REVOLUTA and WRKY53 connect early and late leaf development in Arabidopsis

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ABSTRACT
As sessile organisms, plants have to continuously adjust growth and development to ever-changing environmental conditions. At the end of the growing season, annual plants induce leaf senescence to reallocate nutrients and energy-rich substances from the leaves to the maturing seeds. Thus, leaf senescence is a means with which to increase reproductive success and is therefore tightly coupled to the developmental age of the plant. However, senescence can also be induced in response to sub-optimal growth conditions as an exit strategy, which is accompanied by severely reduced yield. Here, we show that class III homeodomain leucine zipper (HD-ZIPIII) transcription factors, which are known to be involved in basic pattern formation, have an additional role in controlling the onset of leaf senescence in Arabidopsis. Several potential direct downstream genes of the HD-ZIPIII protein REVOLUTA (REV) have known roles in environment-controlled physiological processes. We report that REV acts as a redox-sensitive transcription factor, and directly and positively regulates the expression of WRKY53, a master regulator of age-induced leaf senescence. HD-ZIPIII proteins are required for the full induction of WRKY53 in response to oxidative stress, and mutations in HD-ZIPIII genes strongly delay the onset of senescence. Thus, a crosstalk between early and late stages of leaf development appears to contribute to reproductive success.

KEY WORDS: REVOLUTA, HD-ZIPIII, WRKY53, Leaf senescence, Hydrogen peroxide signaling

INTRODUCTION
Senescence is the final stage of leaf development and involves the concerted reallocation of nutrients from the leaves to developing parts of the plant, especially fruits and seeds. Thus, leaf senescence has a major impact on yield quantity and quality, e.g. salvaged nitrogen (N) from wheat leaves accounts for up to 90% of the total grain N content (Kichey et al., 2007). In order to minimize loss of nutrients, plants induce leaf senescence in response to endogenous cues such as plant age and altered hormone homeostasis. However, external factors, such as the availability of water or light quality can also induce senescence, referred to as premature senescence (Ballaré, 1999). Although age-induced senescence tends to maximize seed production, premature senescence describes an exit strategy that is induced in response to sub-optimal growth conditions and is often correlated with severely decreased yields.

The onset and progression of leaf senescence is accompanied by immense changes in the leaf transcriptome. It is estimated that about 20% of all genes are altered in expression upon induction of senescence, implying an important role for transcriptional regulators (Balazadeh et al., 2008; Breeze et al., 2011; Buchanan-Wollaston et al., 2005; Zentgraf et al., 2004). NAC and WRKY transcription factors are over-represented in the senescence transcriptome (Guo et al., 2004) and some members of these two transcription factor families have been shown to play central roles in regulating senescence (Balazadeh et al., 2010, 2011; Besseau et al., 2012; Breeze et al., 2011; Miao et al., 2004; Uauy et al., 2006; Ulker et al., 2007; Yang et al., 2011). WRKY proteins are plant-specific transcriptional regulators that contain a DNA-binding domain of ~60 amino acids. This domain contains a WRKYGQK motif at the N terminus and a zinc-finger structure at the C terminus, and is called the WRKY domain. Diverse processes, such as the response to pathogens or wounding but also leaf senescence, are controlled by WRKY transcription factors (Rushton et al., 2010). WRKY53, a key player in age-induced leaf senescence, regulates a complex network of downstream targets that promote vast physiological changes associated with the reallocation of nutrients and the induction of cell death (Lin and Wu, 2004; Miao et al., 2004). Owing to its important function, WRKY53 expression, activity and protein stability are tightly controlled (Zentgraf et al., 2010). When leaf senescence is induced, the WRKY53 locus is activated by histone modifications H3K4me2 and H3K4me3 (Ay et al., 2009; Brusslan et al., 2012), whereas DNA methylation remains low and unchanged (Zentgraf et al., 2010). Several promoter-binding proteins have already been characterized for WRKY53 regulation, including WRKY53 itself, other WRKYs and the activation domain protein (AD protein), which has some similarity to HPT kinases and works as an activator of WRKY53 expression (Miao et al., 2008; Potschin et al., 2014). In addition, a mitogen-activated protein kinase kinase kinase (MEKK1) was characterized to bind directly to the DNA of the WRKY53 promoter. The binding region of MEKK1 appears to be involved in the switch from leaf age-dependent to plant age-dependent expression of WRKY53 (Hinderhofer and Zentgraf, 2001; Miao and Zentgraf, 2007). MEKK1 can directly phosphorylate the WRKY53 protein, thereby increasing its DNA-binding activity (Miao and Zentgraf, 2007). As almost all WRKY factors contain WRKY factor-binding sites (W-boxes) in their proximal promoter regions, a complex regulatory WRKY network exists. Besides the transcriptional regulation, WRKY53 protein stability is strongly controlled by a HECT E3-ubiquitin ligase (Miao and Zentgraf, 2010). Moreover, gene expression changes are accompanied by hormonal changes. Although the plant hormones cytokinin and auxin act to delay senescence (Kim et al., 2011;
Li et al., 2012), ethylene, abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) strongly promote leaf senescence (Li et al., 2012). Besides hormone homeostasis, elevated hydrogen peroxide levels also trigger senescence (Bieker et al., 2012; Smykowski et al., 2010).

