The regulation of WRKY53 by other transcription factors during leaf senescence in *Arabidopsis thaliana*

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1 Summary

Leaf senescence is the final stage of leaf development. It serves to relocate nutrients from the old leaf tissue to new, young tissue, such as new developing leaves, flowers or seeds. The old senescing tissue will ultimately die. Senescence can be optically recognized by the loss of chlorophyll. The leaves lose their green colour, leaving a yellow colour through the now visible carotenoids. However, when this becomes apparent, the senescence program already started long before and molecular changes were already initiated.

Senescence is a highly coordinated process accompanied by extreme changes in gene expression suggesting a strong involvement of transcription factors. These extreme changes in gene expression are mediated by many different transcription factor families, in which WRKY transcription factors, especially WRKY53, are particularly important. WRKY53 shows its highest expression during early senescence events and has been characterized as a positive regulator of leaf senescence. WRKY factors bind W-boxes in the promoters of their target genes, and since WRKY factors themselves also contain many W-boxes in their promoters, it is assumed that they regulate each other in a WRKY regulatory network. This thesis describes the decoding of the WRKY network in the senescence process. Several WRKYs were selected according to known expression data, and binding of these candidates to *WRKY53* W-boxes with their native surrounding sequences was tested. In addition, I performed transient transformation assays of Arabidopsis protoplasts with a *WRKY53* promoter:*GUS* construct and *35S:WRKY* overexpression effector constructs of these WRKKYs. Two interesting candidates were identified, namely WRKY18 and WRKY25, which appear to be involved in the regulation of *WRKY53* and senescence.

WRKY18 binds directly to several W-boxes of the *WRKY53* promoter and represses the expression of a *WRKY53* promoter-driven reporter gene in a transient transformation system using Arabidopsis protoplasts. Accordingly, phenotypic analyses of *wrky18* mutant and *WRKY18* overexpressing plants revealed a negative effect of WRKY18 on senescence induction and progression. In addition, a direct protein-protein interaction between WRKY53 and WRKY18 was demonstrated. In summary, WRKY18 acts as a negative regulator in senescence and is involved in the WRKY53 regulatory network.

In contrast to WRKY18, WRKY25 positively regulates the expression of *WRKY53*. However, the senescence phenotype of *wrky25* and *WRKY25* overexpressors is opposite than expected. WRKY25 is also involved in the senescence process as a negative regulator indicating that complex regulatory cues are in place. Overexpression of *WRKY25* also mediates higher tolerance to H_2O_2 stress and the intracellular H_2O_2 content is lower compared to wild type. The knockout mutants show the opposite effect, indicating that WRKY25 is also involved in controlling intracellular redox conditions. Moreover, WRKY25 action itself is redox-dependent as under non-oxidizing conditions it functions as a much stronger positive regulator of *WRKY53* expression in transiently transformed Arabidopsis protoplasts. In summary, WRKY25 is a negative senescence regulator, which mediates a higher tolerance to H_2O_2 and is involved as a redox-sensitive transcription factor in the WRKY53 regulatory network.

Taken together, this work characterized new regulators of the senescence process. Two additional WRKY factors can be assigned to the WRKY network. In addition, a non-WRKY protein, REVOLUTA (REV), has been described to participate in this WRKY network.

2 Zusammenfassung

Die Blattseneszenz ist das letzte Stadium der pflanzlichen Blattentwicklung. Sie dient dazu, Nährstoffe aus dem altem Blattgewebe in neues, junges Gewebe, wie sich entwickelnde Blätter, Blüten oder Samen einzulagern. Das alte seneszente Gewebe wird letztendlich absterben. Die Seneszenz kann optisch anhand des Chlorophyllverlustes erkannt werden. Die Blätter verlieren ihre grüne Farbe, zurück bleibt eine gelbe Färbung durch die sichtbar werdenden Carotinoide. Wenn dies jedoch offensichtlich wird, hat das Seneszenzprogramm bereits lange zuvor begonnen und molekulare Veränderungen wurden bereits eingeleitet.

Die Seneszenz ist ein hoch koordinierter Prozess, der von extremen Änderungen in der Genexpression begleitet wird, was auf eine starke Beteiligung von Transkriptionsfaktoren hindeutet. Diese extremen Änderungen in der Genexpression werden von vielen verschiedenen Familien von Transkriptionsfaktoren vermittelt, wobei die WRKY-Transkriptionsfaktoren, insbesondere WRKY53 dabei besonders wichtig sind. WRKY53 zeigt seine stärkste Expression während früher Seneszenz Ereignisse und wurde als positiver Regulator der Blattseneszenz charakterisiert. WRKY-Faktoren binden W-Boxen in den Promotoren ihrer Zielgene und da WRKY-Faktoren selbst auch viele solcher W-Boxen in ihren Promotoren enthalten, wird vermutet, dass sie sich in einem WRKY-regulatorischen Netzwerk gegenseitig regulieren. Diese Arbeit befasst sich mit der Entschlüsselung des WRKY-Netzwerkes im Seneszenz Prozess. Dazu wurden verschiedene WRKYs anhand bekannter Expressionsdaten ausgewählt und eine Bindung dieser Kandidaten an die *WRKY53* W-Boxen mit ihren nativ umgebenden Sequenzen wurde getestet. Zusätzlich führte ich transiente Transformationstests von Arabidopsis-Protoplasten mit einem *WRKY53* Promotor:*GUS*-Konstrukt und *35S:WRKY*-Überexpressions-Effektorkonstrukten dieser WRKYs durch. Dabei wurden zwei interessante Kandidaten identifiziert, nämlich WRKY18 und WRKY25, die an der Regulation von *WRKY53* und der Seneszenz beteiligt zu sein scheinen.

