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The Polycomb protein LHP1 regulates *Arabidopsis thaliana* stress responses through the repression of the MYC2-dependent branch of immunity

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SUMMARY

Polycomb repressive complexes (PRCs) have been traditionally associated with the regulation of developmental processes in various organisms, including higher plants. However, similar to other epigenetic regulators, there is accumulating evidence for their role in the regulation of stress and immune-related pathways. In the current study we show that the PRC1 protein LHP1 is required for the repression of the MYC2 branch of jasmonic acid (JA)/ethylene (ET) pathway of immunity. Loss of *LHP1* induces the reduction in H3K27me3 levels in the gene bodies of *ANAC019* and *ANAC055,* as well as some of their targets, leading to their transcriptional upregulation. Consistently, increased expression of these two transcription factors leads to the misregulation of several of their genomic targets. The *lhp1* mutant mimics the *MYC2*, *ANAC019*, and *ANAC055* overexpressers in several of their phenotypes, including increased aphid resistance, abscisic acid (ABA) sensitivity and drought tolerance. In addition, like the *MYC2* and *ANAC* overexpressers, *lhp1* displays reduced salicylic acid (SA) content caused by a deregulation of *ICS1* and *BSMT1*, as well as increased susceptibility to the hemibiotrophic pathogen *Pseudomonas syringae* pv. tomato DC3000. Together, our results indicate that LHP1 regulates the expression of stress-responsive genes as well as the homeostasis and responses to the stress hormones SA and ABA. This protein emerges as a key chromatin player fine tuning the complex balance between developmental and stress-responsive processes.

Keywords: Polycomb, H3K27me3, LHP1, MYC2, stress, pathogen, drought, abscisic acid, aphid, salicylic acid.

INTRODUCTION

An appropriate regulation of responses to environmental and developmental cues is essential for the survival of all organisms as individuals and species (Huot *et al.*, 2014). Plants, as multicellular sessile organisms, face innumerable biotic and abiotic stressors throughout their life cycles, ranging from drought, extreme temperatures and heavy metals, to herbivory and microbial pathogens, *inter alia*. For instance, it is well known that upon the recognition of a pathogenic microorganism, plants activate a network of signaling cascades that lead to a massive transcriptional reprogramming and a series of defense responses in order to restrain the infection (Nürnberger and Brunner, 2002; Bent and Mackey, 2007; Boller and Felix, 2009; Bigeard *et al.*, 2015). However, it is also known that the excessive and unnecessary activation of immune responses can have detrimental effects on physiology, as evidenced by the existence of several autoimmune mutants, many of which display compromised development and growth (van Wersch *et al.*, 2016). Plants must therefore fine tune the expression of stress-responsive

genes, and there is increasing evidence indicating that chromatin dynamics plays a crucial role in this process (Smale *et al.*, 2014; Mehta *et al.*, 2015; Probst and Mittelsten Scheid, 2015; Espinas *et al.*, 2016; Lämke and Bäurle, 2017; Ramirez-Prado *et al.*, 2018a,b). The characterization of mutants for various chromatin modifiers has elucidated the role of several of these proteins in the regulation of both developmental and stress-responsive pathways. Several epigenomic regulators with diverse functions, including histone mark writers, readers and erasers, were identified as positive and negative regulators of immunity, or shown to play an important role in the regulation of the interplay between the diverse hormonal pathways that constitute the plant immune system (Bu *et al.*, 2008, Ding and Wang, 2015; Espinas *et al.*, 2016; Ramirez-Prado *et al.*, 2018a,b).

Polycomb group (PcG) proteins were first identified as repressors of the Homeotic (*Hox)* genes in *Drosophila melanogaster,* regulating the identity of the body segments of this organism (Lewis, 1978; Kassis *et al.*, 2017). These proteins combine with each other in order to form multisubunit Polycomb repressive complexes (PRCs), named PRC1 and PRC2. It is generally accepted that the PRC2 complex performs the deposition of the repressive H3K27me3 mark on its targets, which are recognized by PRC1 in order to further stabilize the repression of gene expression via histone ubiquitination (Sanchez-Pulido *et al.*, 2008; Bratzel *et al.*, 2010; Endoh *et al.*, 2012; Zhou *et al.*, 2017); nevertheless, several reports in *Drosophila* and other organisms, have challenged this initial model, evidencing that the relationship between PcG proteins is much more complex than formerly thought. There is growing evidence indicating that PRC1 and PRC2 can bind independently to chromatin or, in contrast, generate binding sites for each other (Schwartz and Pirrotta, 2013, 2014; Di Croce and Helin, 2013).

The transcriptional repression mediated by PRCs plays an important role in many biological processes in various organisms including the inactivation of the X chromosome in animals (Wutz, 2011), prevention of senescence (Jacobs *et al.*, 1999), imprinting (Terranova *et al.*, 2008; Wolff *et al.*, 2011), and the maintenance of stem cell identity (Rajasekhar and Begemann, 2007). In contrast with *D. melanogaster*, *Arabidopsis thaliana* counts with three different core PRC2 complexes and each one preferentially regulates the expression of genes at specific developmental stages (Wang *et al.*, 2016). Embryonic development is regulated principally by FIS-PRC2 while vegetative development is controlled by the EMF2–PRC2 complex. Flowering is another biological process widely recognized for being controlled by PcG proteins, mainly by components of the PRC1 and VRN–PCR2 complex in which LHP1 (LIKE HETEROCHROMATIN PROTEIN 1) and CLF (CURLY LEAF) are major players, respectively (Wang *et al.*, 2016). A recent study showed that the formerly thought embryo-specific H3K27me3 methyltransferase MEA (MEDEA) is induced by biotic stress and involved in the repression of immune responses in Arabidopsis through the targeting of defense genes, thus indicating that PRCs activity is not limited, at least in plants, to the regulation of developmental transitions but that they can also contribute to stress responses (Roy *et al.*, 2018).

LHP1 (also known as TFL2 and TU8), the only Arabidopsis homolog of the *Drosophila melanogaster* HETEROCHROMATIN PROTEIN1 (HP1), is considered as a component of the PRC1 complex, as it co-localizes with and binds to H3K27me3 marks through its chromodomain (Gaudin *et al.*, 2001; Turck *et al.*, 2007; Zhang *et al.*, 2007). This protein interacts with several of the PRC1 components, including BMI1 and RING1; nevertheless, the *lhp1* mutant developmental phenotype is similar to that of *clf,* and recent studies have described a stable physical interaction between LHP1 and the PRC2 complex, challenging this classification (Derkacheva *et al.*, 2013). In recent publications, we and others depicted the importance of LHP1 in the spreading of H3K27me3, independently of PRC1. Indeed, we found that loss of this protein leads to narrower H3K27me3 peaks on the 5′ region of genes, indicating its importance for the spread of this methylation mark over the gene body of its targets and the stabilization of a repressive environment (Veluchamy *et al.*, 2016; Wang *et al.*, 2016). It is well known that LHP1 represses the transcription of numerous genes that participate in flowering and floral organ identity, such as *Flowering Locus T* (*FT), Flowering Locus C (FLC), AGAMOUS (AG)*, and *APETALA3 (AP3)*. In fact, Arabidopsis *lhp1* mutants present early flowering and altered flower development (Gaudin *et al.*, 2001), which could be partly explained by increased levels of *FT* and the ectopic expression of several MADS-BOX transcription factors (TFs) such as *AG*, *AP2*, and *SEP3* along the floral whorls (Ohto *et al.*, 2003; Takada and Goto, 2003). Furthermore, this mutation leads to a highly pleiotropic phenotype, characterized by reduced plant size and shortened internode length, as well as downward curled leaves and early senescence (Gaudin *et al.*, 2001).