Here, we identify REVOLUTA (REV), a transcription factor known to regulate polarity-associated growth processes in embryos, leaves, stems, vasculature and roots (Carlsbecker et al., 2010; McConnell et al., 2001; Smith and Long, 2010), as a direct regulator of WRKY53 expression. During early leaf development, REV is involved in establishing the dorsoventral axis of leaves by specifying the domain that will later develop into the upper side of the leaf (Byrne, 2006). REV, also known as INTERFASCICULAR FIBERLESS (IFL), has been shown to play multiple roles in meristem organization, leaf polarity set-up and vascular development (Otsuga et al., 2001; Talbert et al., 1995; Zhong and Ye, 1999). Using a ChIP-Seq approach, we identified REV-binding sites in the WRKY53 promoter and by qRT-PCR demonstrate that REV promotes WRKY53 expression. Conversely, plants that carry loss-of-function mutations in REV and other HD-ZIPIII genes show lower levels of WRKY53 expression, confirming that HD-ZIPIIIIs are also required for WRKY53 expression. By performing a detailed expression analysis using both REV and WRKY53 GUS-reporter lines, we reveal that both genes have partially overlapping patterns of expression. In wild-type plants, WRKY53 expression is strongly induced in response to hydrogen peroxide. However, in rev mutant plants and in transgenic plants with reduced HD-ZIPIII activity, this response is significantly dampened. Furthermore, the ability of REV to bind to the WRKY53 promoter is also dependent on the redox environment and, under oxidative conditions, less binding is observed. In line with the lower WRKY53 expression levels, rev mutant plants are considerably delayed in age-induced leaf senescence, suggesting a role for HD-ZIPIIIIs in this physiological process. Taken together, we conclude that REV is a positive regulator of WRKY53 expression, which influences the onset of leaf senescence in response to changes in the cellular redox state. Obviously, early and late leaf development are tightly linked by transcriptional networks between HD-ZIPIII and WRKY factors, in which disturbed early development is coupled to extended life span of leaves and delayed senescence.

RESULTS

REVOLUTA is a positive regulator of WRKY53 expression, a major factor controlling age-induced leaf senescence

REVOLUTA is a member of the class III homeodomain leucine zipper (HD-ZIPIII) transcription factor family that regulates various polarity-associated growth processes during development (Carlsbecker et al., 2010; McConnell et al., 2001; Smith and Long, 2010), but plays an additional role in shade-induced growth promotion (Bou-Torrent et al., 2012; Brandt et al., 2012). REVOLUTA expression is controlled by the microRNAs miR165 and miR166 at the post-transcriptional level (Rhoades et al., 2002), and by the association with small leucine-zipper-type microProteins at the post-translational level (Kim et al., 2008; Staudt and Winkel, 2011; Winkel et al., 2007). Using a genome-wide chromatin-immunoprecipitation sequencing approach (ChIP-Seq), we recently identified binding regions for REV across the Arabidopsis genome (Brandt et al., 2012). This analysis revealed binding of REV to the promoter of the WRKY53 transcription factor (Fig. 1A).Transient promoter-GUS experiments in Arabidopsis protoplasts revealed an induction of WRKY53 expression after co-transformation of 35S::REVd, a dominant microRNA-resistant version of REV (Fig. 1B). Quantitative ChIP-PCRs confirmed the binding of REV to the ChIP-Seq identified binding motifs (Fig. 1C). For better control of REV activity, we constructed transgenic plants expressing REVd fused to the rat glucocorticoid receptor carrying an N terminal FLAG epitope. In response to dexamethasone (DEX) induction, the chimeric FLAG-GR-REVd fusion protein translocates to the nucleus, where it can associate with DNA and alter the expression of target genes. In response to DEX induction, REV can significantly upregulate WRKY53 expression (Fig. 1D), while seedlings carrying mutations in REV and plants with globally reduced HD-ZIPIII activity show reduced levels of WRKY53 mRNA (Fig. 1E), thus supporting a new role for REV as a direct and positive regulator of WRKY53.

REVOLUTA and WRKY53 have overlapping patterns of expression

REVOLUTA, as well as the other class III HD-ZIP transcription factors of Arabidopsis, have a distinct expression pattern, confining their expression to the adaxial domain of developing leaves, the xylem part of the vasculature, the pro-vasculature and the shoot apical meristem. Both WRKY53 and REV are expressed in young seedlings (Fig. 2A,B). Even though REV function was initially described for polarity-associated growth processes during early leaf development, REV is still expressed at later stages of development (supplementary material Fig. S1) and an additional function in shade avoidance has recently been assigned to REV (Brandt et al., 2012). In comparison with the vascular expression pattern of REV, WRKY53 shows a broader less-specific pattern of expression and is most highly expressed in old leaves (Miao and Zentgraf, 2007). In genetic backgrounds with reduced REV mRNA [rev-5 (Fig. 2C), 35S::miR165a (Fig. 2D)] or with reduced REV protein activity (35S::ZPR3; Fig. 2E), the spatial expression of WRKY53 is more restricted to hydatodes and overall expression levels appear to be much lower in leaf tissue. In older seedlings, expression of both genes is found in vascular strands (Fig. 2F-M). Surprisingly, high co-expression is observed in the root vasculature at all investigated stages of development. It is not known whether WRKY53 has an additional function in root development but it might be important to note that the expression in the root vascular appears to be independent of HD-ZIPIII function (Fig. 2B-E).

