Short title: WHY1 regulates SA homeostasis

Dual-localized WHIRLY1 affects salicylic acid biosynthesis via coordination of ISOCHORISMATE SYNTHASE1, PHENYLALANINE AMMONIA LYASE1 and S-ADENOSYL-L-METHIONINE-DEPENDENT METHYLTRANSFERASE1

Wenfang Lin1, Hong Zhang1, Dongmei Huang1, Dirk Schenke 3, Daguang Cai 3, Binghua Wu*2, Ying Miao*1

1 Fujian Provincial Key Laboratory of Plant Functional Biology, College of Life Sciences, Fujian Agriculture and Forestry University, 350002 Fuzhou, China; 2 College of Horticulture Science, Fujian Agriculture and Forestry University, 350002 Fuzhou, China; 3 Department of Molecular Phytopathology, Christian-Albrechts University of Kiel, Germany

*Corresponding author

Fujian Provincial Key Laboratory of Plant Functional Biology, Fujian Agriculture and Forestry University, Fuzhou 350002, China; Email: ymiao@fafu.edu.cn, binghua.wu@fafu.edu.cn Phone: 0086 59186392987

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors is: Ying Miao (ymiao@fafu.edu.cn), Binghua Wu (binghua.wu@fafu.edu.cn).
Author contributions

Y.M. designed the study. W.F.L. performed SA measurements, immunoblots, phenotyping, and RT-qPCR. D.H. performed, ChIP-seq, ChIP-qPCR, H.Z. performed plasmid constructs and promoter activation activity and the mutants screening. B.H.W performed microarray data analyses. W.F.L. and Y.M. analyzed the data. Y.M. wrote the paper. D.S. and D.C. critically read the paper.

One-sentence summary: Arabidopsis nuclear- and chloroplast-localized WHIRLY1 adjusts SA content in cells via ICS1, PAL1, and BSMT1 to regulate plant senescence in a developmental-dependent manner.

Abstract

Salicylic acid (SA) influences developmental senescence and is spatiotemporally controlled by various mechanisms, including biosynthesis, transport, and conjugate formation. Altered localization of Arabidopsis WHIRLY1 (WHY1), a repressor of leaf natural senescence, in the nucleus or chloroplast causes a perturbation in SA homeostasis, resulting in adverse plant senescence phenotypes. WHY1 loss-of-function mutation resulted in SA peaking 5 days earlier compared to wild-type plants, which accumulated SA at 42 days after germination. SA accumulation coincided with an early leaf-senescence phenotype, which could be prevented by ectopic expression of the nuclear WHY1 isoform (nWHY1). However, expressing the
plastid WHY1 isoform (pWHY1) greatly enhanced cellular SA levels. Transcriptome analysis in the WHY1 loss-of-function mutant background following expression of either pWHY1 or nWHY1 indicated that hormone metabolism-related genes were most significantly altered. The pWHY1 isoform predominantly affected stress-related gene expression, whereas nWHY1 primarily controlled developmental gene expression. Chromatin immunoprecipitation-qPCR (ChIP-qPCR) assays indicated that nWHY1 directly binds to the promoter region of isochorismate synthase1 (ICS1), thus activating its expression at later developmental stages, but indirectly activated S-adenosyl-L-methionine-dependent methyltransferase1 (BSMT1) expression via ethylene response factor 109 (ERF109). Moreover, nWHY1 repressed expression of phenylalanine ammonia lyase-encoding gene (PAL1) via R2R3-MYB member 15 (MYB15) during the early stages of development. Interestingly, rising SA levels exerted a feedback effect by inducing nWHY1 modification and pWHY1 accumulation. Thus, the alteration of WHY1 organelle isoforms and the feedback of SA are involved in a circularly integrated regulatory network during developmental or stress-induced senescence in Arabidopsis.

Keywords: dual-localized WHIRLY1, SA biosynthesis, plant senescence, feedback loop, Arabidopsis thaliana

Introduction

Salicylic acid (SA) is crucial for plant growth, responses to pathogens, programmed
cell death, and environmental responses. Its content is temporally and spatially controlled by various mechanisms, including biosynthesis, transport, and conjugate formation. For example, leaf development in *Arabidopsis* is regulated by SA biosynthetic/signaling genes. Early leaf senescence is a result of SA overproduction in mutants such lines overexpressing isochorismate synthase (ICS1) and phenylalanine ammonia lyase (PAL) (Love et al., 2008; Rivas-San et al., 2011), whereas the hypersensitive response (a fast form of programmed cell death) has been intensively investigated in the S-adenosyl-L-methionine-dependent methyltransferase mutant (*bsmt1*; Vlot et al., 2009). There are two main SA biosynthetic pathways in plants: the phenylalanine ammonia lyase (PAL) pathway and the isochorismate (IC) pathway, which both depend on the primary metabolite chorismate (Dempsey et al. 2011). In the PAL pathway, the chorismate-derived L-phenylalanine is converted into SA via either benzoate intermediates or coumaric acid through a series of enzymatic reactions involving PAL, benzoic acid 2-hydroxylase (BA2H), and other uncharacterized enzymes (Leon et al. 1995b). The cytosolic PAL pathway produces approximately 10% of defense-related SA and in Arabidopsis four PAL enzymes have been identified. In the IC pathway, chorismate is converted in a two-step process to SA via isochorismate involving isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL). In *Arabidopsis*, two ICS enzymes have been described to convert chorismate to isochorismate, but in recent studies another isochorismate synthase was identified (Rekhter et al. 2019; Torrens-Spence et al. 2019). This
pathway accounts for ~90% of the SA production generated by the plastid-localized ICS1 inducible by pathogens and UV light (Wildermuth et al. 2001; Garcion et al. 2008). Endogenous SA undergoes a series of chemical modifications including hydroxylation, glycosylation, methylation, and amino-acid conjugation. These modifications directly affect the biochemical properties of the SA derivatives, and play a pivotal role in SA catabolism and homeostasis to regulate leaf senescence (Zhang et al. 2013). It has been shown that SA affects regulation of gene expression during leaf senescence (Morris et al. 2000; Vogelmann et al. 2013; Zhang et al. 2013; 2017) and plays a role in advancing flowering time in Arabidopsis thaliana (Martínez et al. 2004) as well as in inhibiting seed germination (Alonso-Ramirez et al. 2009; Lee et al. 2013). Although SA biosynthesis and its function in both local and systemic acquired resistance (SAR) against microbial pathogens and in plant development are well understood (Park et al. 2007; An and Mou, 2011), the underlying molecular mechanism of free SA homeostasis in cells is less clear.

WHIRLY family proteins are localized to both the nucleus and organelles, and perform numerous cellular functions in both locations (Krause et al. 2005; Grabowski et al. 2008). In the nucleus, WHIRLY1 (WHY1) protein was found to regulate the expression of genes related to defense and senescence by binding to their respective promoters (Desveaux et al. 2000; Desveaux et al. 2004; Xiong et al. 2009; Miao et al. 2013; Krupinska et al. 2013). WHY1 protein binds for example to the promoter of WRKY53 and repressed WRKY53 and WRKY33 expression in a developmental-dependent
manner during early senescence in *Arabidopsis* (Miao et al. 2013; Ren et al. 2017), whereas it activates the *HvS40* gene during natural and stress-related senescence in barley (*Hordeum vulgare*) (Krupinska et al. 2013) and *PsbA* gene expression in response to chilling treatment in tomato (*Solanum lycopersicum*; Zhuang et al. 2018).