In addition to its well characterized role during plant development, there are hints that LHP1 could participate to the regulation of stress responses. Indeed, LHP1 was identified as a partner of LIF2, a heterogeneous nuclear ribonucleoprotein Q (hnRNP-Q) with three RNA recognition motifs. Both proteins seem to co-localize on stress-responsive genes, suggesting a role in the regulation of their expression (Molitor *et al.*, 2016). The *lif2* mutant has been further characterized as a regulator of immune responses in Arabidopsis (Le Roux *et al.*, 2014); however, the specific role of LHP1 in the regulation of stress responses has not been elucidated to date. In the current study we show that LHP1 negatively regulates the MYC2-dependent branch of

immunity by targeting and repressing the expression of *ANAC019* and *ANAC055,* — encoding jasmonic acid (JA) and abscisic acid (ABA)-induced TFs in Arabidopsis (Tran, 2004; Bu *et al.*, 2008) — as well as some of their targets, thereby playing a role in immunity and drought response. This protein emerges as a key player in the epigenetic regulation of the balance between the activation and repression of developmental and environment-responsive physiological programs. This work also provides evidence for the acquired role of the highly conserved PRCs in the regulation of plant-specific processes, such as the control of phytohormone balance and responses, biotic stress responses and drought stress.

RESULTS

LHP1 controls the transcriptomic network downstream of MYC2 through direct transcriptional repression of *ANAC019* and *ANAC055*

As an initial approach, we mined the previously published transcriptome analysis of the *lhp1* mutant, focusing on genes that are upregulated in *lhp1* as the main role of PRCs is the repression of gene expression (Veluchamy *et al.*, 2016). A Gene Ontology (GO) analysis with the *AGRIGO* software revealed a significant enrichment in GO terms related to stress responses in this data set, including *response to chemical stimulus, to other organism, to water deprivation, to JA stimulus, to insect and hyperosmotic salinity tolerance* (Figure 1a), suggesting that LHP1 is involved in the regulation of biotic and abiotic stress responses.

In order to deepen our understanding on the molecular mechanisms by which LHP1 participates in the regulation of several stress-related genes, we analyzed the 823 upregulated genes in *lhp1* with the TF2Network tool, which predicts potential regulators for a set of co-expressed genes (Kulkarni *et al.*, 2017). This analysis showed that a representative subset of these genes co-expresses with *ANAC055* (10%) and *ANAC019* (9%) (Figure 1b), encoding TFs that act under the control of MYC2, a master regulator of the interplay between the ABA-, JA-, and ethylene (ET)-responsive pathways, as well as of diverse immune and stress-responsive mechanisms in Arabidopsis (Dombrecht *et al.*, 2007; Bu *et al.*, 2008; Jiang *et al.*, 2009; Kazan and Manners, 2013). Together with *ANAC072*, *ANAC019* and *ANAC055* are induced by JA, herbivory, ABA and drought in a MYC2-dependent fashion, acting as activators and repressors of several of their targets, including *VSP1* and *VSP2*, which encode defense peptides against insect attack (Liu *et al.*, 2005; Bu *et al.*, 2008; Chen *et al.*, 2012; Jaouannet *et al.*, 2015).

To determine if the MYC2-dependent signaling pathway is upregulated in the *lhp1* mutant, we quantified the expression of several of its known regulators and targets, including *MYC2*, *ANAC019*, *ANAC055*, *ANAC072* and *VSP1* by RT-qPCR. Through this assay, we were able to determine that *ANAC019*, *ANAC055*, and *VSP1* are significantly upregulated in *lhp1* compared with the wild-type (WT) (Figure 1c); however, the expression of *MYC2* and *ANAC072* was found to be unaltered in the mutant (Figure 1c). To understand the molecular mechanism by which the expression of only some of the genes in this signaling pathway is affected in *lhp1,* and taking into account that LHP1 participates in the repression of hundreds of genes through the stabilization of the H3K27me3 mark, we mined our previously generated H3K27me3 chromatin immunoprecipitation (ChIP)-seq data on *lhp1* and wild-type plants, as well as the ChIP-seq data obtained with an anti-GFP antibody in a plant expressing an LHP1-GFP fusion protein (Veluchamy *et al.*, 2016). We visualized the H3K27me3 levels in the gene bodies of the studied loci in both genetic contexts, as well as LHP1 binding to these genes, and found that neither *MYC2* or *ANAC072* present this histone mark or LHP1 binding along their gene bodies (Figures 2a,b and S1a,b), indicating that they are not epigenetically controlled by PRCs. Conversely, *ANAC019, ANAC055,* and *VSP1* were identified as LHP1 targets and their methylation levels were found to be reduced in the *lhp1* mutant (Figures 2c–e and S1c–e), suggesting that the *lhp1* mutation leads to reduced H3K27me3 levels in the gene bodies of these loci, and promoter regions in the case of *ANAC055* (Figure 2c), rendering them more accessible to the transcriptional machinery, independently of changes in the expression levels of *MYC2*. To confirm these observations experimentally, we quantified H3K27me3 levels on the former loci in *lhp1* and WT plants by ChIP-qPCR, observing a significant reduction in H3K27me3 levels on *ANAC019*, *ANAC055*, and *VSP1* in the *lhp1* mutant (Figure S2), indicating that LHP1 promotes the deposition of this histone mark on their gene bodies. H3K27me3 levels were found to be significantly low in *MYC2* and *ANAC072* in contrast with *ANAC019*, *ANAC055*, and *VSP1*, in both WT and *lhp1* backgrounds (Figure S3), confirming that *MYC2* and *ANAC072* are not controlled by this histone mark.

As *MYC2, ANAC019, ANAC055, ANAC072*, and *VSP1* are ABA-responsive genes (Jiang *et al.*, 2009), we assessed the binding of LHP1 to these genes and their H3K27me3 levels through ChIP-qPCR before and after ABA treatment, using the Arabidopsis line expressing the LHP1–GFP fusion protein. We were able to confirm that both LHP1 binding and H3K27me3 levels decrease in *ANAC055*, *ANAC019*, and *VSP1* upon ABA treatment (Figure 3), suggesting that LHP1 contributes to their direct repression through their binding and stabilization of H3K27me3 at these loci. Conversely, as previously observed, histone methylation levels and LHP1 binding are significantly low in *MYC2* and *ANAC072,* and there is no significant change of these parameters on these loci in response to ABA (Figure 3a,c), confirming that LHP1 does not interact with and repress these two stress-