Using publicly available microarray data (http://bar.utoronto.ca), we also analyzed at which stages of development and in response to which treatments REV and WRKY53 are co-expressed (supplementary material Fig. S2). We find evidence for co-expression during early developmental stages but not during the later stages of leaf development. This discrepancy suggests that REV mRNA is not upregulated at late stages of leaf development but residual protein could respond to a cellular signal and induce the expression of REV-regulated senescence targets. However, our GUS expression analyses using REV::GUS plants indicate that REV is still expressed to certain extends in older leaves (supplementary material Fig. S1).

In order to identify other direct REV targets that show an expression pattern resembling WRKY53, we surveyed recently published timecourse microarray datasets (Reinhart et al., 2013) that revealed 119 genes to be upregulated in response to REVOLUTA induction. Our ChIP-Seq datasets resulted in the identification of 286 high confidence REV-binding sites (corresponding to 552 potentially regulated genes) across the entire Arabidopsis genome (Brandt et al., 2012). By comparing both datasets, we could identify 18 of the 119 REV-regulated genes (15% of the REV upregulated set) to have REV-binding sites in their respective promoters (Table 1). WRKY53 is among these 18 genes and we investigated whether other senescence-related genes could be identified in this dataset.
genome-wide survey with a high temporal resolution classified thousands of genes as differentially expressed senescence genes (DESGs) (Breeze et al., 2011). Interestingly, REV was also classified as a DESG, showing a dip of expression at the onset of leaf senescence. Furthermore, nine out of the 18 potential direct REV targets (Table 1) were also classified as DESGs, implying that REV might have an additional function in late developmental stages.

**WRKY53** expression is modulated in response to oxidative stress in a REVOLUTA-dependent manner

**WRKY53** expression is strongly upregulated in response to hydrogen peroxide as part of the age-induced senescence-promotion pathway (Miao et al., 2004). Because REV is a novel upstream regulator of **WRKY53** expression and possesses a domain that is suggestive of sensing changes in the redox state of the cell, we investigated whether REV is required for the induction of **WRKY53** expression in response to oxidative stress. Therefore, we grew Col-0 wild-type plants and mutant plants with reduced HD-ZIPIII activity (**rev**-5, 35S::miR165a and 35S::ZPR3) on soil for 3 weeks in long-day conditions. In order to elicit oxidative stress, plants were sprayed with hydrogen peroxide solutions of different concentrations (0.01%, 0.1% and 1%) and plant material was harvested before and after spraying. Subsequent RNA isolation, cDNA synthesis and quantitative PCR analysis revealed a strong induction of **WRKY53** in response to H$_2$O$_2$ application in Col-0 wild-type plants. These changes of **WRKY53** mRNA levels were significantly dampened in **rev** mutants (**rev**-5) and in plants with reduced HD-ZIPIII activity (35S::ZPR3), indicating that REV activity is required for high-level **WRKY53** induction in response to oxidative stress signaling (Fig. 3).
subsequent oxidation. The increase in intracellular H2O2 was similar 1 h after heat treatment and 1 h after spraying 0.1% H2O2 but dropped more rapidly in the H2O2-treated samples. This indicates that external application of 0.1% H2O2 leads to intracellular changes in the range of an oxidative burst in stress response (supplementary material Fig. S3).

REVOLUTA is a redox-sensitive transcription factor

REV is a positive regulator of WRKY53 expression and is required for high level of WRKY53 induction in response to oxidative stress. This could be either due to an upregulation of REV mRNA in response to oxidative stress or to a response of the REV protein to altered redox conditions. To test whether REV mRNA is upregulated in response to hydrogen peroxide treatment, we treated Col-0 wild-type plants with H2O2 and performed quantitative RT-PCRs. We detected no induction of REV mRNA but a slight decrease in response to high levels of hydrogen peroxide (supplementary material Fig. S4), excluding the idea that REV is transcriptionally upregulated in response to oxidative stress.

It has been shown that proteins of the class II homeodomain leucine-zipper (HD-ZIPII) family from sunflower interact with DNA in a redox-sensitive manner (Tron et al., 2002). To test whether REV shows also redox-dependent DNA binding, we performed redox-sensitive DPI-ELISA experiments. Therefore, crude lysate of E. coli cells expressing HIS-tagged REV protein were prepared and incubated with streptavidin plates pre-loaded with biotinylated oligonucleotides containing the REV-binding site 1 of the WRKY53 promoter (W53-BS1). ELISA plates were then washed and subsequently incubated with HRP-tagged anti-HIS antibodies. Enhanced signal was detected in the control binding reaction (HIS-REV lysate versus a lysate from BL21 cells expressing the empty vector control), indicating that HIS-REV binds to the W53-BS1 element (Fig. 4A). As observed for the sunflower HD-ZIPII proteins (Tron et al., 2002), REV also showed enhanced binding in response to reducing conditions (10 mM DTT), whereas in response to oxidative conditions (10 mM H2O2) DNA-binding was reduced (Fig. 4A). This negative effect is reversible as the subsequent addition of 10 mM DTT was able to restore REV DNA binding.