WRKY18 bindet direkt verschiedene W-Boxen des *WRKY53*-Promotors und unterdrückt die Expression eines *WRKY53*-Promotor-gesteuerten Reportergens in einem transienten Transformationssystem unter Verwendung von Arabidopsis-Protoplasten. Dementsprechend zeigten phänotypische Analysen von *wrky18*-Mutanten und *WRKY18*-überexprimierenden Pflanzen einen negativen Effekt von

WRKY18 auf die Seneszenzinduktion und -progression. Zusätzlich konnte eine direkte Protein-Protein Interaktion zwischen WRKY53 und WRKY18 nachgewiesen werden. Zusammenfassend nimmt WRKY18 als negativer Regulator an der Seneszenz teil und ist am WRKY53-regulatorischen Netzwerk beteiligt.

Im Gegensatz zu WRKY18 reguliert WRKY25 die Expression von WRKY53 positiv. Der Seneszenz-Phänotyp von *wrky25*-Mutanten und *WRKY25*-Überexpressoren ist jedoch entgegengesetzt zu dem, was erwartet wurde. WRKY25 ist auch als negativer Regulator am Seneszenzprozess beteiligt, was darauf hindeutet, dass komplexe regulatorische Wechselwirkungen stattfinden. Die Überexpression von *WRKY25* vermittelt auch eine höhere Toleranz gegenüber H_2O_2-Stress und die intrazelluläre H_2O_2-Menge ist im Vergleich zum Wildtyp geringer. Die Knockout Mutanten zeigen den gegenteiligen Effekt, was darauf hinweist, dass WRKY25 auch an der Kontrolle der intrazellulären Redox-Bedingungen beteiligt ist. Darüber hinaus ist die WRKY25-Wirkung selbst redoxabhängig, da es unter nicht oxidierenden Bedingungen ein viel stärkerer positiver Regulator der *WRKY53*-Expression in transient transformierten Arabidopsis-Protoplasten ist. Zusammenfassend ist WRKY25 ein negativer Seneszenzregulator, der eine höhere Toleranz gegenüber H_2O_2 vermittelt und als Redox-sensitiver Trankriptionsfaktor am WRKY53-regulatorischen Netzwerk beteiligt ist.

Insgesamt charakterisiert diese Arbeit neue Regulatoren des Seneszenz Prozesses. Zwei zusätzliche WRKY Faktoren konnten dem WRKY-Netzwerk zugeordnet werden. Zudem konnte für ein nicht-WRKY Protein, REVOLUTA (REV), eine Beteiligung an diesem WRKY-Netzwerk beschrieben werden.

3 Introduction

Senescence is the final stage of plant development and ultimately leads to the death of the plant. It is a recycling process, which transfers nutrients such as mineral, nitrogen and carbon sources from old, dying tissue into developing parts of the plant, like new leaves, flowers and seeds (Figure 1). These nutrients result from the degradation of macromolecules, which is highly coordinated. Before anthesis, they are only redistributed from older leaves into non-reproductive organs, while after anthesis the newly developing reproductive organs are activated. Hence, there is a switch from sequential leaf senescence to monocarpic leaf senescence, which has a critical impact on yield quality and quantity. For agriculture, senescence is of great importance and a better understanding could have economic benefits. It is a process, that ensures the better fitness of the plant and thus has an effect on the survival of the next generation (Kim et al., 2018; Zentgraf et al., 2010).

The whole senescence process is classified into the senescence of leaves, flowers and fruits and can be controlled in the onset and/or in the intensity and the rate of progression. This work is limited to the leaf senescence of *Arabidopsis thaliana*.

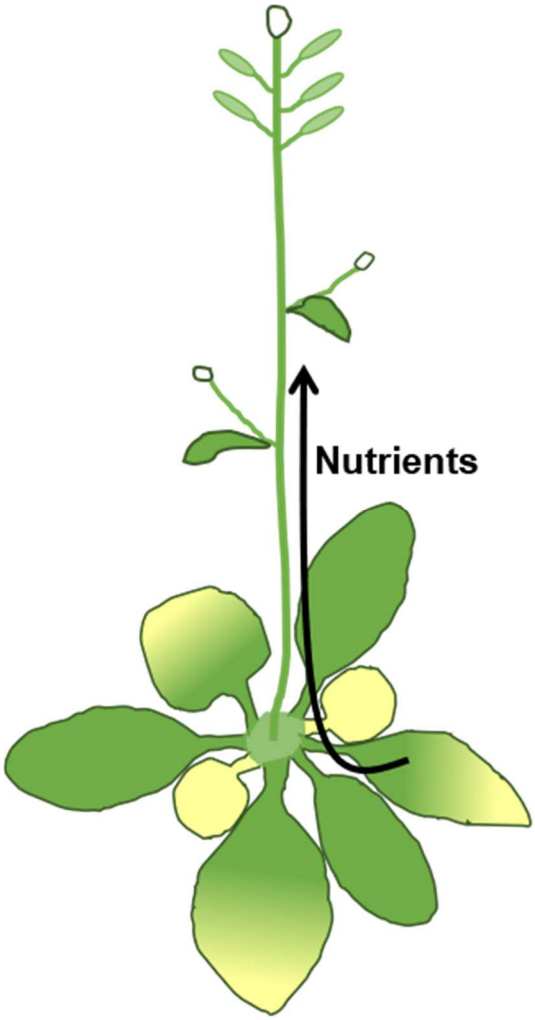

Figure 1: Recycling process of nutrients into developing tissue for *Arabidopsis thaliana*.