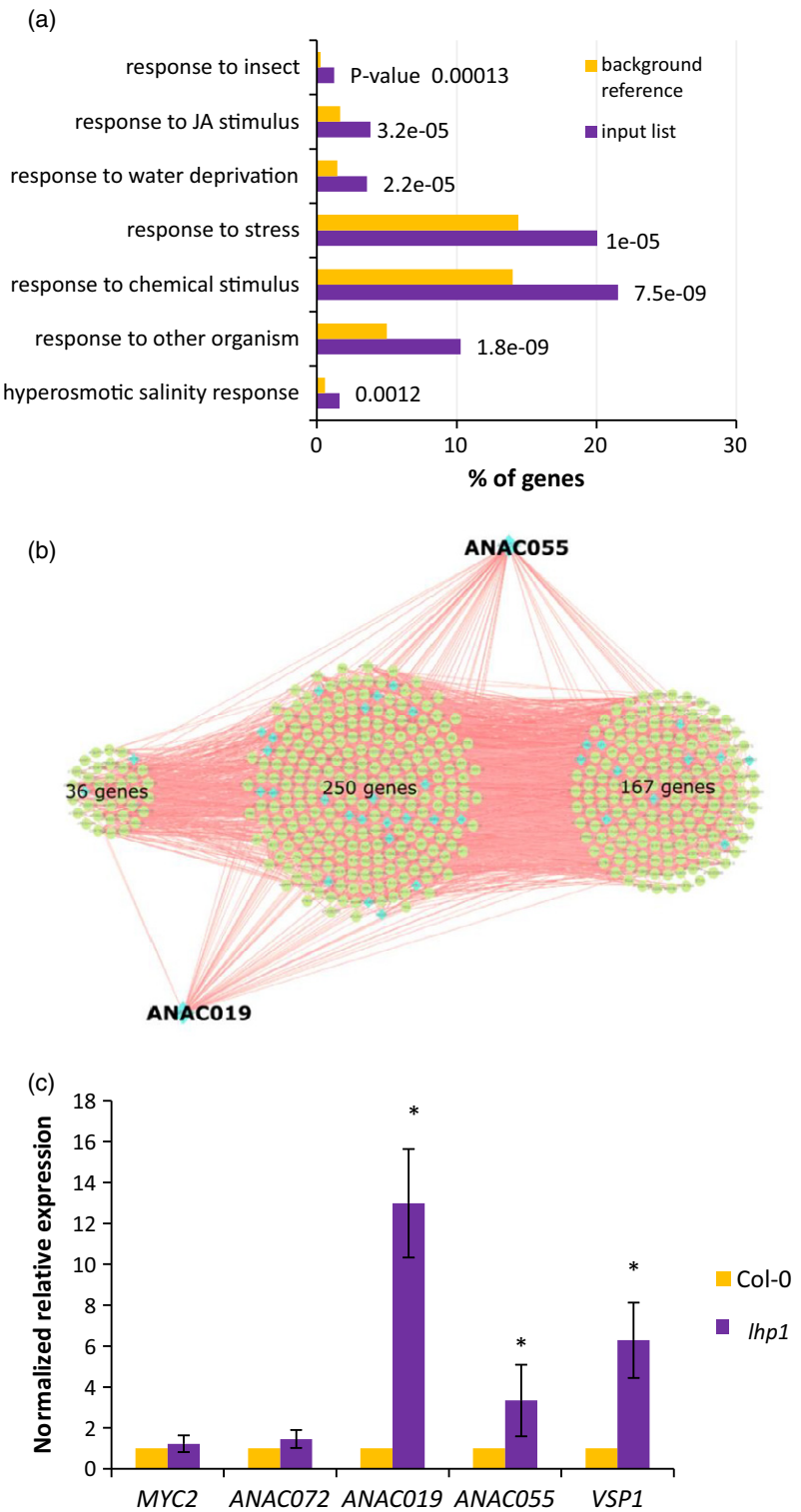

Figure 1. Expression of stress-responsive genes is altered in the *lhp1* mutant. (a) Bar chart representing the functional annotation of some stress-related upregulated genes in the *lhp1* mutant. (b) TF2Network representing *ANAC019* and *ANAC055*-related genes in the upregulated gene set of the *lhp1* mutant. Known transcription factors are represented as blue diamonds and other genes as green circles. Genes on the right cluster contain PWMs for ANAC055, on the left for ANAC019 and in the middle PWMs for both TFs. Red lines represent co-expression. (c) Normalized relative expression of genes involved in the MYC2 branch of immunity in 14-day-old WT and *lhp1* plants. The bars represent standard deviation (SD) between three biological replicates and *statistical significance (*t*-test, *P*-value < 0.05).

responsive genes. Taken together, the previous results indicate that LHP1 contributes to the regulation of the transcriptomic network downstream MYC2 at different levels, through the transcriptional repression of *ANAC019* and *ANAC055,* as well as *VSP1*.

The *lhp1* mutant presents increased ABA sensitivity and drought tolerance

It is generally accepted that MYC2 is a positive regulator of ABA signaling, as it has been previously shown that its mutation and overexpression make Arabidopsis plants less

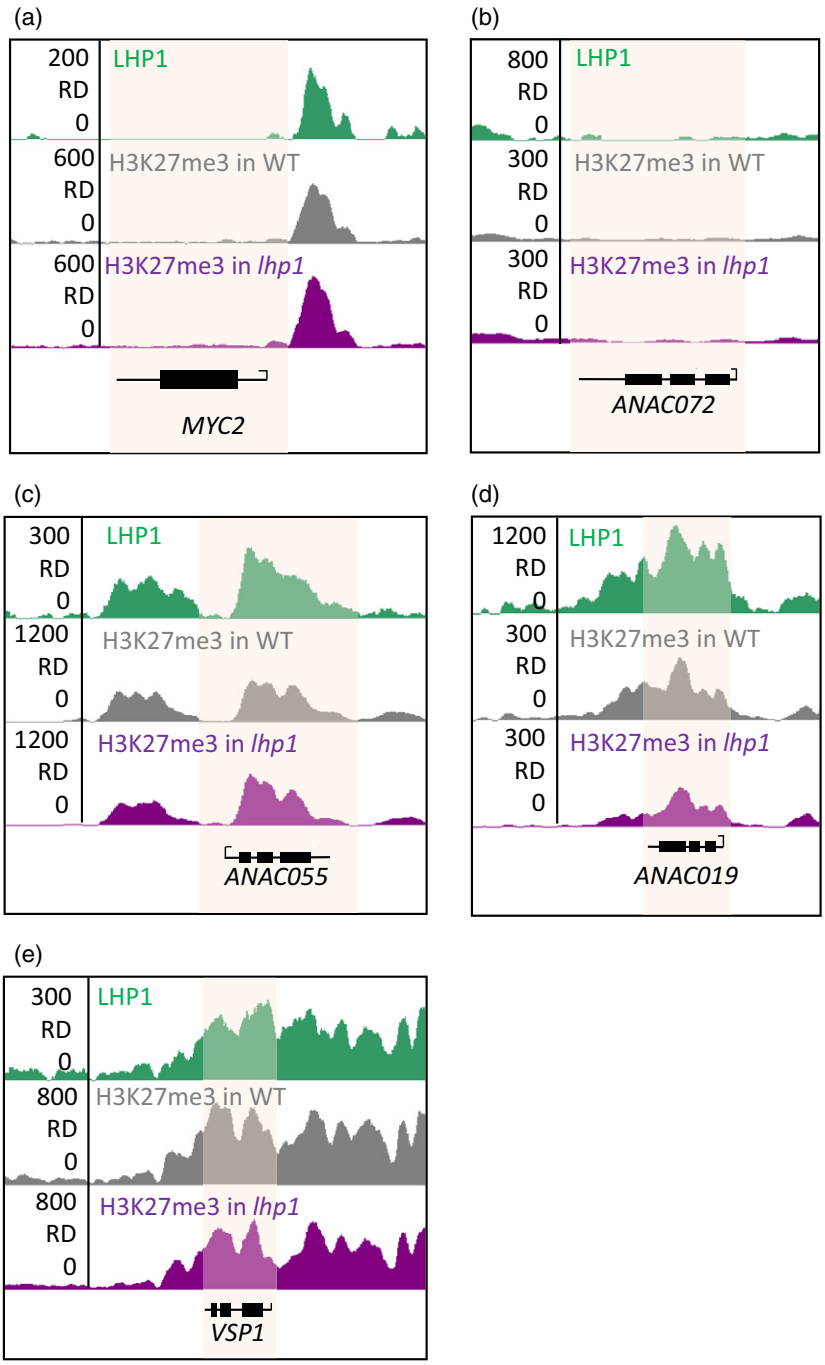

Figure 2. LHP1 targets and controls H3K27me3 levels of some genes in the MYC2 signaling pathway. Visualization of ChIP-seq results showing LHP1 binding and H3K27me3 levels in some MYC2-dependent genes in WT and *lhp1*. (a) *MYC2*, (b) *ANAC072*, (c) *ANAC055*, (d) *ANAC019*, (e) *VSP1*. Result from a single biological replicate.

and more sensitive to this hormone, respectively (Abe *et al.*, 2003b; Lorenzo *et al.*, 2004). For this reason, we germinated WT, *lhp1*, and *tfl2-6* (another LHP1 mutant line) seeds in half-strength Murashige and Skoog (½MS) medium supplemented with different ABA concentrations, and evaluated the germination rate, root length and seedling greening of the different genotypes in response to this phytohormone. We observed that both *lhp1* mutant lines present increased ABA sensitivity, as shown by the delayed and lower germination rates in the presence of this hormone (Figures 4a and S4), as well as a stronger ABA-induced reduction in embryo greening and root length relative to the WT (Figures 4b,c and S4).