We examined the possibility of whether the C-terminal PAS-domain of REV might act as a redox sensor domain. Redox-DPI-ELISA experiments with HIS-REV lacking the PAS-domain (HIS-REVΔPAS) showed the same redox-sensitive behavior as observed for HIS-REV (Fig. 4B). However, without the PAS-domain, REV-DNA binding was strongly enhanced, supporting the idea that the PAS-domain regulates REV activity via a steric masking mechanism, as proposed by Magnani and Barton (2011). It is conceivable that the observed redox effects in the ELISA system

Table 1. Identification of potentially direct REV target genes by comparing ChIP-Seq and microarray experiments with an inducible version of REV

<table>
<thead>
<tr>
<th>AGI</th>
<th>Name</th>
<th>Microarray</th>
<th>ChIP-Seq</th>
</tr>
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<tr>
<td>AT2G41940</td>
<td>ZFP8</td>
<td>2.0</td>
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</tr>
<tr>
<td>AT5G47370</td>
<td>HAT2</td>
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<tr>
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<td>DV311/RTFL8</td>
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<td>1626</td>
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<td>HAT14</td>
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<td>272</td>
</tr>
<tr>
<td>AT5G19590</td>
<td>Plant VAMP protein</td>
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</tr>
<tr>
<td>AT4G18700</td>
<td>CIPK12</td>
<td>1.3</td>
<td>465</td>
</tr>
<tr>
<td>AT4G27730</td>
<td>OPT6</td>
<td>3.0</td>
<td>169</td>
</tr>
<tr>
<td>AT4G03510</td>
<td>RMA1</td>
<td>7.0</td>
<td>33</td>
</tr>
<tr>
<td>AT1G17970</td>
<td>RING/U-Box protein</td>
<td>5.1</td>
<td>1173</td>
</tr>
<tr>
<td>AT5G14730</td>
<td>DUF1645</td>
<td>5.8</td>
<td>726</td>
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<td>AT2G45450</td>
<td>ZPR1</td>
<td>13.1</td>
<td>400</td>
</tr>
<tr>
<td>AT5G05890</td>
<td>CPD</td>
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<td>202</td>
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<tr>
<td>AT1G74940</td>
<td>DUF581</td>
<td>2.2</td>
<td>106</td>
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<td>AT3G06390</td>
<td>HAT3</td>
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<tr>
<td>AT4G23810</td>
<td>WRKY53</td>
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<td>450</td>
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<tr>
<td>AT5G16030</td>
<td>Unknown protein</td>
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<td>789</td>
</tr>
<tr>
<td>AT1G49200</td>
<td>RING/U-Box protein</td>
<td>14.4</td>
<td>18</td>
</tr>
<tr>
<td>AT2G02080</td>
<td>IID4</td>
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<td>528</td>
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<tr>
<td>AT3G13810</td>
<td>IID11</td>
<td>0.4</td>
<td>1643</td>
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‡Senescence-associated gene not included in the Breeze et al. (2011) analysis.

*Differentially expressed genes during senescence (Breeze et al., 2011).
Three-week-old plants were treated with different concentrations of H$_2$O$_2$ (0%, 0.01%, 0.1%, 1% H$_2$O$_2$). In 12-day-old seedlings, we detected REV binding to binding site 2 (fragment II) and no binding was observed to binding site 1 (fragment III). When treated with hydrogen peroxide prior DEX induction, binding to binding site 2 was significantly affected (Fig. 4D), indicating that REV DNA binding is indeed redox sensitive. The same experiment with 7-week-old plants revealed that, at later developmental stages, both binding sites are occupied by REV and the binding seems to be enhanced but exhibits the same redox sensitivity (Fig. 4E). Taken together, we demonstrate that REV shows a stage-specific redox-dependent DNA-binding behavior and that oxidizing conditions decrease the ability to bind DNA in vitro and in vivo.

Mutations in the REVOLUTA gene or the overall reduction of HD-ZIPIII activity delay the onset of leaf senescence

One function of the WRKY53 protein is the regulation of the onset of senescence, documented by the phenotype of the wrky53 mutant showing delayed senescence. As REV is an activator of WRKY53 expression, we expected rev mutant plants to also display a delayed senescence phenotype. Our analysis revealed that plants carrying mutations in REV or plants with greatly reduced HD-ZIPIII activity are significantly delayed in senescence, while overall development is not retarded, which clearly confirms a role of HD-ZIPIII proteins in this process (Fig. 5; supplementary material Figs S5, S6). Furthermore, the phenotype of rev5 was even stronger than that of wrky53, indicating that WRKY53 might not be the only senescence-associated gene regulated by REV.