3.1 Senescence

Senescence can be divided into three different phases: initiation, reorganization and termination. The initiation of leaf senescence can be triggered by internal and external signals. Examples of internal signals are the nominal age of the leaves or tissue or the state of development. Extreme temperatures, drought, wounding or pathogen infection are examples of external signals. Usually, plants grow continuously until they reach their maximum size, which can cause senescence. In *Arabidopsis thaliana*, the onset of senescence is usually the time when flower development is initiated and vegetative meristem activity is terminated (Thomas, 2013). The right time to initiate senescence is important. Starting too late can lead to incomplete mobilization of nutrients. Starting too early would result in lower carbon assimilation and nitrogen uptake (Masclaux-Daubresse et al., 2010). The only visible feature of senescence is the yellowing of the leaves during the reorganization phase, resulting from the degradation of chlorophyll. Furthermore, changes in cell structure, metabolism and gene expression can be

observed in the cells (Noodén and Leopold, 1988). There is a degradation of RNA, proteins and membranes, which are broken down into their respective components: proteins to small peptides and amino acids; RNA to low molecular weight nitrogen compounds; and membranes to lipids and then to sugars. However, the intensive degradation of macromolecules can increasingly lead to toxic intermediates, whereas antioxidative enzymes such as peroxidases and catalases play a decisive role (Zimmermann et al., 2006). Natural antioxidants such as anthocyanin increase during senescence and are believed to protect against oxidative stress induced damage (He and Giusti, 2010). The photosynthetic activity decreases early accompanied by the degradation of mRNAs encoding proteins of the photosynthesis machinery (Hensel et al., 1993). In contrast, there is an increased expression of genes involved in the degradation and mobilization of nutrients. In addition, the vascular system is affected last because it is required for the transport of nutrients (Gan and Amasino, 1997). The degradation of the nucleus and the DNA only takes place in the termination phase, since controlled transcription and the energy supply of the cell must be maintained. Cell death begins with the destruction and collapse of vacuoles, which leads to shrinkage and degradation of the cytoplasm, nuclear condensation and fragmentation and breakdown of the cell wall (Kuriyama and Fukuda, 2002).

Massive changes in gene expression accompany the process of leaf senescence. Genes that are up-regulated during senescence are called senescence-associated genes (SAGs). So far, a large number of SAGs have been found (Buchanan-Wollaston et al., 2003). These include degradative enzymes such as RNases (Taylor et al., 1993), proteases (Lohman et al., 1994; Hensel et al., 1993) and lipases (Ryu and Wang, 1995), but also genes that are involved in the transport of nutrients. In contrast, genes that are down-regulated during senescence are called senescence-down-regulated genes (SDGs). These include genes that are necessary for photosynthesis (Hensel et al., 1993).

3.2 Regulation of senescence

3.2.1 Transcription factors during senescence

These extreme changes in gene expression imply a strong participation of transcription factors (TFs) (Balazadeh et al., 2008; Breeze et al., 2011). TFs can act as activators or repressors of certain target genes. Breeze et al., 2011 generated microarray data for *Arabidopsis thaliana* leaf material in a high time resolution to identify gene

expression changes during leaf senescence. They found that 6323 genes were differentially expressed, including members of the NAC and WRKY transcription factor families. In fact, the NAC and WRKY family of transcription factors constitute the largest and second largest group of TFs expressed during senescence, followed by C2H2 zinc finger and AP2/EREBP TFs (Guo et al., 2004). For some WRKY and NAC transcription factors, there is already experimental evidence for their participation in senescence regulation. WRKY6 is known to be associated with senescence and defence (Robatzek and Somssich, 2002), WRKY22 participates in the dark-induced senescence (Zhou et al., 2011), WRKY54 and WRKY70 co-operate as negative regulators of senescence (Besseau et al., 2012), and WRKY53, WRKY75, and WRKY45 were characterized as positive regulators (Miao et al., 2004; Li et al., 2012; Chen et al., 2017). AtNAP and ORE1, transcription factors of the NAC family, are positive regulators of senescence (Guo and Gan, 2006; Kim et al., 2009). JUNGBRUNNEN1 (JUB1) is a negative senescence regulator that also lowers intracellular hydrogen peroxide (H_2O_2) levels (Wu et al., 2012).

NAC transcription factors not only participate in the senescence process, but also in regulating plant growth and development including abiotic stress responses (Shao et al., 2015). They function, for example, in flower development (Sablowski and Meyerowitz, 1998), cell division (Kim et al., 2006) and salt stress (Jiang and Deyholos, 2006).