ABA is considered as one of the main plant hormonal regulators of diverse abiotic stress responses, such as drought and salinity (Tuteja, 2007). Furthermore, besides being induced by ABA, MYC2 has been reported to promote osmotic and oxidative stress tolerance (Abe *et al.*, 2003a; Dombrecht *et al.*, 2007), and *ANAC019* and *ANAC055* have been described as positive regulators of

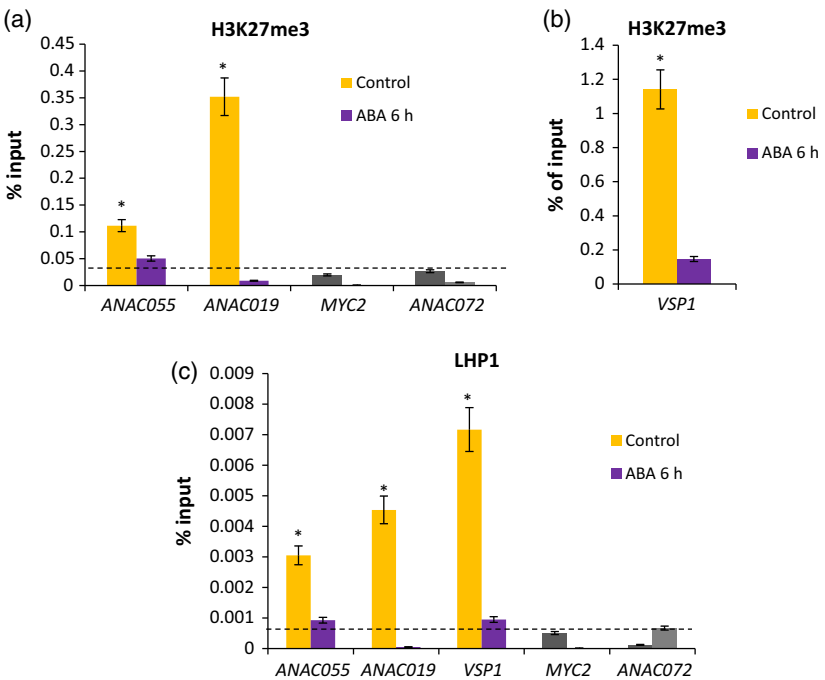

Figure 3. LHP1 directly participates in the dynamic induction of MYC2-dependent genes. (a) Relative abundance of H3K27me3 on selected gene body regions of *MYC2* and MYC2-dependent NAC genes under control conditions and in response to 50 μM ABA. (b) Relative abundance of H3K27me3 on a selected gene body region of *VSP1* under control conditions and in response to 50 μM ABA. (c) Relative LHP1 binding to selected regions of *MYC2* and gene bodies of MYC2-induced genes under control and ABA-induced conditions. *Represents statistical significance regarding the WT (*t*-test, $P < 0.05$) and bars represent standard deviation between two biological replicates. The dotted line indicates the background level.

drought tolerance and their overexpression to increase tolerance to this environmental stress (Tran, 2004). To test if the *lhp1* mutation and the consequent induction of *ANAC019* and *ANAC055* affected Arabidopsis drought tolerance, we carried out a drought sensitivity assay in WT, *lhp1* and *tfl2-6* plants, measuring survival rates of each genotype after a 21-day drought period and rewatering. We observed that plants carrying loss of function alleles of *LHP1* display significantly higher survival rates and vigor than the WT after a prolonged drought period (Figure 5), indicating that LHP1 represses ABA-mediated responses to drought, at least partially through the repression of *ANAC019* and *ANAC055*.

LHP1 regulates defense against phloem-feeding aphids

The activation of the MYC2-dependent signaling pathway has been linked to diverse physiological processes involved in biotic stress responses. The MYC2-dependent induction of *VSP1* and *VSP2* has been associated with increased insect resistance, as these proteins have been shown to present anti-insect activity and to delay insect development (Ellis *et al.*, 2002; Liu *et al.*, 2005; Jaouannet *et al.*, 2015). For this reason, we carried out an infection assay in WT and *lhp1* plants with the green peach aphid *Myzus persicae*. Resistance to these insects was assessed by measuring the number of nymphs produced by the applied aphids of each genotype 5 days post-challenging. Through this assay, we were able to conclude that, coherent to the increased *VSP1* expression in *lhp1,* the mutant displayed increased resistance to this insect, as evidenced by the reduced aphid progeny when fed on *lhp1* compared with WT plants (Figure 6). This result indicated that LHP1 is a negative regulator of the accumulation of VSP1, and thereby of insect resistance, a phenomenon occurring most likely through the repression of *ANAC019* and *ANAC055* and the direct repression of the *VSP1* locus.

LHP1 regulates SA homeostasis and defense against *Pseudomonas syringae* pv. tomato DC3000

The role of *ANAC019* and *ANAC055* is not limited to the regulation of insect and drought responses, as their induction by MYC2 increases susceptibility to some bacterial pathogens (Zheng *et al.*, 2012). It has been previously reported that the bacterial toxin coronatine (COR), from diverse *Pseudomonas syringae* strains, triggers COR-induced stomatal reopening through the *COI1–MYC2–ANAC019/055* pathway, facilitating bacterial penetration (Zheng *et al.*, 2012). Conversely, their induction has been associated to the transcriptional repression of the SA biosynthetic gene *ICS1,* which contributed to around 90% of the SA in Arabidopsis (Wildermuth *et al.*, 2001), and the activation of *BSMT1,* involved in the conversion of SA into its inactive and volatile version meSA, leading to lower SA levels and increased disease susceptibility (Chen *et al.*, 2003; Zheng *et al.*, 2012). We mined our *lhp1* RNA-seq data to determine if the mutant displayed altered *ICS1* and *BSMT1* expression, finding that similar to the *ANAC* overexpressers, *lhp1* presents a downregulation or upregulation of these loci, respectively. We validated the transcriptomic data by RT-qPCR, confirming the deregulation of these two genes in *lhp1* (Figure 7a). Furthermore, we visualized the H3K27me3 levels in *lhp1* and the LHP1

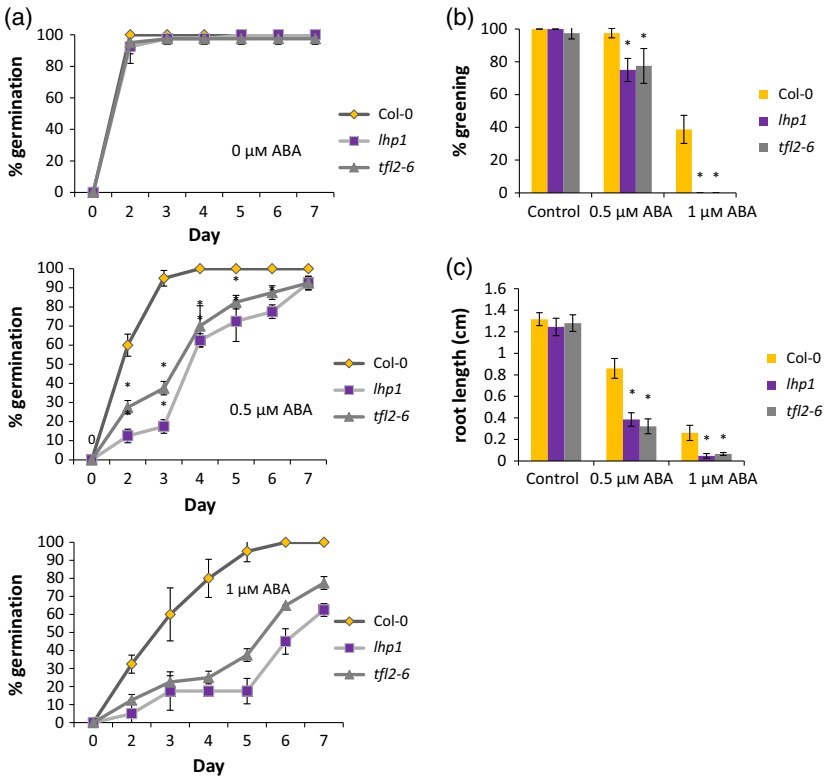

Figure 4. *lhp1* mutation alters ABA sensitivity. (a) Germination rates for WT, *lhp1* and *tfl2-6* lines in ½MS, ½MS + 0.5 μM ABA or ½MS + 1 μM ABA media. (b) Embryo greening rate of seedlings germinated on ½MS medium supplemented with different ABA concentrations. (c) Root length of 7-day-old plants germinated in control or ABA-supplemented ½MS medium. Only one biological replicate is shown. *Represents statistical significance compared with the WT (*t*-test, $P < 0.05$) and bars standard deviation. Here, 80 plants/genotype/treatment were used on each experiment.

binding to these genes, finding that *ICS1* is neither an LHP1 target nor displays H3K27me3 on its genes body (Figure 7b), suggesting that its repression in the *lhp1* mutant may occur directly through the activity of ANAC019 and ANAC055; conversely, *BSMT1* is an LHP1 target and the methylation levels along its gene body seem to be slightly reduced in the *lhp1* mutant (Figure 7b), suggesting that LHP1 participates in the repression of this gene through its targeting. We assessed the H3K27me3 levels in *BSMT1* in the WT and *lhp1* through ChIP-qPCR, confirming that the *lhp1* mutation leads to a reduction in levels of this histone mark in this gene, contributing to its derepression in the mutant (Figure 7c). Likewise, we performed ChIP-qPCR of both loci before and after ABA treatment on plants expressing the LHP1–GFP fusion protein, evaluating H3K27me3 and LHP1 binding levels. As predicted from the previous visualization and results, *BSMT1* was found to display a significant enrichment in this histone mark and in LHP1 binding, which are both reduced in response to ABA (Figure 7d,e), allowing the induction of this gene in an LHP1-dependent manner. This result is coherent to previous experimental data showing that *BSMT1* expression is induced by ABA treatment (Figure S5). By contrast, *ICS1* does not present significant LHP1 binding or H3K27me3 deposition along its gene body (Figure 7d,e), confirming that this locus is not epigenetically regulated by PRCs. The *ICS1* and *BSMT1* regions targeted for ChIP-qPCR are represented in Figure S6.