In the nucleus, WHY1 protein also modulates telomere length by binding to their AT-rich region (Yoo et al. 2007) and affects microRNA synthesis (Swida-Barteczka et al. 2018). Moreover, in chloroplasts, WHY1 plays a role in organelle genome stability, facilitating accurate DNA repair (Cappadocia et al. 2010; 2012; Lepage et al. 2013) and affects RNA editing/splicing (Prikryl et al., 2008; Melonek et al. 2010). The intracellular localization of WHY1 and/or the developmental stage of the plants may contribute to its various functions (Ren et al. 2017). Furthermore, WHY1 has been reported to be involved in (a)biotic stress signaling pathways, e.g. in response to chilling (Zhuang et al. 2018), high light (Kucharewicz et al. 2017), N deficiency (Comadira et al. 2013), reactive oxygen species (Lin et al. 2019; Lepage et al. 2013), hormones such as SA and abscisic acid (Xiong et al. 2009; Isemer et al. 2012), and defense signaling, being e.g. required for SA- and pathogen-induced PR1 expression (Desveaux et al. 2005).

In this study, we extend the roles of the dual-localized WHY1 protein to SA biosynthesis via regulating *PAL1* and *ICS1* expression and SA modification via affecting *BSMT1* gene expression, in a developmental-dependent manner. Moreover, the cellular SA level affected the distribution and status of WHY1 protein in the
nucleus and in plastids, suggesting a feedback mechanism to regulate SA content. Further, globally analysis of gene expression in WHY1 loss-of-function mutants and pWHY1 or nWHY1 gain-of-function mutant indicated that the levels of hormone metabolism-related genes were significantly altered. Our results provide evidence that the dual-localized WHY1 protein functions in both the nucleus and chloroplasts to fine-tune SA dynamics affecting plant development in *Arabidopsis*.

Results

**WHY1 changes the gene expression level of PAL, ICS, and BSMT1, and alters SA contents during plant development**

To explore how WHY1 is involved in the SA metabolism pathways (Figure 1a), we used the *why1-1* mutant previously deployed in several of our studies (Miao et al. 2013; Ren et al. 2017; Lin et al. 2019). This *why1-1* mutant displays an early-senescence phenotype (Miao et al. 2013), similar to the *bsmt1* mutant (Vlot et al. 2009) and the SA 3-hydroxylase mutant (*s3h*; Zhang et al. 2013). We analyzed the expression levels of *ICS*, *PAL*, and *BSMT1*, encoding proteins with both benzoic acid (BA) and SA carboxyl methyltransferase activities, and genes for SA glycoside/glucose ester modification enzymes such as *UGT71B1*, *UGT89B1*, or *UGT74F2* (Dempsey et al. 2011) in the *why1* mutant compared to WT during plant development 28–42 days after germination (dag). Interestingly, *WHY1* loss-of-function mutation increased the transcript level of *PAL1* and *PAL2* at 37 dag, but greatly decreased the transcript level of *BSMT1* at 35 and 37 dag and of *ICS1* at 42 dag.
(Figure 1b), whereas the transcript levels of \textit{UGT71B1}, \textit{UGT74F2}, \textit{UGT89B1}, and \textit{S3H} were not altered in the \textit{why1} mutant during plant development (Supplemental Figure S1).

Thus, we tested whether SA contents also changed in the \textit{why1} mutant during plant development. The SA contents, including conjugated and free type of SA, of the \textit{why1} and WT plants were measured with a HPLC assay during the period from 28 dag to 58 dag of plant development. Our results indicate that \textit{WHY1} loss-of-function mutation made both conjugated SA and free SA peak 5 days earlier (at 37 dag) than that in wild type (at 42 dag) (Figure 1c, d).

In order to genetically confirm this hypothesis, we produced the \textit{why1pal1}, \textit{why1sid2}, \textit{why1pal1sid2}, and \textit{why1bsmt1} double/triple mutants (Supplementary Figure S2) and measured the SA contents in these mutants during plant development (Figure 1e). Interestingly, the early SA peak disappeared in the \textit{why1pal1} line at 37 dag, showing a similar SA profile as the wild type, whereas SA accumulation in \textit{why1} mutants combined with \textit{bsmt1} mutation were not that strongly affected, displaying the same early-senescence phenotype as the \textit{why1} line. However, SA accumulation in \textit{why1} combined with \textit{sid2 (ics1)} was inhibited at 42 dag. The \textit{why1pal1sid2} triple mutant showed a delayed-senescence phenotype and, again, had no earlier SA peak, with SA maintained at low levels even at 37 and 42 dag, suggesting that PAL activity is crucially important for SA accumulation during the early stages of plant development. Thus, we genetically confirmed that SA content in cells is affected by \textit{WHY1},
nWHY1/pWHY1 affects the gene expression level of *PAL1*, *ICS1*, and *BSMT1* as well as SA content during plant development

WHY1 is known to be dual-localized to the nucleus and plastids (Grabowski et al. 2008). To clarify which isoform of WHY1 affects SA metabolism and its dynamics, we complemented the *why1* mutant line with *pWHY1*, *nWHY1*, or *pnWHY1* under 35S promoter control (Lin et al. 2019) and analyzed the subsequent transcript levels of *PAL*, *ICS*, and *BSMT1* at 28–42 dag. Complementation with *nWHY1* or full-length WHY1 (*pnWHY1*) restored the transcript levels of *PAL1*, *PAL2*, and *BSMT1* to comparable with the level in the wild type, whereas the *nWHY1/why1* line had even lower *PAL1* expression level at 37 dag and 42 dag compared to WT. Surprisingly, complementation with *pWHY1* resulted in a 2-fold increase in transcript level of *PAL1* and repressed the transcript level of *BSMT1* at 37 dag, and also significantly increased the transcript level of *ICS1* at 42 dag (Figure 2a). Measurement of SA contents in the complemented *why1* mutant background from 28 to 42 dag showed that, until 37 dag, both *nWHY1/why1* and *pnWHY1/why1* lines exhibited significantly restored wild-type SA accumulation compared to the *why1* line, and that the SA content in the *nWHY1/why1* mutant was even lower at 42 dag. However, *pWHY1* significantly pronounced SA accumulation during the whole period of development (Figure 2b), indicating that nWHY1 somehow repressed SA accumulation via suppression of *PAL1* expression. On the other hand, *pWHY1* might pronounce SA
accumulation via repressing $BSMT1$ during early developmental stages and promoting $ICS1$ at later developmental stages.

**Hormone-related gene enrichment in “compartmental WHY1” transgenic plants**

In order to globally understand the differences and similarities in the transcriptome response following pWHY1 and nWHY1 expression, a microarray sequencing analysis was deployed. Phenotypic differences were observed in the short-term response and thus, to avoid long-term secondary artifacts caused by continuous expression, an estradiol-inducible promoter was used to generate “inducible compartmental WHY1” transgenic plants ($VEX:pWHY1/why1$ and $VEX:nWHY1/why1$) as described in Ren et al. (2017). We found that WHY1 protein level increased about 14-fold following a 2-h induction with 20 μM estradiol (Ren et al. 2017). The total RNA isolated from the 35-day-old rosette leaves of inducible $VEX:pWHY1/why1$ and $VEX:nWHY1/why1$ plants before (0 h) and after estradiol application (2 h), as well as total RNA from $why1$ and WT plants, was used for transcriptome analysis by ATH1 Arabidopsis GeneChip microarrays with two biological replicates. Comparison of the transcriptome of inducible pWHY1 plants to that of non-inducible pWHY1 plants revealed a complex genetic reprogramming with 1165 and 4560 transcripts being at least 2-fold up- and down-regulated, respectively. Comparison of inducible nWHY1 plants to that of non-inducible nWHY1 plants also revealed a complex genetic reprogramming with 920 and 3965 transcripts up- and down-regulated, respectively. Transcriptomic comparison of the $why1$ mutant to WT plants identified 4432 and 1190
transcripts up- and down-regulated, respectively (Supplementary Figure S3).