WRKY transcription factors are, like the NACs, unique to the plant kingdom. Besides senescence, they are involved in the regulation of many plant processes including the responses to pathogen infestation (Hu et al., 2012), abiotic stresses (Jiang and Deyholos, 2009; Rushton et al., 2010) and trichome development (Johnson et al., 2002). WRKY factors are named after their characteristic DNA-binding domain (DBD), the WRKY domain. This domain consists of approximately 60 amino acids and contains a strongly conserved WRKYGQK motif at the N-terminus and a zinc-finger structure at the C-terminus; it binds the promoters of certain target genes through a so-called W-box (TTGACC/T). This sequence is the minimal core element necessary for binding of a WRKY protein to DNA (Ciolkowski et al., 2008). There are often several W-boxes in one promoter, and interestingly there are also W-boxes in the promoters of *WRKY* genes, suggesting strong transcriptional networking between WRKY factors. One member of the WRKY family, namely WRKY53, has emerged as an important

regulator of senescence events (Zentgraf and Doll, 2019). WRKY53 shows an interesting pattern of expression: it first increases with the leaf age, but then becomes strongly activated in all rosette leaves during bolting (Hinderhofer and Zentgraf, 2001). After its increased expression, it acts in a highly controlled network of other WRKY factors and SAGs to initiate the senescence process. Transgenic Arabidopsis overexpressors of *WRKY53* and knockout Arabidopsis mutants of *wrky53* clearly show that WRKY53 acts as a positive regulator of senescence (Miao et al., 2004; Xie et al., 2014). During onset of senescence, the locus of *WRKY53* is activated by histone modifications H3K4me2 and H3K4me3 (Ay et al., 2009; Brusslan et al., 2012). In contrast, it was recently found that the JmjC-domain containing protein JMJ16 is a specific H3K4 demethylase that negatively regulates leaf senescence. The repression of positive regulators of senescence, including *WRKY53*, is achieved by demethylating their histones H3 at Lys4 and thereby inactivating their transcription (Liu et al., 2019). In addition, the single-stranded DNA-binding protein WHIRLY1 (WHY1) represses the enrichment of H3K4me3, but enhances the enrichment of H3K9ac at the *WRKY53* locus (Huang et al., 2018). WHY1 functions as an upstream suppressor of *WRKY53*, and the *why1* mutant plants display an accelerated senescence phenotype (Miao et al., 2013). Furthermore, Chen and co-workers showed that WRKY53 acts in complex with a histone deacetylase (HDA9) to remove H3 acetylation marks from certain promoter regions, and to suppress the expression of key negative senescence regulators (Chen et al., 2016). Recently, the transcription factors WRKY18 and WRKY53 were found to bind in complex with HISTONE ACETYLTRANSFERASE 1 (HAC1) the promoter regions of sugar response genes and activate their expression through acetylation of H3K27ac (Chen et al., 2019). Several additional proteins are known to be able to bind the promoter of *WRKY53* and regulate its expression. WRKY53 can bind its own promoter and affect its expression in a negative feedback loop (Miao et al., 2004). The *WRKY53* promoter is also bound by a mitogen-activated protein kinase kinase kinase (MEKK1) that positively regulates *WRKY53* expression. An interaction of MEKK1 with WRKY53 also occurs on the protein level, which leads to a phosphorylation of WRKY53 resulting in enhanced DNA binding activity of WRKY53. Thus, MEKK1 has a dual function in the regulation of WRKY53: it binds the promoter of *WRKY53* to regulate transcript levels and phosphorylates the protein WRKY53, thereby increasing its DNA binding activity (Miao et al., 2007). Another positive regulator of *WRKY53* is the ‘activation domain protein’ (AD-Protein), which can

phosphorylate itself with a kinase domain thereby enhancing its binding to the *WRKY53* promoter (Miao et al., 2008). GATA4 is another transcription factor that activates or represses the promoter of *WRKY53* depending on the binding site and phenotypic analyses of *gata4* mutants show a delayed senescence, suggesting a positive regulation of senescence by GATA4 (unpublished group work). A negative regulator of *WRKY53* is the jasmonic acid (JA)-inducible protein EPITHIOSPECIFYING SENESCENCE REGULATOR (ESR/ESP), that functions in pathogen resistance (Miao and Zentgraf, 2007). *WRKY53* and *ESR/ESP* are antagonistically regulated in response to salicylic acid (SA) and JA, and both proteins can negatively influence the expression of the other gene. Phenotypic analyses show that ESP/ESR is a negative senescence regulator. ESP/ESR can interact with WRKY53 in the nucleus, through which it also gets there. Thus ESP/ESR has a different function depending on its localization: in pathogen resistance as a cytoplasmic protein and in senescence regulation via WRKY53 as a core protein (Miao and Zentgraf, 2007). For WRKY53, as an important regulator of early senescence events, it is also known that it is degraded via UPL5, a HECT E3 ubiquitin ligase, which ensures that onset of senescence takes place at the correct time (Miao et al., 2010).