We assessed the physiological effect of the deregulation in *ICS1* and *BSMT1* expression by quantifying SA levels before and after infection with *Pst* DC3000, in both, *lhp1* and WT plants. In agreement to the previous result, *lhp1* displays lower SA levels than the WT in mock conditions, but also 24 h after pathogen infection (Figure 8a). This result suggested increased susceptibility of the *lhp1* mutant to this bacterial pathogen, as SA positively regulates immune responses to *Pst* DC3000 (Halim *et al.*, 2006). In order to confirm this hypothesis, we quantified the susceptibility of the *lhp1* and *tfl2-6* mutants to *Pst* DC3000 following a protocol modified from Ross and Somssich (2016). From this assay, we found that both mutant lines displayed increased bacterial abundance (Figures 8b and S7) and symptom development relative to the WT (Figure 8c), confirming the increased susceptibility to this bacterial pathogen induced by the *lhp1* mutation. Therefore, we propose that LHP1 positively regulates resistance to hemibiotrophic bacterial pathogens through the regulation of SA biosynthesis and metabolism.

DISCUSSION

LHP1 regulates hormone homeostasis in Arabidopsis

Phytohormones are involved in the regulation of almost every physiological and developmental process occurring in the plant life cycle from germination, flowering and senescence, to responses to environmental cues. In this

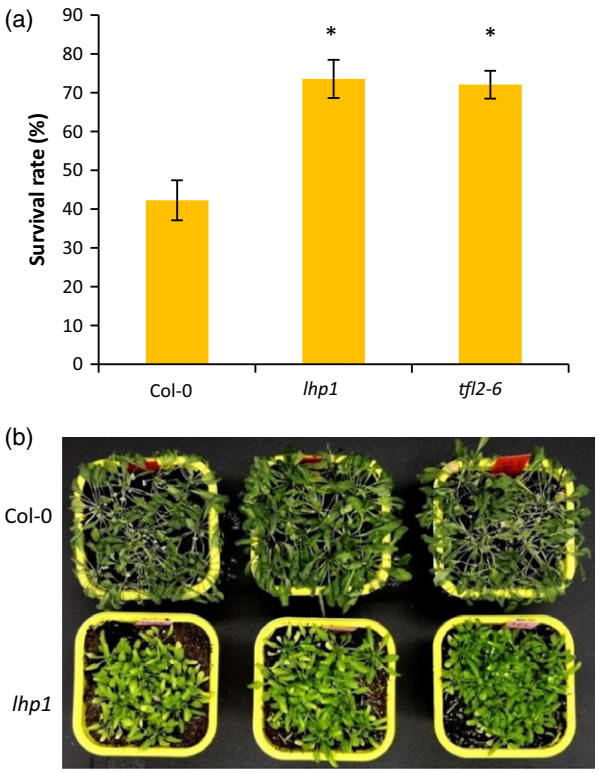

Figure 5. Drought tolerance of the *lhp1* and *tfl2* mutants. (a) Survival rate (no. surviving plants/no. total plants × 100) of WT, *lhp1* and *tfl2-6* plants after 21 days of drought treatment followed by rewatering. (b) Image illustrating WT and *lhp1* plants after 21 days of drought treatment followed by rewatering. *Represents statistical significance regarding the WT (*t*-test, $P < 0.05$) and bars represent standard deviation between two biological replicates. Here, 60 plants/genotype were used for each biological replicate.

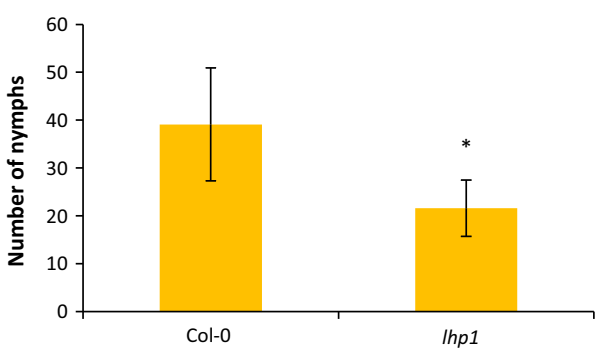

Figure 6. *The lhp1* mutant displays increased resistance to *Myzus persicae*. Number of nymphs produced by aphids on 4-week-old WT and *lhp1* plants. Here, 10 plants were used on three independent biological replicates. *Represents statistical significance regarding the WT (*t*-test, $P < 0.05$) and bars represent standard deviation calculated from all the plants in all replicates.

way, the delicate balance between hormone biosynthesis, transport and responses determines plant fitness, as the prioritization of some physiological processes over others can have a more or less detrimental effect under specific environmental conditions. It has been previously reported that the *lhp1* mutation affects auxin content and responses in Arabidopsis through the repression of several *YUCCA* genes and the misregulation of several auxin-responsive genes (Kim *et al.*, 2004; Rizzardi *et al.*, 2011). Notably, LHP1 has been shown to participate in the formation of an auxin-driven chromatin loop, and thereby to control root development (Ariel *et al.*, 2014), however the direct physiological effect of the auxin deregulation in *lhp1* has not been addressed in depth. Interestingly, the deregulation of auxin biosynthesis has been related to the *lhp1* altered glucosinolate content, as the biosynthesis of these crucifer-specific phytoanticipins is biochemically linked to that of auxin (Ludwig-Muller *et al.*, 1999), suggesting that the LHP1 protein may also participate in stress responses. Consistently, in a recent study, the role of the PRC2 proteins CLF and SWN in ABA-induced senescence was characterized, showing that these two histone methyltransferases repress ABA-induced senescence-associated genes. From an initial screening the authors concluded that the double *clf swn* but not *lhp1* display increased ABA sensitivity (Liu *et al.*, 2018). However, in this study the researchers performed a qualitative evaluation of the ABA-induced reduction in aerial growth and senescence after treating 10-day-old plants with ABA, while in the current study we quantified germination rate, embryo greening and root growth on ABA-supplemented medium, in which the increased sensitivity of the *lhp1* mutant became evident (Figure 4). Previously, it has been shown that the PRC1 triple mutation *atbmi1a/b/c* derepresses various ABA-responsive genes, such as *FUS3, ABI3* and *ABI4* (Merini *et al.*, 2017). In contrast, according to our transcriptomic data, these genes are not deregulated in *lhp1,* suggesting that different PRC proteins repress different aspects of ABA signaling.