Overexpression of the small leucine-zipper-type microProtein ZPR3, which largely reduces the activity of HD-ZIPIIIIs, led to a further enhancement of the senescence phenotype, which was ameliorated in the wrky53 mutant background (supplementary material Fig. S3). This confirms that the senescence phenotype is...
mediated by deregulation of WRKY53 expression through HD-ZIPIIs but also suggests that additional HD-ZIPIIs are involved, as the senescence phenotype of 35S::ZPR3 plants is much stronger compared with rev5 mutants (Fig. 5; supplementary material Figs S5,S6). Consistent with the phenotype, two typical senescence-related physiological parameters, the decrease in chlorophyll content and the increase in lipid peroxidation, were also delayed in wrky53, rev5 and rev5 wrky53 mutants (Fig. 6A,B). Furthermore, the mRNA expression levels of SENESCENCE ASSOCIATED GENE 12 (SAG12) and SAG13, which are commonly used as senescence marker genes, were significantly reduced at the late developmental stages in wrky53, rev5 and rev5 wrky53 mutants compared with Col-0 wild-type plants (Fig. 6C,D). Taken together, these results confirm that REV acts upstream of WRKY53 in the control of age-induced senescence.

Depletion of REV delays the onset of leaf senescence more efficiently than depletion of WRKY53. To further investigate the possibility that REV acts upstream of several senescence-associated genes, we focused our attention on the potential direct REV targets classified as DESGs (Table 1). Here, we decided to investigate three groups of genes: (1) genes whose expression decreases with age (HAT3 and AT1G49200); (2) genes whose expression increases with age (AT1G74940 and IDD11); and (3) genes whose expression decreases with age but rises during senescence (AT5G47180 and ZFP8). In the first group of genes, we found that expression in wrky53, rev5 and rev5 wrky53 mutants is maintained at a higher level towards the onset of senescence (weeks 5 and 6), whereas expression levels are dropping rapidly in wild-type plants (Fig. 7A,B). For the second group of genes whose expression increases with age in wild-type plants, we detected elevated levels in wrky53, rev5 and rev5

Fig. 5. Genetic interaction of REV with WRKY53. (A) Rosette leaves of 6- and 7-week-old representative plants were sorted according to their age: whole rosettes were also photographed upside down to visualize the older leaves. (B) For a quantitative evaluation of leaf senescence, plants were harvested in a weekly rhythm and leaves of at least six plants were categorized into four groups according to their leaf color: (1) ‘green’; (2) leaves starting to become yellow from the tip as ‘yellow-green’; (3) completely yellow leaves as ‘yellow’; and (4) dry and/or brown leaves as ‘brown/dry’. The percentages of each group with respect to total leaf numbers are presented. Error bars indicate s.d. Student’s t-test was performed comparing leaf counts of wrky53, rev5 and rev5wrky53 with Col-0 numbers, *P<0.05, **P<0.005, ***P<0.0005. n=7-15.
wrky53 mutants at early developmental stages (weeks 4 and 5) and decreased levels at the late stages (Fig. 7C,D). Expression of the third group of genes is also altered at various time points in wrky53, rev5 and rev5 wrky53 mutants compared with Col-0, but in all lines the transcriptional increase during senescence is diminished (Fig. 7E,F), further corroborating the idea that loss of REV function profoundly alters the senescence transcriptome, which might be causative for the strong senescence phenotype of rev mutant plants.

Loss-of-function wrky53 mutant plants do not show obvious developmental defects during early leaf development, indicating that WRKY53 is not required for REV function at these stages of development. However, the severe 35S::ZRP3-induced leaf phenotype is ameliorated in the wrky53 mutant background, suggesting that the action of other HD-ZIPIIIs involves WRKY53 also at early stages (supplementary material Fig. S7). Nonetheless, WRKY53 protein levels are most likely very low during these early

Fig. 6. Molecular senescence parameters. (A) Chlorophyll contents of number 5 leaves from Arabidopsis Col-0, wrky53, rev5 and rev5 wrky53 plants. Left axis indicates atLeaf+ values. Plant age is indicated in days after seeding (DAS). (B) Lipid peroxidation in Col-0, wrky53, rev5 and rev5 wrky53 plants. Values represent mean of at least three biological replicate±s.d. Comparison of means and the determination of statistical differences was carried out using Student’s t-test (*P<0.05, **P<0.005 and ***P<0.0005). (C,D) qRT-PCR expression analysis of the senescence marker genes SAG12 and SAG13. All values were normalized to GAPDH expression. Error bars indicate s.d. of four technical replicates.

Fig. 7. qRT-PCR of other REV target genes differentially expressed during senescence. Quantitative real-time PCR profiling of putative REV target genes at late developmental stages in wild-type and mutant plants (4-, 5-, 6-, 7- and 8-week-old plants). (A-F) Expression changes over time of HAT3, AT1G49200, AT1G74940, IDD11, AT5G47180 and ZFP8. The Y-axis represents the relative expression level normalized to GAPDH. Error bars indicate s.d. of four technical replicates.
stages of development due to the degradation of WRKY53 by the HECT domain ubiquitin ligase UPL5, which is highly expressed in young leaves (Miao and Zentgraf, 2010). Taken together, we discovered that HD-ZIPIII factors interact with WRKY53 genetically to promote age-induced leaf senescence, and disruption of early leaf development correlates with delayed senescence and extended life span of leaves.

