of the SDD1 gene as a direct target of GTL1.

In summary, the current data suggest that fine-tuning of GTL1 activity plays an important role in defining the balance between growth, defense and developmental adaptations to biotic and abiotic stress conditions. Given its involvement and role in these processes, further studies are warranted into the regulation of GTL1 at the post-translational level.

**GTL1 contribute to RIN4-mediated Effector-triggered immunity**

RPM1-INTERACTING PROTEIN 4 (RIN4) interacts with AvrRpm1 and Pseudomonas syringae pv maculicola 1 (RPM1) [5, 65], whereby the association of AvrRpm1 provokes the phosphorylation of RIN4 by RIN4-interacting receptor-like protein kinase (RIPK) [13] enhancing its activity as a negative regulator of plant defense. However, phosphorylated RIN4 induces the activation of the R-protein RPM1 triggering the RPM1-dependent defense response [13]. MPK4 is a crucial regulator of defense against virulent pathogens and PTI, but the protein kinase is also implicated in ETI regulation [37]. Consistent with a role of GTL1 in ETI, resistance of gtl1 plants infected with the avirulent Pst AvrRpm1 and Pst AvrRpt2 strain was compromised in the gtl1 background, indicating that GTL1 is a positive regulator of RPM1/ RPT2-mediated ETI. Interestingly, mpk4 mutants complemented by the constitutively active MPK4 (CA-MPK4) exhibit distinct responses to different avirulent Pseudomonas strains. CA-MPK4 lines are affected in pathogen resistance mediated by TIR-NB-LRR, but not CC-NB-LRR, receptors. In this regard, CA-MPK4 lines retained WT-like resistance to Pst DC3000 AvrRpm1 recognised by CC-NB-LRR receptors, whereas we showed that the mpk4 mutant is more resistant, while the mpk4/gtl1 mutant partially restored susceptibility. Therefore, we postulate that MPK4 functions as a negative regulator of GTL1 in AvrRpm1 -triggered RIN4-mediated immunity. In summary, we reason that GTL1 is embedded in the MPK4 pathway and coordinates SA-metabolism and homeostasis which directly impacts basal immunity, PAMP- and effector-triggered immunity.

**Materials and methods**

**Plant material and growth conditions**

Experiments were performed by the usage of Arabidopsis thaliana of the Columbia accession grown on soil in plant growth chambers (Percival Scientific) under short-day conditions (8h light/ 16 h dark) at 22°C. Nicotiana benthamiana were grown under long-day conditions (16 h light + 8 h darkness) at 28°C. gtl1-2 (Salk_005965), gtl1-5 (Salk_044308) and mpk4-2 (Salk_056245) seeds were obtained from NASC.

**Accession numbers**

GTL1 (AT1G33240), MPK4 (AT4G01370).

**Additional Materials and Methods**

See S1 Materials and Methods
Supporting information

S1 Fig. Domain map of GTL1 and in-vitro kinase assay. A) Schematic representation of GTL1; NTH-Myb-like, N-terminal trihelix domain; CC, Coiled coil domain; CTH-Myb-like, C-terminal trihelix domain; red bar, putative MAPK interaction domain.

B) In in-vitro kinase assays followed by LC/MS-MS, MPK4 does not phosphorylate GTL1 at the previously reported phosphopeptide nor at another site.

S2 Fig. Phenotypical and functional characterization of gtl1 and GTL1ox lines. A) Overview of the phenotype and shoot-fresh weight of WT, gtl1-2, gtl1-5, GTL1ox1 and GTL1ox2 plants. The shoot fresh weight was analyzed of 2 week-old plants in 3 biological replicates. Error bars, mean ± SEM, statistical significance was analyzed by Student’s test; n.s, non-significant against WT. Scale bar = 1 cm.

B) The allelic GTL1 mutants gtl1-2 and gtl1-5 were challenged by leaf infiltration with PstDC3000. Plants, of three biological replicates, were leaf-infiltrated with a bacterial suspension at OD$_{600}$ 0.005, the density of colony-forming units (cfu) was analyzed 2 and 72 hours post inoculation (hpi). Error bars, mean ± SEM, statistical significance was analyzed by Student’s test, asterisks indicate significant differences compared to treated WT, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

C) Pathogen-treatment of GTL1ox1 and GTL1ox2 lines refers to Fig 1F.

D) Negative control for ROS-burst assay (Fig 2E)

E) Expression of PAD3 and CAD8 after SA application. 14 day-old WT seedlings were treated with 1μM SA for 6 hours. Error bars, mean ± SEM, Asterisks indicate significant differences compared to untreated WT, * $p \leq 0.05$, *** $p \leq 0.001$.

S3 Fig. Expression of CBP60g, CAD8, PAD3 and FRK1. A-B) Expression of CBP60g (A), PAD3 (B) and CAD8 (C) in gtl1-2 and mpk4.

D) Expression of FRK1 after SA-treatment. 14 day-old WT seedlings were treated with 1μM SA for 6 hours. Error bars, mean ± SEM, statistical significance was analyzed by Student’s test, letters above bars represent significance groups, $p \leq 0.01$.

S4 Fig. Expression of CBP60g, EDS5 and PAD3 and further pathogen-treatments of GTL1ox lines. A) GTL1_GFP fusion protein and single GFP in Arabidopsis root of independent transgenic lines used for ChIP.

B-D) Expression of CBP60g (B) and EDS5 (C) and PAD3 (D) in gtl1-2, GTL1ox1 and GTL1ox2 after flg22 application (1μM, flg22 for 1hr). Statistical significance was analyzed by Student’s test. Letters above bars represent significance groups, $p \leq 0.05$; n.s, non-significant.

E-F) Pathogen-treatment of GTL1ox1 and GTL1ox2, refers to Fig 6A and 6B.

G) WT, gtl1-2, mpk4-2 and mpk4/gtl1 double mutant. The shoot fresh weight was analyzed of 7 week-old plants in 3 biological replicates. Error bars, mean ± SEM, statistical significance was analyzed by Student’s test. Letters above bars represent significance groups, $p \leq 0.001$.

S1 Table. Transcriptome composition of gtl1-2 /WT of 14 day-old seedlings. GO terms of up- and down-regulated genes. Isolation of GTL1 in the in vivo MPK4-Tandem Affinity Purification approach combined with an LC-MS/MS analysis before and after flg22 application.

(XLSX)
S2 Table. Genes dedicated to cluster I and II, GO term analysis, Matrix-cluster. (XLSX)

S3 Table. Transcriptome composition of gtl1-2/WT of 14 day-old seedlings, 1 hr after flg22-treatment, p ≤ 0.0001, Go term analysis of down-regulated genes. (XLSX)

S1 Materials and Methods. (DOCX)

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