To visualize gene expression reprogramming in the VEX:pWHY1 VEX:nWHY1 and the why1 plants, their entire nuclear transcriptome was subjected to MapMan analysis allowing the identification of biological processes with significant alterations (Thimm et al., 2004). The hormone metabolism pathways were significantly overrepresented after induction of pWHY1, nWHY1, or by WHY1 loss-of-function mutation, in particular affecting auxin, jasmonic acid (JA), and ethylene metabolism, as well as SA metabolism (Figure 3, Supplemental Datasets S1–S4). The regulation of secondary metabolism and stress were also significantly enriched after induction of pWHY1 expression (Fig. 3a). These stresses were associated with biotic- and abiotic-stress responses that were related to redox imbalance, which were mostly up-regulated by pWHY1 (Figure 3a). By contrast, the regulation of RNA, development, and signaling terms were significantly enriched after induction of nWHY1 expression. Since the opposite regulation of signaling, development, RNA, and transport terms was observed in WHY1 loss-of-function mutant plants (Figure 3a), these changes could be attributed to the inducible expression of pWHY1 or nWHY1 (Figure 3a). Globally, a net enrichment of biological processes linked to hormone metabolism was found within the most significantly differential expressed genes after induction of pWHY1 or nWHY1 or deletion of WHY1 (Figure 3b). A net enrichment for biological processes linked to hormone metabolism, secondary metabolism, and photosynthetic stress was found within the most differentially expressed genes in the inducible pWHY1 line.
(Figure 3b). A net enrichment for biological processes linked to RNA regulation, development, or signaling was found within the most differentially expressed genes in the inducible nWHY1 line (Figure 3b). Finally, a net enrichment for biological processes linked to photosynthesis and signaling or development or RNA regulation was found within the most differentially expressed genes in the why1 line (Figure 3b).

Among the differentially expression genes, 153 were common to the inducible pWHY1 and nWHY1 lines. Among these, 42 hormone-related genes exhibited up- or down-regulated expression in the pWHY1 or why1 lines, including metabolism- and signaling-related genes for SA, JA, IAA, and ethylene (Figure 3, Supplementary datasetS2, S4). The 24 most expressed or suppressed genes in pWHY1, nWHY1, or why1 plants, which encode key components of the SA metabolism pathway including ICS1, ICS2, PAL1, PAL2, UGT71B1, UGT89B1, UGT74F2, BSMT1, as well as SA signaling–related genes, or senescence/cell death–related genes are shown in the heatmap (Figure 3c).

**WHY1 directly binds at the promoter region of ICS1 and indirectly affects PAL1 and BSMT1 expression in a developmental-dependent manner**

WHY1 was first reported as a transcription factor in the nucleus (Marechal et al. 2000). To investigate whether WHY1 directly regulates ICS1, PAL1/PAL2, and BSMT1 gene expression, we analyzed our previous ChIP-seq dataset and above microarray dataset and found that ICS1, MYB15, and ERF109 are direct targets of WHY1 (Miao
et al. 2013; and Figure 4a), whereas PAL1 and BSMT1 are not. A search for
transcription factor binding motifs in promoter regions of ICS1, MYB15, ERF109,
PAL1, and BSMT1 genes was conducted with PlantCARE (Lescot et al. 2002), which
identified two w-boxes, six MYC elements, and four MYB motifs in the promoter of
PAL1; 6xERE elements in the BSMT1 promoter (Figure 4b; Supplementary Figure S3);
and several GTNNNNAAT and AT-rich motifs in the ICS1, MYB15, and ERF109
promoters (Supplementary Data-5). In order to clarify the relationship among these
motifs, we firstly confirmed WHY1 binding at the target genes by chromatin
immunoprecipitation qPCR (ChIP-qPCR) using leaf material collected 37 and 42 dag
from plants expressing HA-tagged WHY1 under its native promoter (P\textit{why1}:WHY1-HA),
as described in previous work (Miao et al. 2013). The putative cis elements found in
WRKY53, ICS1, MYB15, ERF109, and WRKY33 promoters, included several
GTNNNNAAT or AT-rich motifs (Figure 4b; Supplementary Dataset S5), were
enriched 5–20 fold (Figure 4c). The regions containing GTNNNNAAT and AT-rich
motives of MYB15, ERF109, and WRKY53 were enriched 10–15 fold at 37 dag,
whereas fragments of ICS1 and WRKY33 could not be detected at 37 dag, but
together with MYB15 and WRKY53 a high enrichment was observed at 42 dag
(Figure 4c). Furthermore, the expression levels of these genes were analyzed by
reverse transcription quantitative PCR (RT-qPCR) at 37 and 42 dag in \textit{why1} and WT
plants. In the knockout-mutant background, WHY1 binding negatively correlated with
gene expression of ERF109 at 37 dag and ICS1 at 42 dag, and positively correlated
with MYB15 expression at both 37 and 42 dag. Whereas WRKY53 expression was positively correlated in why1 plants at 37 dag, WRKY33 was up-regulated at 42 dag. Thus, WHY1 appears to exert either negative effects on gene expression (WRKY53, WRKY33 and MYB15) or cause activation of its target genes, such as ERF109 and ICS1, depending on the developmental stage.

In order to further verify the activation or repression activity of WHY1, the promoter sequences of WRKY53, ICS1, MYB15, ERF109, PAL1, and BSMT1 were cloned into dual-luciferase vectors and applied in a transient expression assay using Nicotiana benthamiana leaves (Hellens et al., 2005). In addition to measuring promoter activation or repression by WHY1, MYB15 and ERF109 were also included in the analysis to investigate indirect effects of WHY1 in the nucleus. The coding sequences of WHY1, MYB15, and ERF109 were cloned under the control of the Arabidopsis ACTIN1 promoter (ACTIN:WHY1-HA, ACTIN:MYB15-HA, and ACTIN:ERF109-HA) (Figure 5a), and co-infiltrated with the reporter vector to drive LUCIFERASE (LUC) expression (Hellens et al., 2005). We then measured the LUC and RENNILASE (REN) luminescence ratio (i.e. LUC/REN ratio) in infiltrated leaves. To assess any basal activation or repression of putative promoters, a mini-GAL4 promoter vector was used in each co-infiltration experiment as a control; the WRKY53 promoter was used as a positive control. The results showed that WHY1 activated the promoters of ICS1 and ERF109, but it repressed the promoters of MYB15 and WRKY53, displaying the opposite expression pattern of the why1 knockout plants (Figure 4b). The transcription
factors MYB15 and ERF109 were respectively able to activate PAL1, PAL2, and BSMT1 gene expression (Figure 5b-c). Therefore, WHY1 directly activates ICS1 expression and indirectly affects PAL1, PAL2, and BSMT1 gene expression via MYB15 and ERF109, respectively.