**Functional analyses of root-specific co-expression patterns of REV and WRKY53**

It is unknown which tissues are involved in the perception of senescence signals and conversion of these into the senescence triggers. We find co-expression of REV and WRKY53 during the early stages of leaf development. Later in development, co-expression was very obvious in the vasculature of the leaves and in the root vascular cylinder (Fig. 2L,M), although both REV and WRKY53 are expressed throughout development (supplementary material Fig. S1). This is in agreement with the finding that REV is involved in the induction of WRKY53 expression by hydrogen peroxide and that very high levels of hydrogen peroxide were observed in vascular tissue indicated by DAB staining of leaf sections (Zimmermann et al., 2006). Moreover, it remains tempting to speculate that the root might also act as a senescence sensor; however, whether roots play a role during onset and progression of senescence has not yet been determined and whether and to what extent hydrogen peroxide is transported through the vasculature over long distances is also not known so far. Auto-propagating waves of reactive oxygen species (ROS) that rapidly spread from the initial site of exposure to abiotic stress to the entire plant are involved in conferring systemic acquired acclimation, also allowing a much faster transcriptome and metabolome reprogramming of systemic tissues in response to abiotic stress (Mittler et al., 2011; Suzuki et al., 2013).

To further investigate the spatial aspects of REV and WRKY53 expression, we decided to perform grafting experiments with Col-0 wild-type, rev5 and wrky53 mutant plants. When the aerial parts of Col-0 were grafted onto either wrky53 or rev5 rootstocks, no significant delays in the onset of senescence were observed. However, the converse grafting of the aerial parts of either wrky53 or rev5 to Col-0 rootstocks significantly delayed the onset of senescence where the latter again showed a much stronger effect (Fig. 8A,B). The grafting experiments revealed that the root seems not to be involved in the REV/WRKY53-mediated senescence pathway and that depletion of REV and WRKY53 in only aerial tissue strongly affects senescence.

**DISCUSSION**

Plants induce leaf senescence to provide carbon, nitrogen and mineral resources to the developing fruits or seeds. Senescence is induced in response to plant age but environmental signals such as light, the availability of water and temperature strongly influence this process. A high-resolution temporal transcript profiling of senescing Arabidopsis leaves gives insight into the temporal order of gene activation and repression (Breeze et al., 2011). Approximately 6500 genes are up- or downregulated during the course of leaf senescence, implying an important role for transcription factors in this process. Transcription factors themselves are transcriptionally upregulated in senescing leaves the largest groups being NAC, WRKY, C2H2-type zinc-finger, AP2/EREBP and MYB proteins (Guo and Gan, 2005). Here, we show that HD-ZIPIII factors, which are known to be involved in basic patterning processes, have an additional role in the latest step of leaf development, the regulation of senescence. REV is a direct and positive regulator of WRKY53 expression and mutations in REV and other HD-ZIPIII genes delay the onset of leaf senescence. Interestingly, the delay of the onset of leaf senescence in plants lacking REV is stronger compared with plants lacking only WRKY53, implying that REV acts also upstream of other senescence-associated genes. In plant lines with even more reduced HD-ZIPIII activity, achieved by overexpression of
miRNA165a (35S::miR165a), rosette leaves were so strongly downward curled that it was impossible to determine the onset of senescence. The loss of several HD-ZIPII genes, as in the case of the phb phv rev triple mutant, causes severe developmental defects, including consumption of the apical stem cells (Emery et al., 2003; Prigge et al., 2005). The severity of these developmental defects largely precludes a thorough analysis of the general role of HD-ZIPIII proteins at later stages of development. Nevertheless, our findings clearly suggest that the role of HD-ZIPIIIIs in promoting senescence is more complex and involves regulation of several senescence-associated target genes. In the rev5/wrky53 double mutant, leaf yellowing and chlorophyll loss were less severe at later stages than in the rev single mutant, whereas senescence-associated gene expression was more severely affected for some senescence-related genes. This clearly points towards a complex network that is altered in different aspects if one or more components are depleted from the system. It was already shown that WRKY53 acts as an upstream regulator, downstream target and protein-interaction partner of WRKY18, which is a negative regulator of leaf senescence, illustrating the complexity of the network and possibly explaining the partially intermediate phenotype of the double mutant (Potschin et al., 2014).

The mechanism by which REV promotes senescence appears to involve transcriptional regulation of direct target genes. Here, we have identified nine genes that are potential direct REV targets that are also differentially expressed during senescence. One of these target genes is HAT3, which has been shown to play an important role downstream of REV in the process of setting up polarity in the young leaf primordium (Bou-Torrent et al., 2012; Brandt et al., 2012; Turchi et al., 2013). In young seedlings, HAT3 expression depends partly on the presence of REV, which is supported by lower levels of HAT3 mRNA in rev mutant seedlings (Brandt et al., 2012). During senescence, HAT3 mRNA levels decrease with plant age (Fig. 7A). In rev mutant seedlings, however, HAT3 mRNA is more abundant compared with wild type (Fig. 7A). Moreover, the expression levels of several other senescence-related target REV genes changed in a complex way (Fig. 7B-F). These findings suggest that the transcriptome of rev mutant plants is profoundly altered, resulting in stage-dependent mis-expression of many differentially expressed senescence-associated genes.