LHP1 participates in the repression of the MYC2 signaling pathway

In the present study, based on the correlation between ChIP, transcriptomic and phenotypic data, we propose that LHP1 is a repressor of the MYC2 signaling pathway through the targeting of *ANAC019*, *ANAC055* and their target *BSMT1*. MYC2 has been extensively studied and characterized as a master regulator of the interplay between hormone signaling, including ABA, JA, ET and SA, as well as responses to diverse biotic and abiotic stresses, such as drought, or defense against insects and pathogens (Dombrecht *et al.*, 2007; Zheng *et al.*, 2012; Kazan and Manners, 2013). We prove that LHP1 binds to *ANAC019* and *ANAC055* and that in response to ABA the LHP1 binding and H3K27me3 levels on the gene bodies of these loci decrease, leading to their derepression and the subsequent expression and repression of several of their stress-responsive targets. Furthermore, LHP1 targets and participates in the regulation of the expression of *VSP1*

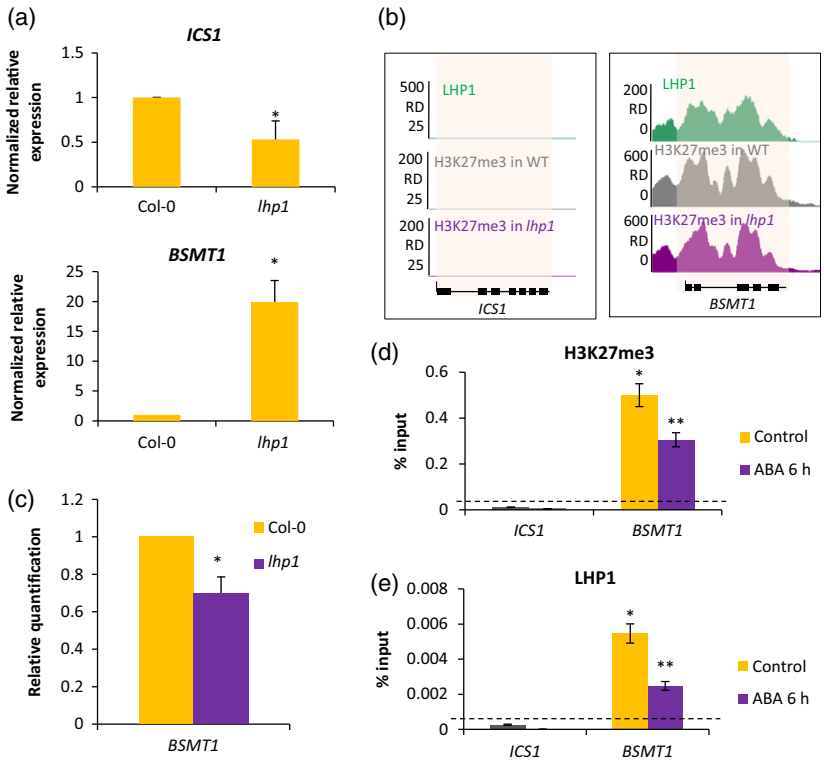

Figure 7. LHP1 regulates genes involved in SA homeostasis. (a) Relative expression of SA biosynthetic *(ICS1)* and metabolic *(BSMT1)* genes in *Ihp1*. *Represents statistical significance regarding the WT (*t*-test, $P < 0.05$) and bars represent standard deviation between three biological replicates. (b) Visualization of WT and *Ihp1* H3K27me3 levels as well as LHP1 binding on *ICS1* and *BSMT1*. (c) Relative abundance of H3K27me3 on a selected gene body region of *BSMT1* in Col-0 WT and *Ihp1* mutant background. *Represents statistical significance regarding the WT (*t*-test, $P < 0.05$) and bars represent standard deviation between two biological replicates. (d) Relative abundance of H3K27me3 on *ICS1* and *BSMT1* in control conditions and in response to ABA. The dotted line indicates the background level. (e) Relative LHP1 binding to *ICS1* and *BSMT1* in response to ABA. The dotted line indicates the background level. *Represents statistical significance regarding the WT (*t*-test, $P < 0.05$) and bars represent standard deviation between two biological replicates.

and *BSMT1* in response to ABA, acting at several levels in this pathway (Figure 9).

As previously discussed, the *Ihp1* mutant displays increased ABA sensitivity, to which the upregulation of *ANAC019* and *ANAC055* in *Ihp1* may be contributing. However, it caught our attention that the exact molecular mechanisms by which the MYC2-dependent pathway affects ABA sensitivity has not been depicted to the date: apart from the MYC2-dependent induction of the ABA-responsive genes *RDR22* and *ADH1*, the precise physiological phenomenon behind the altered ABA sensitivity of the *MYC2, ANAC019*, and *ANAC055* mutants and overexpressers remains obscure (Abe *et al.*, 2003a; Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007). Conversely, it has been proposed that the increased drought tolerance observed in the *ANAC* overexpressers may be due to the detoxification of toxic aldehydes through the glyoxalase pathway, and it is thought that the glyoxalase I7 (GLYI7, *RAFL06-15-P15*) may contribute to this process, as its expression is induced in these lines and the overexpression of related proteins has been shown to improve salinity and drought tolerance in rice, tobacco, and alfalfa (Oberschall *et al.*, 2000; Singla-Pareek *et al.*, 2003; Tran, 2004). According to our RNA-seq data, *GLYI7* is also upregulated in *Ihp1* (Log2FoldChange = 3.4221), and could contribute to drought tolerance in this mutant. However, the effect of this specific enzyme on drought tolerance has not been experimentally addressed in Arabidopsis (Schmitz *et al.*, 2018), and there may be several other deregulated genes in the *ANAC* overexpressors and in *Ihp1* that contribute to the observed phenotype.

In addition, *ANAC019* and *ANAC055* upregulation in *Ihp1* coincides with the repression of *ICS1* and the upregulation of *BSMT1* in this mutant (Figure 7a), a phenomenon that has been described as a consequence of the induction of these TFs (Zheng *et al.*, 2012). However, in this study we provide evidence for the role of LHP1 in the direct repression of *BSMT1* (Figure 7c–e), highlighting the role of this protein in the fine-tuning of this signaling pathway at different levels. The altered expression levels of *ICS1* and *BSMT1* in *Ihp1* are coherent with the reduced SA content in the mutant and its increased susceptibility to *Pst* DC3000, suggesting that LHP1 is a positive regulator of SA biosynthesis and defense responses against this hemibiotrophic pathogen. Furthermore, the observed repression of SA-mediated immunity in *Ihp1* is coherent with the increased ABA sensitivity of the mutant, as ABA signaling is known for its repressive effect on SA accumulation and SA-mediated immunity, a phenomenon to which it contributes through the activation of the MYC2-dependent signaling pathway (Cao *et al.*, 2011; Lievens *et al.*, 2017; Mine *et al.*, 2017).

It has been known for several years that the MYC2 branch of immunity is regulated at the transcriptional and post-translational level, with the JAZ proteins inhibiting the activity of MYC2 (Santner and Estelle, 2007). In

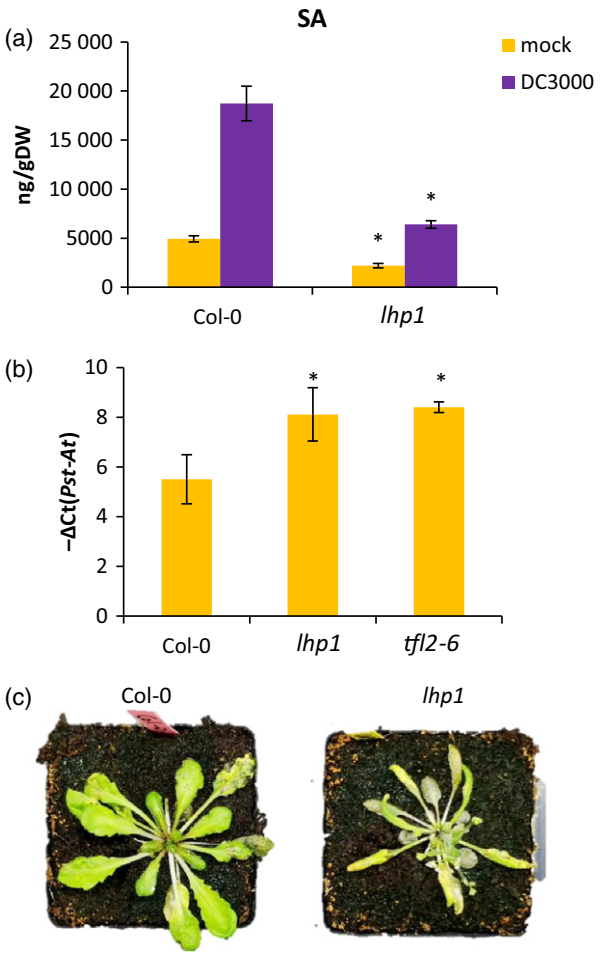

Figure 8. SA homeostasis and SA-mediated immunity are altered by the *lhp1* mutation. (a) SA levels in WT and *lhp1* leaves in mock and 24 hpi samples with *Pst* DC3000. Bars represent the SD between three technical replicates. *Represents statistical significance regarding the WT (*t*-test, $P < 0.05$). (b) Resistance levels of *lhp1* and *tfl2-6* to *Pst* DC3000 calculated based on the ΔCt method. Here, 30-day-old plants were spray inoculated and leaf samples collected 3 dpi. Bacterial quantification was performed following the qPCR protocol described by Ross and Somssich (2016). *Represents statistical significance regarding the WT (*t*-test, $P < 0.05$) and bars represent SD between technical replicates. Only one biological replicate is shown. (c) Image illustrating symptom development in WT and *lhp1* plants at 7 dpi with *Pst* DC3000.