**WHY1 and MYB15/ERF109 regulate leaf senescence and ROS accumulation**

Since WHY1 is a repressor of plant senescence at early stage (35–42 dag) of plant development (Miao et al. 2013), we compared the phenotypes of the pal1, sid2, myb15, and erf109 mutants (Supplementary Figure S4) with that of the why1 mutant to analyze if the effects of WHY1 on SA metabolism influence senescence. pal1 and sid2 plants have already been reported to have a delayed-senescence phenotype, whereas oePAL1, oeSID2, and bsmt1 plants show an early-senescence phenotype (Love et al., 2008; Rivas-San et al., 2011; Vlot et al., 2009; Huang et al. 2010). We analyzed all mutants with respect to a visible senescent yellow leaf ratio (Miao and Zentgraf, 2007) and reactive oxygen species (ROS) production by nitro blue tetrazolium chloride (NBT) staining and diaminobenzidine (DAB) staining assays under normal growth conditions. The results showed that pal1, sid2, myb15, and erf109 each displayed visibly delayed senescence and less ROS production, which was in contrast to bsmt1 plants that showed slightly earlier senescence and higher ROS accumulation similar to why1 and pWHY1 plants (Figure 6a-b).

Furthermore, the transcript levels of senescence-related genes such as WRKY53,
SAG12, SAG13, SAG101, and PAD4 were measured by RT-qPCR and indicated as a heatmap (Figure 6c). These genes were up-regulated in why1 and pWHY1 plants, similar to in PAL1-overexpressing (oePAL1) plants; however, the same genes were down-regulated in pal1, myb15, and sid2 similar to in nWHY1 plants (Figure 6c).

Interestingly, in the BSMT1-overexpressing (oeBSMT1) line, the transcript levels of senescence-related genes SAG12 and WRKY53 were up-regulated whereas the transcript levels of SAG13 and SAG101 were down-regulated, which was the reverse of the expression trend observed in bsmt1 and erf109 (Figure 6c). However, the transcript level of PAD4 was up-regulated in the both bsmt1 and oeBSMT1. This indicates that BSMT1 is involved in alternative signaling pathways that function in developmental senescence or stress-related senescence.

**SA level feedback affects distribution of the WHY1 protein in plastids and the nucleus**

WHY1 is required for SA- and pathogen-induced PR1 expression (Desveaux et al. 2005). WHY1 distribution is affected by protein modification (Ren et al. 2017) and cellular H₂O₂ level (Lin et al. 2019). To determine if SA feedback affects WHY1 expression, we quantified WHY1 transcription by RT-qPCR in response to exogenous MeSA in WT plants treated for 1, 4, 6, and 8 hours. Unexpectedly, MeSA treatment did not affect WHY1 expression level (Figure 7a). Thus, MeSA treatment probably affects WHY1 protein function or distribution in plastids or the nucleus. Nuclear and plastid proteins isolated from 35-day-old WT rosettes after MeSA treatment for 1, 3, and 6
hours were immunodetected with a specific monoclonal antibody against WHY1 (Lin et al. 2019; Supplementary Figure S4), and antibodies against Histone 3 and photosystem II (PSII) protein were used as markers for pure nuclear and plastidial protein preparations (Figure 7b-c; Supplementary Figure S5). A water treatment served as control for MeSA application. Interestingly, the results indicated that during the MeSA treatment time course, WHY1 accumulation decreased in plastids and the nuclear isoform of WHY1 was altered in its status, with small nWHY1 (29 kDa) levels slightly increasing while large nWHY1 (37 kDa) levels decreased (Figure 7b-c). We investigated the 4-h time point in more detail to quantify these alterations. We captured and calculated three replicates of protein band signals by the software program Image J and statistically analyzed their significance, demonstrating that WHY1 accumulated significantly less in plastids, and the small nuclear isoform of WHY1 was significantly increased, whereas large isoform nWHY1 (37 kDa) levels were significantly decreased after a 4-h MeSA treatment (Figure 7d; Supplementary Figure S6). Thus, exogenous MeSA treatment affects WHY1 accumulation in plastids and alters the modification status of nWHY1 in the nucleus, a similar response as that observed in response to H$_2$O$_2$ treatment (Lin et al., 2019). Furthermore, we analyzed WHY1 distribution between plastids and the nucleus under SA-deficient conditions. The nuclear and plastid fractions isolated from the single sid2 and pal1 mutants and the double sid2 pal1 mutant were subjected to immunoblotting using the WHY1-specific peptide antibody. The results demonstrated that pWHY1 in the sid2,
pal1, and sid2 pal1 mutants significantly accumulated in plastids when compared to WT (Figure 7e, g). Accordingly, the large nuclear WHY1 isoforms (37 kDa) were highly abundant and the small nuclear WHY1 proteins (29 kDa) were less abundant in the pal1 and sid2 pal1 mutants, but not in the sid2 single mutant (Figure 7f, g). This indicates that the ICS1 pathway plays a prominent role in modification of nWHY1 protein.

Discussion

It has become increasingly clear that the dual location of proteins mediates diverse intercellular signaling processes, as, for example, described for MAP kinase (Bobik et al. 2015; Chan et al. 2016), CIPK14 (Ren et al. 2017) as well as hormone (ABA, SA) (Koussevitzky et al. 2007; Caplan et al. 2015; Kacprzak et al. 2019) and ROS (hydrogen peroxidase and singlet oxygen) signaling (Lin et al. 2019; Duan et al. 2019, Lv et al. 2019). Proteins with dual subcellular localization can affect transcription and display various functions in intracellular signaling (Lin et al., 2019; Isemer et al., 2012; Sun et al., 2011; Nevarez et al., 2017; Pesaresi and Kim, 2019; Wu et al., 2019; Woodson et al., 2011/2013). This study revealed that dual-localized WHY1 protein directly activates ICS1 expression in the nucleus at later stages of plant development, whereas it indirectly controls PAL1 and BSTM1 expression via alteration of MYB15 and ERF109 transcription at early stages of plant development. Therefore, WHY1 influences cellular SA content during plant development. A SA-level feedback in turn affects WHY1 distribution causing a shift into the nucleus and preferential
accumulation of the smaller 29 kDa form. This loop of nWHY1 integrating SA
dynamics via PAL1/ICS1 and BSMT1 plays a pivotal role in controlling leaf
senescence.