3.2.2 Hydrogen peroxide during senescence

Interestingly, most of the regulators of *WRKY53* are responsive to hydrogen peroxide (H_2O_2). The expression of *WRKY53* itself, *MEKK1* and the *AD-Protein* is induced by H_2O_2 (Miao et al., 2004; Miao et al., 2007; Miao et al., 2008), in contrast it is known that *UPL5* expression is repressed (Miao et al., 2010). *GATA4* expression is activated or repressed by H_2O_2, depending on plant age (unpublished group work). In general, there are many senescence-associated transcription factors induced by H_2O_2 (Balazadeh et al., 2008). H_2O_2 is a reactive oxygen species (ROS) that causes oxidative stress in cells. Oxidative stress reflects an imbalance between generation and degradation of ROS, caused either by an increased formation of ROS, or by the loss of antioxidant protection systems. Death of single cells or the whole organism can be the result. Indeed, there is a strong correlation between life-span and tolerance to oxidative stress (Zentgraf, 2009). To avoid too high concentrations of H_2O_2, there are enzymes in the plant cell that can detoxify it. They include several forms of catalases (CAT) and ascorbate peroxidases (APX). However, H_2O_2 is also important for the plant and serves as a signal molecule during senescence. During bolting of Arabidopsis plants, there is an increase in H_2O_2 concentration, which is considered to act as signal

to induce senescence (Miao et al., 2004; Zimmermann et al., 2006). The expression of *CAT2* is down-regulated by 'G-Box Binding Factor 1' (GBF1) at bolting time, leading to an initial increase in H_2O_2 concentration (Smykowski et al., 2010). As a result of this increase, the cytosolic APX1 activity is inhibited, leading to an even higher H_2O_2 peak. By a so far unknown mechanism, APX1 activity is inhibited by its own substrate H_2O_2 during the period of bolting. This temporary H_2O_2 increase serves as a signal to induce the expression of various SAGs, like *WRKY53*, and thus contributes to the initiation of senescence. The up-regulation of the SAG *CAT3* with progression of senescence and restoration of APX1 activity can reduce H_2O_2 concentration again. The complex initiation of senescence is summarised in Figure 2.

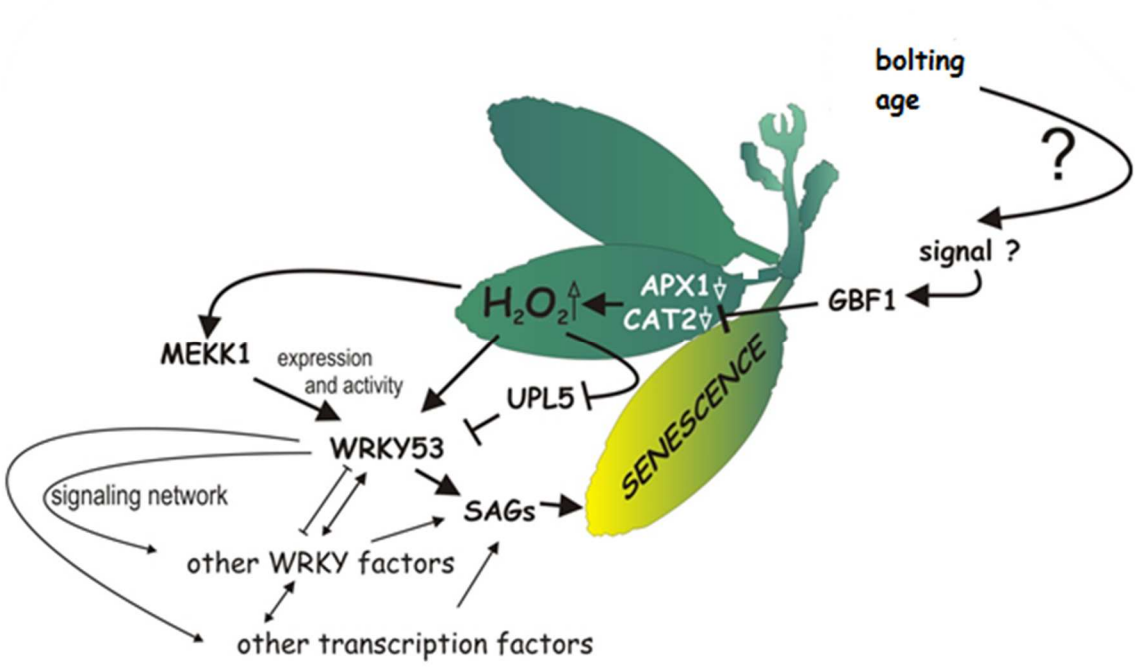

Figure 2: A Model for the initiation of senescence. A hydrogen peroxide peak occurs due to the down-regulation of *CAT2* and the post-transcriptional inactivation of APX1 during bolting of Arabidopsis. Hydrogen peroxide then serves as a signal molecule inducing the expression of several SAGs including *WRKY53*. WRKY53 is part of a signalling network, leading to the initiation of senescence (modified after U. Zentgraf).