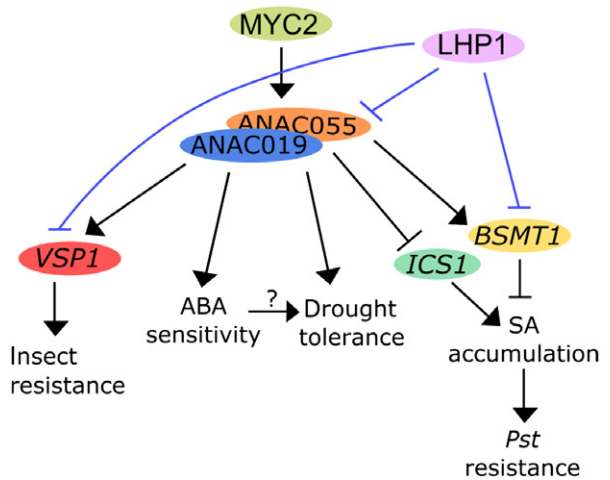

Figure 9. Molecular model representing the role of LHP1 in the MYC2-dependent branch of immunity. LHP1 represses the expression of *ANAC019* and *ANAC055* through the stabilization of H3K27me3 levels in their gene bodies. In the absence of LHP1, these two TFs are upregulated, inducing the expression of *VSP1* and *BSMT1,* while repressing *ICS1*. *ANAC019* and *ANAC055* upregulation contributes *to* ABA hypersensitivity and drought tolerance of *lhp1*, as well as decreased SA levels and increased susceptibility to the hemibiotrophic pathogen *Pst* DC3000. LHP1 also participates in the direct repression of *BSMT1* and *VSP1*.

addition, it has been reported that the SWI/SNF chromatin remodeler SPLAYED (SYD) is necessary for the transcriptional induction of MYC2 and MYC2-dependent genes in response wounding (Walley *et al.*, 2008). Interestingly, SYD has also been shown to counteract PRCs in the repression of the flowering genes *APETALA3* and *AGAMOUS* (Wu *et al.*, 2012), supporting the hypothesis that this chromatin remodeler also counterbalances the repression of the MYC2 immune pathway by LHP1, highlighting the diverse layers of complexity of this process. As it has been previously shown, the misregulation of this pathway has a significant effect on tolerance to diverse stresses (Abe *et al.*, 2003b; Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007; Kazan and Manners, 2013; Song *et al.*, 2014), therefore keeping it under tight regulation may be beneficial for plants.

PcG proteins differentially regulate defense responses in Arabidopsis

As previously discussed, the MEA H3K27me3 histone acetyltransferase has been shown to be an immune repressor, and its mutation reported to render Arabidopsis highly resistant to both a necro- and a hemibiotrophic pathogen (Roy *et al.*, 2018). Interestingly, the *mea-6* mutant displays normal SA content compared with the WT under control conditions, while its induction is significantly higher when challenged with *Pst* DC3000. This phenomenon is different from the one occurring in *lhp1,* in which SA levels are constitutively lower, suggesting that LHP1 and MEA regulate immunity through different mechanisms. Conversely, LIF2, the LHP1 interactor, has been previously reported to co-regulate the expression of hundreds of stress-responsive genes, together with LHP1 (Molitor *et al.*, 2016). However, interestingly, *lhp1* and *lif2* mutants display opposite phenotypes regarding their susceptibility to *Pst* DC3000 and the expression of *MYC2* is downregulated in the latter without affecting SA content (Le Roux *et al.*, 2014), indicating that these interacting proteins contribute to the Arabidopsis immune regulation through differentially regulated targets and mechanisms. This result is coherent

with the fact that LIF2 and LHP1 share only a subset of genomic targets, LIF2 binding being more specialized than that of LHP1 (Molitor *et al.*, 2016).

The stress-related phenotype of the *lhp1* mutant, together with its highly pleiotropic developmental phenotype, highlights LHP1 as a crucial element monitoring the balance between developmental and defense programs. It can be predicted that the derepression of flowering and ABA-responsive pathways comes with a high energetic cost for the *lhp1* mutant, compromising its fitness. The current study contributes to the growing evidence of the role of the chromatin machinery, and more specifically, PRC proteins, in the modulation of adaptive processes in plants, underlining the importance of these complexes in the integration of a plethora of developmental and environment-responsive molecular mechanisms.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and treatments

Arabidopsis WT plants were Columbia-0 (Col-0), and seeds of *lhp1* (SALK_011762 line) and *tfl2-6* (CS6397) mutants were on the same background. Arabidopsis *lhp1* mutant plants complemented with the *ProLHP1:LHP1:GFP* construct have been previously described by Nakahigashi *et al.* (Nakahigashi *et al.*, 2005). Plants were grown in chambers at 20°C on sterile ½MS medium containing 0.8% (w/v) agar under long-days (16 h of light at 20°C, 8 h of darkness at 18°C). Seeds were surface sterilized by treatment with bayrochlore for 20 min, washed, and incubated in sterile-water for 3 days at 4°C to obtain homogeneous germination. For studying ABA-induced changes in LHP1 binding and H3K27me3 level on selected targets, 14-day-old *in vitro* grown plantlets were sprayed with a 50 μM ABA solution or water, as a control. Samples were incubated for 6 h before cross-linking for further experiments. For ABA sensitivity evaluation, seeds were germinated on ½MS medium supplemented with 0, 0.5 or 1 μM ABA in chambers at 20°C under long-days (16 h of light at 20°C, 8 h of darkness at 18°C). Germination was followed during 7 days and at the end of this period embryo greening and root length assessed. Each experiment consisted of three biological replicates.

Drought tolerance evaluation

Arabidopsis WT Col-0, *lhp1*, and *tfl2* seeds were incubated for 3 days at 4°C to obtain homogeneous germination. Seeds were germinated on soil and 5 days post-germination homogeneously distributed on pots in order to reach a similar plant density. Pots with different genotypes were randomly distributed on the growing surface. Plants were grown in an Aralab chamber with controlled light, temperature and humidity (8 h of light at 20°C, 16 h of darkness at 18°C, and 60% humidity). 15 days post-germination, plants were watered, excess water removed from the trays, and pots left to dry for 21 days. After 21 days of drought plants were rewatered and, 3 days after, the plant survival rate was determined for each genotype by calculating the percentage of surviving plants. Each experiment was performed in duplicate obtaining similar results.

Aphid infestation assays

The aphids used in this study are *Myzus persicae* genotype LB01 (from INSA Lyon). They were reared on Chinese cabbage Pe-tsaï (Brassica rapa subsp. Pekinensis) and maintained in cages in controlled conditions at 23°C under 16 h of light, 60–70% of humidity. The experimental method is adapted from the published infestation test protocol (Jaouannet *et al.*, 2015). Plants were grown under short day conditions (8 h light at 22°C (light)/ 21°C (dark), 60–70% of humidity in a mix of soil/sand (2/3 versus 1/3). Four-week old plants were challenged with two 5-day-old apterous aphids (age-synchronized). Aphid progeny was counted after 5 days. We performed three biological replicates of aphid performance assays. Statistical analyses were performed using two-tailed Student's *t*-test.