It still remains unclear to which endogenous or exogenous signals HD-ZIPIIIIs respond in order to promote senescence. The finding that WRKY53 expression is strongly upregulated in response to hydrogen peroxide treatment and that this induction is dampened in hd-zipIII mutant plants implies that HD-ZIPIIIIs might be involved in signal transduction processes in response to changes in the intracellular redox state. Many senescence-associated genes, especially transcription factors of the WRKY and the NAC family, transcriptionally respond to elevated levels of hydrogen peroxide but the mechanism by which the hydrogen peroxide signal is perceived and transmitted is still unclear. Remarkably, the subcellular compartment of hydrogen peroxide production appears to play a role in senescence signaling in which the cytoplasmic H$_2$O$_2$ is more effective in senescence induction than peroxisomal or mitochondrial H$_2$O$_2$ (Bieker et al., 2012; Zentgraf et al., 2012). Thus, sensors and mediators of hydrogen peroxide-induced senescence are most likely cytoplasmic and/or nuclear proteins or molecules. During bolting, intracellular hydrogen peroxide levels increase in leaf tissue. This increase is thought to be mediated by a complex regulation of the hydrogen peroxide scavenging enzymes and promotes the onset of senescence (Bieker et al., 2012; Smykowski et al., 2010).

Analysis of the redox sensitivity of the REV protein revealed a reduced DNA-binding ability of REV in response to oxidative conditions, which appears to be a direct effect on the REV protein and does not involve accessory proteins. These results contradict the finding that upregulation of WRKY53 partially requires HD-ZIPIIIIs and indicate a more complex regulatory mechanism. Most likely, DNA-binding of REV is affected by redox changes and also the transactivation activity or protein-protein interfaces, which will be further dissected in the future. However, two of the direct REV target genes encode EAR-domain proteins that are part of transcriptional repressor complexes (Causier et al., 2012). Among these transcriptional repressors are HAT3 and ZFP8, the mRNA levels of which are altered in the senescence process. Therefore, it seems plausible to conclude that REV is a redox-sensitive transcription factor, which among other targets, regulates genes encoding transcriptional repressors. Decreasing REV DNA-binding activity will result in lower expression levels of these transcriptional repressors, alleviating the repressive activity on their targets. Thus, modulation of REV activity in response to alterations of the intracellular redox state will profoundly affect the REV-regulated transcriptome. It is tempting to speculate that also within the shoot apical meristem, domains with different cellular redox states might exist that could serve as positional signals affecting HD-ZIPIII activity.

Developmental age is a major determinant for the induction of leaf senescence in an optimal growth environment. However, when plants are exposed to situations that strongly permit normal growth, senescence is accelerated in order to bypass these adverse conditions and produce seeds that can withstand these adverse conditions. We have tried to depict the complex interplay between REV and WRKY during early and late development in a model (Fig. 9) in which the regulatory cues of REV involving miRNA-dependent regulation through miR165, miR166 and the LITTLE ZIPPER microProteins ZRP1-4 is connected to the MAP kinase-triggered WRKY transcriptional network. Several intersections can be detected between the formerly independently described players in early and late leaf development in which hydrogen peroxide might play a central role.

Shade causes profound developmental changes in shade-sensitive plants aimed at outgrowing competitor plants. We have previously shown that the leaf regulatory module consisting of HD-ZIPIII and KANADI transcription factors is involved in modulating
growth in response to shade (Brandt et al., 2012). Consistent with this, shade can also trigger leaf senescence (Brouwer et al., 2012), suggesting that leaf patterning, shade avoidance and leaf senescence are interconnected by differential activity of HD-ZIPIII proteins, thus linking early and late leaf development, and adjusting plant growth and development to changing external conditions.

Perspectives
It was recently shown that embryonic growth and patterning of mammals largely depends on cellular senescence as a developmental mechanism to shape organ growth (Muñoz-Espín et al., 2013; Storer et al., 2013). This mechanism partly relates on macrophages, which are mobile cells that invade the tissue to remove senescent cells. In this context, senescent cells also produce secreted compounds that can act as positional signals triggering pattern formation and proliferation in adjacent tissue (Storer et al., 2013). The immune system of plants is substantially different from animals and does not involve macrophage-mediated cell clearing. However, it is conceivable that local cellular senescence could provide positional information to direct growth responses. Our finding that HD-ZIPIIIs, which are known basic patterning factors, can influence senescence processes, suggest not only that early and late leaf development are coupled and processes that influence patterning in the early organ control the concerted degradation of tissue during the late phase of development, but also that physiological processes related to senescence, such as nutrient mobilization or lipid peroxidation, might be part of early leaf patterning processes. Furthermore, the puzzling reduction of DNA-binding activity under oxidizing conditions that contradicts the finding that upregulation of WRKY53 expression by hydrogen peroxide partially requires REVOLUTA prompts us to decipher the redox-dependent changes in the REVOLUTA protein outside the DNA-binding domain in more detail. This, however, will be the subject of further investigations.