SA is crucial for plant growth. During plant development, the first peak of SA appears
at the onset of senescence (Buchanan-Wollaston et al. 2005). It was assumed that SA
was a senescence trigger signal and relative to plant senescence (Morris et al. 2000).
Alteration of SA dynamics resulted in remodeling of plant programmed cell death.
Elucidation of SA biosynthesis and catabolism is important to understand its biological
functions. 10% of SA is synthesized from L-phenylalanine via the PAL pathway in the
cytoplasm, whereas 90% of SA is from chorismate via ICS1/SID2 (ISOCHORISMATE
SYNTHASE1/SALICYLIC ACID INDUCTION DEFICIENT2) in chloroplasts, the latter
of which is responsible for the bulk of SA produced during pathogen infection in
Arabidopsis (Dempsey et al. 2011). Endogenous SA can also undergo a series of
chemical modifications including hydroxylation by salicylate hydroxylase (Yamamoto
et al. 1965; Zhang et al., 2013), glycosylation by glycosyltransferases (Lim et al. 2002;
Dean et al. 2008), methylation by BSMT1 (Park et al., 2007), and amino acid or sugar
conjugation by as shown by Zhang et al. (Zhang et al., 2007; Bartsch et al. 2010). The
microarray data and RT-qPCR results showed that the gene expression levels of
developmental-related transcription factors were up-regulated, and that of
stress-related genes were down-regulated in why1 plants (Figure 7; supplementary
dataset S4). The expression levels of ICS1, PAL1, and BSMT1 were altered
significantly in the *why1* mutant during plant development (Figure 1); this alteration could be rescued completely through complementation with nWHY1 and pnWHY1 (Figure 4). As was previously established, nWHY1 can directly bind to the promoters of many target genes such as *WRKY53, S40, Kenisins*, and *PR10a* (Desveaux et al. 2005; Miao et al. 2013; Krupinska et al. 2017; Xiong et al. 2009) as well as *MYB15, MYC1/2, ICS1*, and several ERF family members, as shown in our WHY1 ChIP-seq dataset (Figure 2; Miao et al., 2013). Moreover, nWHY1 represses the expression of many downstream developmental-related target genes such as *WRKY53, WRKY33, MYB15*, and *TRANSPARENT TESTA GLABRA2 (TTG2)* (Figure 7; Supplementary dataset S1). However, it can also promote the expression of many stress-related genes such as *HvS40* (Krupinska et al. 2013), *PR1* (Desveaux et al. 2005), redox responsive transcription factors (Foyer et al. 2014), *ICS1*, and *ERF109* (Figure 2; Figure 7; Supplementary dataset S1). Several MYB family members can bind to the promoter of *PAL1/PAL2* (Battal et al. 2019). Among these, MYB15 was shown to bind to the promoters of *PAL1* and *ICE1* by ChIP-qPCR. MYB15 mainly plays a virtual role in immunity and cold response (Chezem et al. 2017; Kim et al. 2017; Wang et al. 2019). Our results further confirm that MYB15 can activate *PAL1* expression. ERF-binding cis elements are enriched in the promoter region of *BSMT1*. However, *ERF109* as a target gene of WHY1, which was identified in our ChIP-seq dataset (Miao et al. 2013), was not found to bind to the promoter region of *BSMT1*, as shown in yeast one-hybrid and gel-shift assays (Ximiao Shi, Master thesis, 2018). By
contrast, ERF109 can activate BSMT1 expression as shown by our LUC/REN transit assay (Figure 4), thus supporting our ChIP-seq data. The erf109 and bsmt1 mutants accumulate high levels of anthocyanin in response to high light (Foy et al. 2015); however, the underlying regulatory mechanism is currently unknown. Therefore, the balance module of nWHY1/MYB15-PAL1 and nWHY1/ERF109-BSMT1 at early developmental stages (37 dag) and WHY1/ICS1 regulation at late developmental stages (42 dag) determines SA homeostasis during plant development. An imbalance of PAL1/BSMT1 activity at 37 dag in the why1 mutant and the repression of ICS1 at 42 dag may result in SA accumulation about one week earlier than usual during normal plant development. Thus, nWHY1 impacts SA homeostasis via mediating PAL1 or ICS1 and BSMT1 activity in cells during plant development.

The WHIRLY family is considered to associate with retrograde signaling. Due to their dual location and function in the nucleus and plastids (Krause et al., 2009), it is assumed that WHIRLY1 can move from plastids to the nucleus (Isemers et al., 2012). The plastid isoform of WHIRLY1 affects miRNA biogenesis in the nucleus (Swida-Barteczka et al. 2018). Previously, we showed that the WHY1 protein can be phosphorylated by CIPK14 kinase or oxidized by H$_2$O$_2$, leading to different subcellular localization in the nucleus or in plastids, respectively (Ren et al. 2017; Lin et al. 2019). Here, we further show that WHY1 loss-of-function mutation results in SA production 5 days earlier during plant development, thereby accelerating plant senescence. Complementation with pWHY1 did not revert the SA accumulation phenotype. On the
contrary, pWHY1 further increased SA accumulation during plant development. Consistently, *PAL1* expression is promoted and *BSMT1* expression is repressed at 37 dag, whereas ICS1 is activated at 42 dag (Fig. 4). This phenomenon can be explained by two mechanisms: 1) H$_2$O$_2$ is known to affect SA levels via the ICS1 pathway (Leon et al. 1995; Dat et al. 1998; Chaouch et al. 2010; Guo et al. 2017) and recent data link pWHY1 to ROS production via photosystem I/II (PSI/PSII) (Huang et al. 2017; Lin et al. 2019). Thus, pWHY1 might increase SA level at 42 dag by modulation of the ICS1 pathway via photosystem-induced ROS accumulation to cause an early senescent phenotype. 2) pWHY1 coordinating SA dynamics is feedback controlled by cellular SA levels (Isemer et al. 2012; Caplan et al. 2017); WHY1 isoform changes from plastid to nucleus represses *MYB15* and *PAL1* expression (Huang et al. 2010; Duan et al., 2019) and activates *ERF109* and *BSMT1* expression in response to stress cues such as high light (Estavillo et al. 2011). This demonstrates that dual-localized pWHY1/nWHY1 affects SA contents most likely via connection with PSI/II-mediated ROS affecting leaf senescence.

The distribution of WHY1 between plastids and the nucleus depends on its modification status (Ren et al. 2017) as well as on environmental cues or cellular signals such as H$_2$O$_2$ (Lin et al. 2019) and SA (this work). Although the SA signal cannot promote CIPK14 expression (Wenfang Lin, PhD dissertation, 2017), MeSA treatment feedback alters the nWHY1 protein status (37-kDa or 29-kDa form) (Figure 7), similar to barley WHY1 (Grabowski et al., 2008) and nWHY1 after treatment with
H$_2$O$_2$ in *Arabidopsis* (Lin et al. 2019). The nature of modification resulting in both forms remains unknown. More interestingly, MeSA treatment was shown to reduce WHY1 accumulation in plastids, which was in contrast to H$_2$O$_2$ treatment (Lin et al., 2019). These phenomena are further elucidated in SA-deficient mutants such as *pal1*, *sid2*, and the *pal1 sid2* double mutant. *ICS1* loss-of-function mutation (*sid2*) decreases the modified state of nWHY1 level, *PAL1* loss-of-function mutation (*pal1*) increases the modified state of nWHY1 level, whereas ICS1 or PAL1 loss-of-function mutation increases WHY1 accumulation in plastids. It is known that ICS1 is localized to plastids and is responsible for the bulk production of SA in response to salt or pathogens (Kumazaki and Suzuki, 2019). Plastid-derived SA can be transported from plastid to the nucleus via stromule (Caplan et al. 2015). Combined with global microarray data, it is speculated that this kind of SA signal molecule might influence the nuclear isoform of WHY1, in which the small form (29 kDa) activates stress-related gene expression, such as *S40* and *ICS1* (Krupinska et al., 2013; Figure 7), whereas the large form (37 kDa) represses gene expression, as shown for *WRKY53*, *WRKY33*, and *MYB15* (Miao et al. 2013; Figure 2, 7). Furthermore, it has been reported that phosphorylation of WHY1 by CIPK14 promotes its binding affinity at the promoter of *WRKY53* and *WRKY33* and represses *WRKY53* and *WRKY33* expression (Ren et al. 2017), and that CIPK kinase expression level rapidly increases in response to salt or pathogen stress, accompanying increasing Ca$^{2+}$, H$_2$O$_2$, and SA levels in the cells (Sardara et al., 2017).
Conclusion

We conclude that WHY1 exerts dual functions in plastids and the nucleus. Nuclear WHY1 influences SA content by directly affecting *ICS1* and indirectly affecting *PAL1* and *BSTM1* expression via *MYB15* and *ERF109*. The pWHY1 isoform promotes PAL1/ICS1 expression and represses BSMT1, facilitating high SA accumulation and resulting in early senescence, similar to *bsmt1* mutants. Interestingly, MeSA treatment altered the nWHY1 status (increasing the 29-kDa form of WHY1 while decreasing the 37-kDa form) in combination with declined pWHY1 accumulation. These results indicate that pWHY1/nWHY1 distribution in the nucleus and chloroplast allows cellular balancing of SA and H$_2$O$_2$ contents in a developmental-dependent manner, thereby affecting leaf senescence in *Arabidopsis* (Figure 8).