Pst DC3000 infection

Plants were grown under short-day conditions (8 h light, 16 h darkness, 18°C). Next, 1-month-old plants were spray inoculated with *Pseudomonas syringae* DC3000 at OD_{600} = 0.2, covered for keeping humidity and leave samples collected 72 hpi for bacterial quantification. Leaves were surface-sterilized by incubating them 5 sec in ethanol 70%, followed by 2 5-sec water baths and drying on paper. For each genotype, three adult leaves from different plants were pulled together and frozen at −80°C. DNA extraction was carried out following the method described by Edwards *et al.* (1991) and DNA resuspended in 300 μl of water. DNA concentration for each sample was quantified by NanoDrop spectrometer and diluted to a final concentration of 10 ng μl^{-1}. Abundance of bacterial DNA relative to Arabidopsis DNA was quantified following the ΔCt method described by Ross and Somssich (2016). Eight samples were analyzed by genotype and each experiment was performed in duplicate. Primers used for pathogen quantification are listed in Table S1.

Salicylic acid quantification

Twenty-four hpi with a *Pst* DC3000 suspension or $MgCl_2$, plant true leaves were collected, frozen in liquid nitrogen and lyophilized until completely dehydrated. Dehydrated tissue was ground to a fine powder and, for each sample, 3 mg of dry powder was extracted with 0.8 ml of acetone/water/acetic acid (80/19/1 v:v:v). SA stable labelled isotopes used as internal standards were prepared as described in Le Roux *et al.* (2014). 1 ng of each standard was added to the sample. The extract was vigorously shaken for 1 min, sonicated for 1 min at 25 Hz, shaken for 10 min at 10°C in a Thermomixer (Eppendorf®, and then centrifuged (8000 ***g***, 10°C, 10 min.). The supernatants were collected, and the pellets were re-extracted twice with 0.4 ml of the same extraction solution, then vigorously shaken (1 min) and sonicated (1 min; 25 Hz). After the centrifugations, the three supernatants were pooled and dried (Final Volume 1.6 ml). Each dry extract was dissolved in 100 μl of acetonitrile/water (50/50 v/v), filtered, and analyzed using a Waters Acquity ultra performance liquid chromatograph coupled to a Waters Xevo Triple quadrupole mass spectrometer TQS (UPLC-ESI-MS/MS). The compounds were separated on a reverse-phase column (Uptisphere C18 UP3HDO, 100 × 2.1 mm × 3 μm particle size; Interchim, France) using a flow rate of 0.4 ml min^{-1} and a binary gradient: (A) acetic acid 0.1% in water (v/v) and (B) acetonitrile with 0.1% acetic acid, the column temperature was 40°C. We used the following binary gradient (time, % A): (0 min, 98%), (3 min, 70%), (7.5 min, 50%), (8.5 min, 5%), (9.6 min, 0%), (13.2 min, 98%), (15.7 min, 98%). Mass spectrometry was conducted in electrospray and multiple reaction monitoring scanning mode (MRM mode) in negative ion mode. Relevant instrumental parameters were set as follows: capillary 1.5 kV (negative mode), source block and desolvation gas temperatures 130°C and 500°C, respectively. Nitrogen was used to assist the cone and desolvation

(150 L h^{-1} and 800 L h^{-1}, respectively), and argon was used as the collision gas at a flow of 0.18 ml min^{-1}. For each genotype three technical replicates were performed.

Gene expression analysis by qPCR

Total RNA was extracted from 14-day-old *in vitro* grown seedlings with the NucleoSpin RNA kit (Macherey-Nagel), according to the manufacturer's instructions. First strand cDNA was synthesized from 2 μg of total RNA using SuperScript II Reverse Transcriptase (Thermo Fischer Scientific) according to the manufacturer's instructions. The synthesized cDNA was 100-fold diluted and 2.5 μl mixed with 500 nM of each primer and LightCycler® 480 Sybr Green I master mix (Roche Applied Science) for qPCR analysis. Products were amplified and fluorescence signals acquired with a LightCycler® 480 detection system. The specificity of amplification products was determined by melting curves. *ACT2* was used as internal control for signal normalization. The relative quantification was performed following ΔΔCt method. Data were obtained from duplicates of at least three biological replicates. The sequences of primers can be found in Table S2.

ChIP-qPCR assay

ChIP-qPCR assays were performed on 14-day-old seedlings grown in plates using GFP-Trap Magnetic Agarose (Chromotek gtma-20) anti-H3K27me3 (Millipore 07–449) and Normal Rabbit IgG (Merck 12-370), used as an internal control. Five grams of plantlets were cross-linked in 1% (v/v) formaldehyde at room temperature for 15 min. Cross-linking was quenched with 0.125 M glycine for 5 min. The cross-linked plantlets were ground and nuclei were isolated and lysed in Nuclei Lysis Buffer (1% SDS, 50 mM Tris-HCl pH 8, 10 mM ethylene diamine tetraacetic acid (EDTA) pH 8). Cross-linked chromatin was sonicated using a Covaris S220 (Peak Power: 175, cycles/burst: 200. Duty Factory: 20). The complexes were immunoprecipitated with antibodies, overnight at 4°C with gentle shaking, and incubated for 1 h at 4°C with 40 μl of Protein AG Ultra-Link Resin (Thermo Scientific). For anti-GFP and IgG immunoprecipitations the beads were washed for 6 × 5 min in ChIP Dilution Buffer (1.1% Triton X-100, 1.2 mM EDTA pH 8, 16.7 mM Tris−HCl pH 8 and 167 mM NaCl) and twice in Tris−EDTA (TE). For anti-H3K27me3 immunoprecipitation, the beads were washed 2 × 5 min in ChIP Wash Buffer 1 (0.1% SDS, 1% Triton X-100, 20 mM Tris−HCl pH 8, 2 mM EDTA pH 8, 150 mM NaCl), 2 × 5 min in ChIP Wash Buffer 2 (0.1% SDS, 1% Triton X-100, 20 mM Tris−HCl pH 8, 2 mM EDTA pH 8, 500 mM NaCl), 2 × 5 min in ChIP Wash Buffer 3 (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate,10 mM Tris−HCl pH 8, 1 mM EDTA pH 8) and twice in TE (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8). ChIPed DNA was eluted by two 15-min incubations at 65°C with 250 μl Elution Buffer (1% SDS, 0.1 M NaHCO3). Chromatin was reverse-cross-linked by adding 20 μl of NaCl 5 M and incubated overnight at 65°C. Reverse cross-linked DNA was submitted to RNase and proteinase K digestion, and extracted with phenol−chloroform. DNA was ethanol precipitated in the presence of 20 μg of glycogen and resuspended in 10 μl of nuclease-free water (Ambion) in a DNA low-bind tube. IPs and inputs were 20-fold diluted and 2.5 μl mixed with 500 nM of each primer and LightCycler® 480 Sybr Green I master mix (Roche Applied Science) for qPCR analysis. Products were amplified and fluorescence signals acquired using a LightCycler® 480 detection system. The specificity of amplification products was determined by melting curves. The relative quantification was performed following the ΔΔCt method, and input and IgG values were used to normalize and calculate the % of input. Details for primers used for ChIP-qPCR can be found in Table S3.

ACCESSION NUMBERS

Data have been deposited in the Gene Expression Omnibus (GEO) database: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ihohsmewvbclvsj&acc=GSE76571.

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AUTHOR CONTRIBUTIONS

MB devised the research project. JSRP and MB designed the methodology. JSRP, DL, YH, RBC, MJ and SC performed the experiments. DMM analyzed the data. JSRP, NYRG, CR and MB analyzed the data and wrote the article. DL, HH and AB supervised and complemented the writing. All authors read and approved the final manuscript. MB agrees to be responsible for contact and ensures communication.

SUPPORTING INFORMATION

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

Figure S1. Visualization of a ChIP-seq biological replicate representing LHP1 binding and H3K27me3 levels of some genes in the MYC2 signaling pathway.

Figure S2. *ANAC055, ANAC019* and *VSP1* display decreased H3K27me3 levels in *lhp1*.

Figure S3. *MYC2, ANAC072* and *ICS1* gene bodies are not PRC targets.

Figure S4. *lhp1* and *tfl2-6* development in response to ABA.

Figure S5. *BSMT1* is induced in response to ABA.

Figure S6. *ICS1* and *BSMT1* regions targeted for ChIP-qPCR.

Figure S7. Susceptibility of the *lhp1* and *tfl2-6* mutants to *Pst* DC3000.

Table S1. Primers used for *Pseudomonas syringae* quantification.

Table S2. Primers used for qPCR.

Table S3. Primers used for ChIP-qPCR.

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