MATERIALS AND METHODS

Plant material and growth conditions
The following rev/HD-ZIPIII mutant lines were used in this study: rev-5 (A260V) and rev-6 (R346STOP), two strong ethyl-methylsulfonate (EMS) alleles (Otsuga et al., 2001), phb phv rev triple mutant introgressed in Col-0 (Pigge et al., 2005), 35S::ZPR3 (Wenkel et al., 2007) and 35S::miR165 (Kim et al., 2010). For senescence phenotyping, Arabidopsis thaliana plants were grown in a climatic chamber at 20°C and (4) dry and/or brown leaves as frozen in liquid nitrogen. After homogenization on ice, 500 µl 40 mM Tris (pH 7.0) were added and the samples were centrifuged at 4°C for 30 min. Fluorescence (480 nm excitation, 525 nm emission) of the supernatant was measured in a Berthold TriStar LB941 plate reader.

Chromatin-immunoprecipitation and quantitative PCRs
ChIP and ChIP-qPCRs were carried out as described by Brandt et al. (2012). To quantify gene expression changes, RNA was isolated from seedlings using the roboKlon GeneMATRIX universal RNA purification kit following manufacturer’s recommendations. One microgram of total RNA was reverse transcribed using the Fermentas RevertAid Premium Reverse transcriptase with oligo-dT primers. cDNAs were diluted 10-fold and 3.5 µl were used for RT-PCR reactions. Quantitative measurements were performed on a Bio-Rad CFX384 using the Fermentas SYBR Green qPCR master mix. Relative quantities were calculated using the delta Ct method and normalized relative to a standard curve. Oligonucleotide sequences are listed in supplementary material Table S1. Further descriptions of the methods can be found in the supplementary material. The ChIP-Seq dataset has been published in the Gene Expression Omnibus database (accession number GSE26722).

Redox-DPI-ELISA
Recombinant 6xHis-tagged REV protein with and without the PAS domain was expressed in E. coli and DNA-protein interaction ELISA was basically performed as described previously (Brandt et al., 2010). Crude extracts were pre-incubated with different concentrations of DTT and H2O2 to examine a redox state-dependent binding of REV (for a detailed description, see methods in the supplementary material).

Transformation of Arabidopsis protoplasts and transient promoter-GUS expression
Protoplasts were derived from a cell culture of Arabidopsis thaliana var. Columbia 0 and were transformed with effector and reporter plasmids following roughly the protocol of Negrutiu et al. (1987). The GUS activity assays were carried out as described by Jefferson et al. (1987). A detailed description is presented in the methods in the supplementary material.

Chlorophyll measurements and phenotypic analysis
For assessment of the leaf senescence state, chlorophyll content of leaf 5 was measured using an atLeaf+ chlorophyll meter (http://www.atleaf.com), lipid peroxidation of leaf 6 was measured using the improved thiobarbituric acid/reactive substances assay, as described previously (Hodges and Forney, 2000), and expression of the senescence-associated marker genes SAG12 (At5g45890) and SAG13 (At2g29350) was analyzed by qRT-PCR. A detailed description is presented in the methods in the supplementary material.

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Competing interests
The authors declare no competing financial interests.

Author contributions
K.H., M.P. and J.D. performed senescence phenotyping experiments and redox-ELISA; R.B., T.D. and Y.X. carried out the molecular analysis; D.S. did the gel shift experiment; S.B. measured hydrogen peroxide levels; U.Z. and S.W. designed research, analyzed the data and wrote the article.

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References


Suzuki, N., Miller, G., Salazar, C., Mondal, H. A., Shulaev, E., Cortes, D. F.,
interaction between reactive oxygen species and abscisic acid regulates rapid

gene is necessary for apical meristem development and for limiting cell divisions
in the leaves and stems of Arabidopsis thaliana. *Development* 121, 2723-2735.

regulation of plant homeodomain transcription factors. *J. Biol. Chem.* 277,
34800-34807.

Turchi, L., Carabelli, M., Ruzza, V., Possenti, M., Sassi, M., Penalosa, A., Sessa,
transcription factors control apical embryo development and meristem function.
*Development* 140, 2118-2129.

Gene regulating senescence improves grain protein, zinc, and iron content in

transcription factor of Arabidopsis influences both the plant senescence and

Weigel, M., Varotto, C., Pesaresi, P., Finazzi, G., Rappaport, F., Salamini, F. and
Leister, D. (2003). Plastocyanin is indispensable for photosynthetic electron flow

A feedback regulatory module formed by LITTLE ZIPPER and HD-ZIPIII genes.
*Plant Cell* 19, 3379-3390.

transcription factor VNII2 integrates abscisic acid signals into leaf senescence via

expression profiles of rosette leaves of Arabidopsis thaliana: leaf age versus plant


hydrogen peroxide as signalling molecule for plant senescence. In *Senescence* (T. Nagata, ed.). http://www.intechopen.com/books/senescence/role-of-intracellular-
hydrogen-peroxides-pisignalling-molecule-for-plant-senescence

Zhong, R. Q. and Ye, Z. H. (1999). IFL1, a gene regulating interfascicular fiber
differentiation in Arabidopsis, encodes a homeodomain-leucine zipper protein.
*Plant Cell* 11, 2139-2152.

specific regulation of catalases in Arabidopsis thaliana (L.) Heynh. *Plant Cell
Environ.* 29, 1049-1060.