3.3 Aim of the work

Transcriptional reprogramming is a central feature of senescence regulation that implies an essential role for TFs (Balazadeh et al., 2008; Breeze et al., 2011). WRKY TFs bind W-boxes in their target genes which is a highly conserved consensus sequence (TTGACC/T) required for DNA-protein interaction. Because most WRKY factors own W-boxes in their promoters, it is assumed that there is a strong networking between WRKY factors. WRKY53 has been identified as a positive regulator of leaf senescence with its highest expression during an early stage of senescence. In order

to characterize WRKY factors involved in the regulation of *WRKY53* expression during senescence, I used one *WRKY53* W-box with its native surrounding sequence to characterize binding of selected candidates of the WRKY family to the *WRKY53* promoter. In addition, I performed transient co-transformation assays of Arabidopsis protoplasts with *WRKY53* promoter:GUS and *35S:WRKY* overexpression effector constructs to get further insights into the consequences on *WRKY53* expression *in vivo*. As a result of this preliminary work, WRKY18 and WRKY25 were selected as interesting candidates for further analyses as WRKY18 was the most efficient negative and WRKY25 the most efficient positive regulator. In this work, I mainly aimed to characterize these two candidates in the network driving *WRKY53* expression during senescence. Furthermore, I participated in the detailed characterization of REVOLUTA (REV), a new upstream regulator of *WRKY53* connecting early leaf development and senescence.

4 Results and Discussion

4.1 The WRKY factors and their specific regulatory strategy

WRKY transcription factors are one of the largest TF families in plants. Another important TF family are the bZIP factors, which include GBF1 as an important regulator of senescence induction (Smykowski et al., 2010). The name of the bZIP factors originates from the basic region/leucine zipper domain, which is present in all its members. In addition to their involvement in senescence, the bZIPs participate in, for example, pathogen defence (Alves et al., 2013), abiotic stress signalling (Fujita et al., 2005), and flowering (Abe et al., 2005). Despite having partially overlapping biological functions, both families seem to operate in a different manner to regulate expression of their target genes. WRKYs strongly regulate each other at the transcriptional level in a WRKY network, while bZIPs are predominantly regulated at the post-translational level, via the formation of heterodimers. For comparison, the regulatory mechanisms characterized for WRKYs and bZIPs are described below in more detail.

Almost all TFs bind certain sequence elements of their target genes, the WRKYs bind W-boxes (TTGACC/T), and the bZIPs bind hexamers with an ACGT core. This raises the question of how specificity is achieved between certain factors and their target genes. For the WRKYs, the surrounding sequences of these W-boxes and the overall structure are important for specific binding (Miao et al., 2004; Robatzek and Somssich, 2002; Ciolkowski et al., 2008). Additionally, there are reports of WRKY factors that bind imperfect W-boxes or sequences lacking a W-box (Miao et al., 2004; Potschin et al., 2014; Cai et al., 2008), which increases the sequence diversity. The WRKY domain also mediates DNA-binding specificity through sequence variants for its highly conserved WRKYGQK motif (Brand et al., 2013). Interestingly, WRKY genes contain an enrichment of W-boxes in their promoters compared to the average occurrence of W-boxes in all Arabidopsis genes, which points to a strong mutual regulation in a WRKY network. We found, with the help of the TAIR database, that 72% of the WRKY genes have two or more W-boxes. Such an enrichment was not found for the bZIPs and their bZIP-binding site in their own promoters, indicating a stronger regulation of non-bZIP target genes. Unlike WRKYs, bZIPs must dimerize for DNA-binding. Beside homodimers, many bZIP factors tend to form primarily heterodimers. The type of heterodimerization then determines the DNA-binding specificity. Particular partner selection depends on the monomeric bZIP structure (Vinson et al., 2002; Fong et al.,

2004; Deppmann et al., 2004; Vinson et al., 2006). The heterodimerization is an important control point for the bZIPs, as shown by the *Arabidopsis* interaction viewer on the BAR website (http://bar.utoronto.ca/welcome.htm). Using all 76 WRKYs IDs (TAIR), 170 interactions are identified, while for all 75 bZIPs IDs (TAIR) 389 interactions are obtained, more than the double than for the WRKYs. Furthermore, each monomer contributes to the type of transactivation activity (Miotto and Struhl, 2006). Although WRKYs do not need to dimerize for DNA-binding, it is possible and they can bind to DNA as monomers, dimers, or even as trimers (Xu et al., 2006; Ciolkowski et al., 2008; Liu et al., 2012). In addition, there are even more regulatory mechanisms for both families, like transcriptional gene expression control, subcellular protein localization, and protein modification like phosphorylation or glycosylation. Figure 3 summarises the main differences between the WRKYs and the bZIPs in their regulatory strategies.