Materials and Methods

Plant materials

All *Arabidopsis thaliana* mutants are in Col-0 background. The T-DNA insertion lines *why1* (*Salk_023713*), *sid2*, *pal1*, *bsmt1* (*SAIL_776_B10*), and *myb15* (*myb15-1 SALK_151976, myb15-2 SK2722*) were kindly provided by other scientists. The *erf109* (*SALK_150614*) and *ERF109* over-expression lines (*CS2102255*) were obtained from the Nottingham Arabidopsis stock center (NASC). Homozygous plants were selected and confirmed by PCR or RT-PCR using gDNA and cDNA as templates (Supplementary Fig S2), respectively (*http://signal.salk.edu/ tdnaprimers.2.html*). The
remaining experimental lines used in this study were constructed in our lab, namely
the nWHY1-HA-overexpressing line that produces WHY1 protein localized only to the
nucleus, the pnWHY1-HA-overexpressing line that produces the WHY1 protein
dual-localized to plastids and the nucleus, the complemented PWHY1-HA
(Pwhy1:pnWHY1-HA) line, and the pWHY1-HA line that harbors the construct
containing full-length WHY1 with a nuclear export peptide sequence fused to HA-tag
and produces WHY1 protein localized only to plastids (Miao et al. 2013; Lin et al.;
2019).

Seeds were germinated on wet filter paper followed by vernalization at 4°C for 2 d,
then transplanted to vermiculite and grown in a climatic chamber (100 μE/h, 13 h of
light at 22°C/11 h of dark at 18°C, 60% relative humidity). The rosette leaves were
labeled with colored threads after emergence, as described previously (Hinderhofer
and Zentgraf 2001).

For MeSA treatment, rosette leaves were collected at 1, 2, 3, 4, 6, and 8 h after
spraying with 100 μM MeSA and stored in liquid nitrogen or at -80°C for later use in
RNA or protein isolations. Mock treatments used distilled water instead.

Measurement of SA contents in rosette leaves

SA was extracted from 0.2 g of the 5th leaf from individual plants at different stages
of development and measured by reversed-phase high-performance liquid
cromatography (HPLC) on an Agilent1260 system with a C18 column as previously
described (Verberne et al. 2002), albeit with small modifications: SA was thoroughly
separated from the complex mixture by methanol containing 10% of sodium acetate with pH 6.0 (Lin et al. 2017). Fluorescence detection (excitation at 305 nm and emission at 407 nm) was applied and 3-Hydroxybenzoic acid (3-HBA) was used as an internal standard (Aboul-Soud et al. 2004). Conjugated and free SA was detected at the same time. Three independent biological replicates were performed for each data point.

Staining of ROS

Visualization of \( \text{H}_2\text{O}_2 \) accumulation in leaves was performed using the 3',3'-diaininobenzidine (DAB) staining method according to Zhang et al. (2014) and Huang et al. (2019). Detached rosette leaves were vacuum filtered in 20 mL staining solution containing 1 mg/mL DAB in 50 mM Tris-HCl, pH 5.0 for 10 min, and incubated in darkness at room temperature for 12 h. The leaves were destained by boiling in a mixture of ethanol, glycerol, and acetic acid (3/1/1, v/v/v) for 15 min before imaging.

Detection of superoxide free radicals were performed by the nitroblue tetrozolium (NBT) staining method as described in Lee et al. (2002). The whole rosettes of 5–6-week-old plants were harvested and immersed in 0.1 mg ml\(^{-1}\) NBT solution (25 mM HEPES, pH 7.6). After vacuum infiltration, samples were incubated at 25°C for 2 h in darkness. Subsequently, stained samples were bleached in 70% (v/v) ethanol and incubated further for 24 h at 25°C to remove the chlorophyll.

Imaging was conducted using an Epson Perfection V600 Photo scanner (Epson China, Beijing, China).
Reverse transcription quantitative PCR analysis (RT-qPCR)

RT-qPCR analysis was performed using SYBR Green master mix (SABiosciences, Frederick, MD, USA) according to the manufacturer’s instructions. cDNA synthesis was carried out using a Fermentas first-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) on RNA from 28–55-day-old plants grown under normal light conditions. cDNA was diluted 20-fold prior to qPCR. The Touch 1000 platform (Bio-Rad) was used for qPCR, and the data were analyzed using Bio-Rad software version 1.5. We used GAPC2 or ACTIN as internal reference genes for calculation of relative expression. Primers are listed in Supplemental Table S1. All determinations were conducted in three biological replicates.

Isolation and detection of plastid and nuclear proteins

Chloroplasts and nuclei were prepared and purified as described previously (Ren et al. 2017). Approximately 10 µg proteins of each fraction were separated on 14% (w/v) polyacrylamide gels. After transfer to nitrocellulose membranes, immunodetection followed using specific antibodies against the WHY1 C-terminal peptide CASPNYGGDYEWNR (Faan, Hangzhou, China) (Supplementary Fig S4). To monitor the purity of the chloroplast and nuclear fractions, we used antibodies against the cytochrome b559 apoprotein A or the histone H3 (Cell Signaling, Munich, Germany), respectively (Lin et al. 2019) (Supplementary Fig S5). Immunoblot images were taken using the FluorChem Q machinery (Proteinsimple Company, CA, USA). The digital
data of protein bands were directly captured and the signal intensity of the same area was calculated as absolute means by the Image J software program setup in the FluorChem Q machine. Before we captured the protein band signals, we firstly adjusted all gel backgrounds to be nearly the same level.

ChIP-qPCR assay

Four-week-old rosettes of transgenic plants expressing Pwhy1:WHY1-HA to complement the why1 knockout-mutant background were used for sample preparations. The cross-linked DNA fragments 200–1,000 bp in length were immunoprecipitated by an antibody against the HA-tag (Cell Signaling, Munich, Germany). The enrichments of the selected promoter regions of both genes were resolved by comparing the amounts in the precipitated and non-precipitated (input) DNA samples, which were quantified by qPCR using designed region-specific primers (Supplementary Table S1 and Figure 2). Material from the why1 mutant served as a mock control and was used for normalization to calculate the fold enrichment. The experiments were performed with triplicate biological replicates.

Cloning and Construction of Vectors

The promoter sequence 2 kb upstream of the start codon of MYB15 and the ERF109, WRKY53, PAL1, PAL2, ICS1, and WRKY33 genomic sequences were PCR amplified and then restricted with KpnI and XhoI or XhoI and PstI, respectively, and sub-cloned into the pFLAP vector. The entire cassette was then excised with KpnI and Ascl and cloned into the binary vector pBIN +.
For dual Luciferase assays, promoter sequences were PCR amplified, digested with Ncol and KpnI, and cloned into the pGreenII 0800-LUC binary vector (provided by Roger P. Hellens). DNA constructs used for N. benthamiana agro-infiltration and for agrobacteria-mediated plant transformation were constructed via Goldenbraid cloning (Sarrion Perdigones et al., 2013).

MYB15, ERF109, and WHY1 coding sequences were subcloned into a pUPD vector. In the dual Luciferase assays, MYB15, ERF109, and WHY1 were in the 1α1 vectors, which are based on a pGREENII backbone. For generating the gene-overexpression construct, a CDS fragment was amplified and subcloned into pGEM-T Easy (Promega), excised with BamHI and SalI restriction enzymes, and then cloned under control of the CaMV-35S promoter into pFLAP, before restriction with PacI and AscI and ligation to the pBIN+ binary vector.

Dual-luciferase activity assay

Nicotiana benthamiana plants were grown in climate-controlled rooms (22°C, 16/8 h of light/dark). Plants were grown until they had six leaves and then infiltrated with Agrobacterium tumefaciens GV3101. Plants were maintained in the climate-controlled rooms and, after 4–5 d, 1-cm discs were collected from the fourth and fifth leaves of each plant. Six biological replicates with their respective negative controls were used per assay. The experiment was performed as previously described (Hellens et al., 2005) with minor changes. Agrobacterium was grown overnight in LB and brought to a final O.D.600 of 0.2 in infiltration buffer. Co-infiltrated Agrobacterium carried separate
plasmids: 900 μl of an empty cassette or one that contains the transcription factor driven by the tomato 2-kb ACTIN promoter region, and 100 μl of the reporter cassette carrying one of the test promoters. Leaf discs were homogenized in 300 μl of a passive lysis buffer. 25 μl of a 1/100 dilution of the crude extract was assayed in 125 μl of luciferase assay buffer, and LUC and REN chemiluminescence of each sample was measured in separate wells on the same plate. RLU were measured in a Turner 20/20 luminometer, with a 5-sec delay and 15-sec measurement. Raw data was collected and the LUC/REN ratio was calculated for each sample. Biological samples were pooled together and a Student's t-test was performed against a background control for each experiment as described in the results section. The entire experiment was repeated a second time under similar conditions to confirm the regulatory effect of transcription factors.

Microarray Analysis

Two biological replicates were sampled from leaves of wild-type, VEX:pWHY1/why1, VEXnWHY1/why1, and why1 plants (see our previous paper Ren et al. 2017). Extracted RNA was then amplified and labeled using the standard Affymetrix protocol and hybridized to Affymetrix ATH1 GeneChips according to the manufacturer's guidelines (Katari et al. 2010). Statistical analysis of transcriptome data was carried out using Parke Genome Suite software (www.partek.com). Data preprocessing and normalization were performed using the Robust Microarray Averaging algorithm (Irizarry et al., 2003). Batch effects between the replicates were not found.
Differentially expressed genes were identified by using ANOVA according to false discovery rate, p-value 0.05 and at least a 2-fold change between the genotypes (Supplemental Datasets S1–S4).

Statistical analysis

Quantitative data were determined by at least triplicate biological replicates and the statistical significance was analyzed either using two-way ANOVA or pair-wise multiple t-tests, with the GraphPad Prism software (version 7).

Accession numbers

WHIRLY1 (At1g14410), ICS1 (At1g74710), PAL1 (At2g37040), BSMT1 (At3g11480), MYB15 (At3g23250), ERF109 (At4g34410).

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The authors declare no competing interests.

**Supplemental Data**

Supplemental Figure S1. Gene transcript levels in mutant plants analyzed by RT-qPCR.

Supplemental Figure S2. Transcript levels of *ICS2, UGT71B1, UGT89B1, UGT74F2,* and *S3H* in the *why1* mutant compared to WT during plant development.

Supplemental Figure S3. Venn analysis of transcriptome of nWHY1, pWHY1, pnWHY1, and *why1*.

Supplemental Figure S4. Immunoblot detection to certify specificity of antibody against WHY1 peptide.

Supplemental Figure S5. Immunoblot detection to certify purity of nuclear protein and plastid protein extracts.

Supplemental Figure S6. Immunoblot detection of WHY1 distribution in the organelles after MeSA treatment.

Supplemental Table S1. List of primers used in this study.

Supplementary Dataset S1. List of altered expression genes in the nWHY1 line (induced/non-induced)

Supplementary Dataset S2. List of altered expression genes in the pWHY1 line (induced/non-induced)

Supplementary Dataset S3. List of altered expression genes in the pnWHY1 line
Supplementary Dataset S4. List of altered expression genes in the *why1* line (why1/WT).

Supplementary Dataset S5. Promoter sequences with binding site marked highlight

**Figure Legends**

Figure legends

Figure 1. The various transcript levels of genes encoding key enzymes related to SA metabolism pathway and SA contents in the *why1* line during plant development

a. SA metabolism pathway in the cell. b. The various transcript levels of genes encoding key enzymes related to SA metabolism in the *why1* line during plant development. c. Content of conjugated (C-SA) and free (F-SA) SA in the wild-type and *why1*-mutant plants during the period of 28–55 days after germination (dag); d. Changes of conjugated and free SA contents in a series of double mutants with focus on 37 and 42 dag. e. Senescence phenotype of 37-day-old double mutants.

The relative expression level normalized to GAPC, the wild type at 28 dag (b) was setup as 1. The error bars represent the standard deviation of triplicate biological replicates and triplicate techniques replicates, the values were shown as means ±SD.

Asterisks (*P < 0.05, **P < 0.01) showed significant differences to the wild-type line according to either two-way ANOVA or pair-wide multiple t-tests.

Figure 2. Transcript level analysis of genes encoding key enzymes related to SA and
SA contents in various WHY1 mutants during plant development

Transcript level analysis of genes encoding key enzymes related to SA metabolism pathway (a) and SA contents (b) in the pWHY1/why1, nWHY1/why1, and pnWHY1/why1 transgenic plants compared to wild type from 28 to 42 dag during plant development.

The data represent triplicate biological replicates, the values are shown as means ± SE. Asterisks (*P < 0.05, **P < 0.01) show significant differences to the wild type within the respective conditions according to Student’s t-test.

Figure 3. The VEX:pWHY1, VEX:nWHY1, and why1 plants exhibit a complex nuclear genetic reprogramming.

a. MapMan analysis for gene ontology terms enrichment of the entire VEX:pWHY1, VEX:nWHY1, and why1 nuclear transcriptome.

b. Histogram representing the ratio of differentially expressed genes enrichment changes of selected biological process of the VEX:pWHY1, VEX:nWHY1, and the why1 transcriptome.

c. The heatmap of SA metabolism–related gene expression levels of pWHY1/why1, nWHY1/why1, pnWHY1/why1, and why1 plants. VEX:pWHY1, VEX:pWHY1/why1; VEX:nWHY1, VEX:nWHY1/why1

Figure 4. WHY1 activates/represses target gene expression

a. Enrichment profiles of WHY1 protein in five target genes: ERF109, MYB15, WRKY33, ICS1, and WRKY53 by ChIP-seq; b. Position of promoter motives (GTNNNNAAT plus AT-rich)
of WHY1-target genes; c. Enrichment folds of WHY1 at the promoters of target genes by ChIP-qPCR at 37 and 42 days after germination (dag); d. The expression levels of target
genes at 37 and 42 dag in the why1 mutant compared to WT. The error bars represent SD of triplicate biological replicates. Asterisks indicate significant difference from ACTIN according to two-tail Student’s t-test (* denotes P < 0.05, ** for P < 0.01).