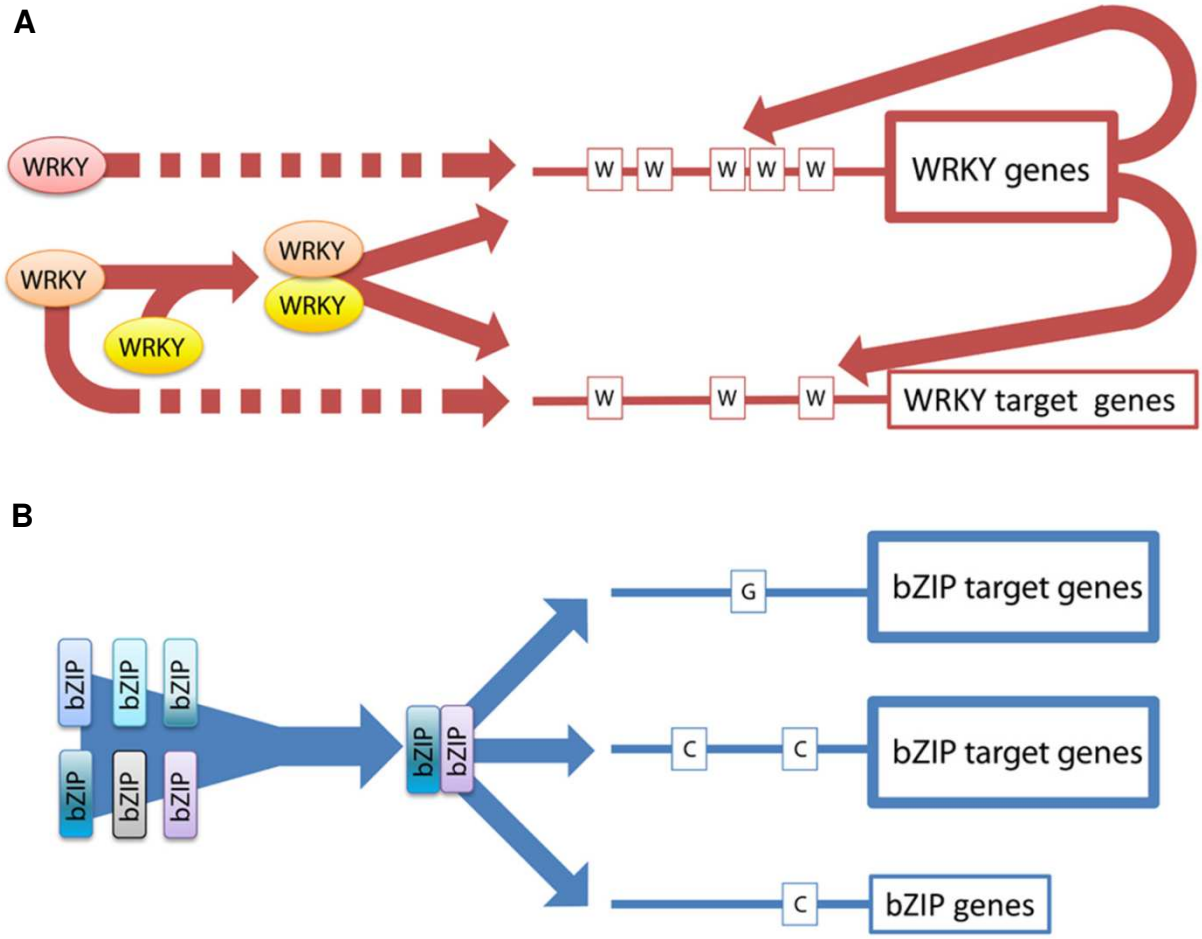

Figure 3: A model of major differences in regulation strategies for the WRKYs and the bZIPs. A. WRKYS strongly regulate each other at the transcriptional level in a WRKY network. They mainly bind and regulate other WRKY promoters, doing so mainly as monomers (dotted line) or dimers. B. bZIPs need to heterodimerize to regulate target gene expression and there is no indication of group-internal network regulation. (Llorca et al., 2014).

The major regulatory mechanisms characterized for WRKY transcription factors in comparison to bZIP transcription factors are described in detail in the following review article:

Llorca, C. M., **Potschin, M.**, Zentgraf, U. (2014). "bZIPs and WRKYs: two large transcription factor families executing two different functional strategies." Frontiers in Plant Science **5**: 169.

4.2 Upstream regulators of *WRKY53*

Various WRKYs were selected based on their expression during senescence, using literature references, genevestigator (https://genevestigator.com/gv/) and eFP Browser expression data (http://bar.utoronto.ca/efp2/Arabidopsis/Arabidopsis_eFPBrowser2) and previous expression data investigated by former members of the Zentgraf group. In addition, at least one WRKY from each group and subgroup of the WRKY family is represented. The WRKY factors are divided into three groups depending on their number of WRKY domains and the zinc-finger structure, while group II is further subdivided into IIa, IIb, IIc, IId, and IIe based on their primary amino acid sequence. These selected candidates were then used for preliminary experiments to find potential upstream regulators of *WRKY53* as an important regulator of early senescence events. Table 1 lists the selected WRKY factors and their group affiliation.

Table 1: Selected WRKY candidates and their group affiliation.

WRKY factor	Group affiliation
WRKY6	IIb
WRKY13	IIc
WRKY15	IId
WRKY18	IIa
WRKY22	IIe
WRKY25	I
WRKY29	IIe
WRKY30	III
WRKY33	I
WRKY38	III
WRKY40	IIa
WRKY60	IIa
WRKY62	III
WRKY70	III

Earlier work of our group showed that *WRKY6*, *WRKY13*, *WRKY15*, *WRKY22*, *WRKY29*, and *WRKY62* are putative target genes of WRKY53 isolated by a genomic pull-down assay, findings that make them interesting candidates for further study (Miao et al., 2004). Thomas Laun, a former member of our group, found in a microarray analyses that the expression of *WRKY6*, *WRKY22* and *WRKY33* is induced by WRKY53, as these genes are upregulated in *WRKY53* overexpressing and downregulated in *wrky53* knockout plants (Laun, 2008). Furthermore, *WRKY25*, *WRKY29*, *WRKY30* and *WRKY60* expression has been altered in either the *WRKY53* overexpressing or *wrky53* knockout plants. The expression of *WRKY15* and *WRKY70* was upregulated in *WRKY53* overexpressing plants and *wrky53* knockout plants, indicating a more complex regulation between these WRKY factors. In addition, Laun (2008) showed that WRKY18, WRKY62 and WRKY53 mutually influence each other's expression and the expression of many other WRKY factors and could therefore be interesting candidates for my further work.