Figure 5. Promoter activation assays using the LUC/REN system

a. Structure of activator and reporter constructs. b. The promoters of ICS1, MYB15, ERF109, WRKY53, and WRKY33 genes were co-infiltrated with a vector containing WHY1 under the regulation of the ACTIN promoter. c. Co-infiltration of MYB15 and ERF109 with the PAL1, PAL2, ICS1, and BSMT1 promoters. Background promoter activity was assayed by co-infiltration with an empty vector of the same type. Shown are means ± SE of six biological replicates. Asterisks denote statistically significant differences from the empty vector calculated using Student’s t-test: *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

Figure 6. Phenotyping of WHY1 loss-of-function mutants and mutants of WHY1 downstream target genes

a. Phenotypes of PAL1, ICS1, MYB15, and BSMT1 loss-of-function mutants at 37 dag compared to why1 mutants. Whole rosette (upper images) and senescent leaves ratio of 5 plants (lower graph); b. ROS accumulation of PAL1, ICS1, MYB15, and BSMT1 loss-of-function mutants at 37 dag compared to why1 mutants by NBT and DAB staining; c. The transcript levels of SAG genes in the PAL1 and BSMT1 loss- or gain-of-function and the MBY15, ERF109, and ICS1 loss-of-function mutants at 37 dag determined through RT-qPCR. The data represent triplicate biological replicates, the values are shown as means ± SE, the wild type was set to 1 in the heatmap.
Figure 7. Changes in plastid and nuclear isoform WHY1 protein levels determined by immunodetection in the *sid2*, *pal1*, or double *sid2 pal1* mutants compared to WT

a. The expression level of WHY1 in the WT plants after MeSA treatment for 1, 2, 4, 6, and 8 h. b. WHY1 immunodetection in nuclear extracts after the treatment of MeSA for 1, 3, and 6 h; c. WHY1 immunodetection in plastid extracts after treatment with MeSA for 1, 3, and 6 h; d and e. WHY1 immunodetection in nuclear extracts and in plastid extracts after MeSA treatment for 4 h (d), and in the *sid2*, *pal1*, or double *sid2 pal1* mutants compared to the wild type (e). Coomassie and silver staining are shown as the protein amount loading controls. nWHY1-L: large size (37 kDa) of nWHY1; nWHY1-S: small size (29 kDa) of nWHY1. The antibody against peptide WHY1 was a commercial product; f-g. Quantification of the alteration of pWHY1 and nWHY1 after MeSA treatment for 4 h (f), in the *sid2*, *pal1*, or double *sid2 pal1* mutants compared to the wild type (g). The protein band signals were captured and calculated by Image J software program (http://www.di.uq.edu.au/ sparqimagejblots). The data show the ratio of average means of three replicates normalized to Histone or PSII. Asterisks indicate significant differences to H₂O treatment (f) and the wild type (g) according to Student's *t*-test. (*P < 0.05, **P < 0.01).

Figure 8. A working model of the senescence pathway performed by the dual-localized WHY1 in response to SA. The nuclear isoforms of WHY1 are represented as both a large molecular mass (37 kDa, bigger letters in the Figure) and a small molecular mass (29 kDa, smaller
letters). WHY1 has dual functions in plastids and the nucleus. WHY1 loss-of-function mutation increases SA accumulation at an early developmental stage (37 dag) through increased PAL1 and repressed BSMT1 expression; elevated SA promotes nuclear WHY1 de-modification and promotes ICS1 and BSMT1 expression thereby balancing SA homeostasis in the cells. High SA levels by ICS1 cause feedback enhancing ROS accumulation, thus promoting senescence. pWHY1 stimulates PAL1/ICS1 expression but represses BSMT1, allowing high levels of SA, leading also to early senescence. Thus, distribution of WHY1 organelle isoforms and the putative feedback of SA form a circularly integrated regulatory network during plant senescence in a developmental-dependent manner. Plastid (Chl) is shown as a green ovary, nucleus (Nuc) as a grey ovary. Lines indicate regulation, wide arrows indicate transfer or translocation, broken lines indicate uncertainty.

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**a**

| VEX:pWHY1 | VEX:nWHY1 |
|------------|------------|
| Functional Group | Representation | P-value | Functional Group | Representation | P-value |
| Hormone metabolism | - | 3.25E-6 | Hormone metabolism | - | 6.25E-6 |
| Secondary metabolism | + | 2.25E-4 | RNA regulation | - | 3.25E-3 |
| Stress | + | 5.32E-4 | Development | - | 2.32E-3 |
| Miscellaneous | + | 9.76E-3 | Miscellaneous | + | 7.76E-3 |
| Photosynthesis | - | 2.13E-2 | Signaling | + | 1.13E-2 |

**b**

| why1 |
|------|
| Functional Group | Representation | P-value |
| Hormone metabolism | + | 2.49E-16 |
| Photosynthesis | + | 6.26E-14 |
| RNA | + | 4.35E-7 |
| Signaling | - | 2.81E-4 |
| Development | + | 4.26E-4 |
| Miscellaneous | - | 2.19E-3 |
| Stress | - | 3.89E-3 |
| Transport | + | 8.94E-3 |

**c**

- **Gene loci**
  - at1g66690
  - at1g88040
  - at5g04380
  - at5g38020
  - at5g37990
  - at5g66430
  - at1g66720
  - at5g37970
  - at3g44860
  - at3g21750
  - at1g73880
  - at2g43820
  - at4g10500
  - at2g37040
  - at3g53260
  - at2g37260
  - at1g66370
  - at3g23250
  - at2g44840
  - at4g34410
  - at1g74710
  - at1g18870
  - at3g11480
  - at4g23810
  - at5g13170
  - at4g30270
  - at5g48990
  - at2g29350
  - at3g48090
  - at3g56240
  - at2g14610

- **Gene names**
  - PAM
  - UGT71B1
  - UGT89B1
  - UGT74F2
  - S3H
  - PAL1
  - PAL2
  - TTG2
  - MYB113
  - MYB15
  - ERF13
  - ERF109
  - ICS1
  - ICS2
  - BSMT1
  - WRKY53
  - SAG29
  - SEN4
  - SAG12
  - SAG13
  - SAG101
  - PAD4
  - PR1

- **Color codes**
  - Hormone metabolism
  - Secondary metabolism
  - Photosynthesis
  - Stress
  - Miscellaneous
  - RNA
  - Protein
  - Signaling
  - Development
  - Transport

**Legend**

- **SA metabolism**
- **senescence**
enrichment profile of WHY1 at the target genes
The image shows a diagram involving proteins and pathways related to plant physiology. The diagram includes the following components:

- **pWHY1**
- **WHY1**
- **nWHY1**
- **H₂O₂**
- **SA**
- **ICS1**
- **PAL1**
- **BSMT1**
- **MeSA**
- **Plant Senescence and Cell Death**

The diagram illustrates interactions such as:

- **pWHY1** and **WHY1** relate through **H₂O₂** and **SA**.
- **pWHY1** interacts with **ICS1**, **PAL1**, and **BSMT1**.
- **nWHY1** interacts with **MYB15**, **ERF109**, and **ICS1**.
- **SA** regulates the expression of genes like **MYB15**, **ERF109**, and **ICS1**.
- **MeSA** is involved in the senescence process.

The diagram suggests a complex regulatory network controlling plant senescence and cell death.
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