Literature references show additional interesting properties of the selected WRKYs and thus support this choice. WRKY6 is known to be senescence-and defence-

associated and positively affects the *'Pathogenesis-related protein 1'* (*PR1*) and *'Senescence-induced receptor-like kinase'* (*SIRK*) expression (Robatzek and Somssich, 2002). *PR1* is expressed during systemic acquired resistance (SAR) and *SIRK* is strongly and specifically expressed during leaf senescence. Interestingly, *SIRK* is also a target of WRKY53 (Miao et al., 2004). WRKY18 was first described in the involvement of the pathogen defence, as *WRKY18* overexpressing plants have a higher resistance to the bacterial pathogen *Pseudomonas syringae* (Chen and Chen, 2002). In addition, WRKY18, WRKY40, and WRKY60 are involved in abiotic stress responses to salt and osmotic stress and in abscisic acid (ABA) signalling. *WRKY18* and *WRKY40* are rapidly induced by ABA, while induction of *WRKY60* by ABA is delayed. Furthermore, ABA-inducible expression of *WRKY60* is almost completely abolished in the *wrky18* and *wrky40* knockout plants, suggesting that *WRKY60* may be a direct target gene of WRKY18 and WRKY40 in ABA signalling (Chen et al., 2010). WRKY22 has already been associated with senescence; participating in dark-induced leaf senescence. Gene expression was suppressed by light and promoted by darkness and hydrogen peroxide (H_2O_2). Mutual regulation existed between *WRKY22* and *WRKY6*, *WRKY53*, and *WRKY70*, respectively (Zhou et al., 2011). In addition, WRKY22 is targeted together with WRKY29 by a MAP kinase cascade (MEKK1, MKK4/MKK5 and MPK3/MPK6) that is activated downstream of the flagellin receptor FLS2, a leucine-rich-repeat (LRR) receptor kinase. Activation of this MAPK cascade confers resistance to both bacterial and fungal pathogens (Asai et al., 2002). WRKY25 was initially characterized to be involved in biotic and abiotic stress responses, it functions as negative regulator after *Pseudomonas syringae* infection, but as positive regulator in mediating resistance against NaCL, heat and oxygenic stress (Zheng et al., 2007; Jiang and Deyholos, 2009; Li et al., 2011). There is further evidence that WRKY25 is involved in oxidative stress: Rizhsky et al. (2004) showed in an older study that the induction of *WRKY25* depends on 'Zinc finger of Arabidopsis thaliana12' (ZAT12) during oxidative stress. ZAT12 is a key regulator of ROS signalling (Rizhsky et al., 2004; Miller et al., 2008) and its expression is in general enhanced during osmotic, drought, salinity, temperature, oxidative or high-light stress and wounding (Davletova et al., 2005; Mittler et al., 2006). APX1 is a key H_2O_2 removal enzyme in plants (Panchuk et al., 2002; Pnueli et al., 2003) and its expression is also dependent on ZAT12 during oxidative stress (Rizhsky et al., 2004). The expression level of *APX1* was reduced more or less in *wrky25* mutants, whereas *ZAT12* was induced to a higher

level compared to wild type (Li et al., 2009). It has been reported frequently that WRKY25 is redundant with WRKY33 e.g.in the positive regulation against salt and heat stress (Jiang and Deyholos, 2009; Li et al., 2011). Andreasson et al. (2005) showed that WRKY25 and WRKY33 interact with 'MAP kinase 4 substrate' (MKS1), that acts downstream of 'MAP kinase 4' (MPK4) in the SA-dependent pathway to activate resistance. WRKY30 can interact on the protein level with WRKY53, and its gene expression is inducible by SA-treatment and H_2O_2 (Besseau et al., 2012). WRKY38 functions with WRKY62 as a negative regulator of plant basal defence. They can interact with Histone Deacetylase 19 (HDA19) that has an opposite role as WRKY38 and WRKY62 in basal resistance to the bacterial pathogen *P. syringae*. Overexpression of *HDA19* resulted in enhanced resistance to *P. syringae*. Therefore, the interaction of WRKY38 and WRKY62 with HDA19 may contribute to the fine-tuning of plant defence responses (Kim et al., 2008). WRKY70 is a negative senescence regulator (Ülker et al., 2007; Besseau et al., 2012) and, in cooperation with WRKY53, positively regulates the basal resistance to *P. syringae* (Hu et al., 2012).

I have now tested these selected WRKY factors whether they are involved in the regulation of *WRKY53*. Therefore, a particular W-box of the promoter of *WRKY53* with its native surrounding sequence, W-box2, was used to characterize the binding of these WRKYs to this *WRKY53* promoter fragment (Figure 4A). This W-box was used because it was shown that WRKY53 itself can bind to it (Miao et al., 2004). For the performed DNA-protein interaction ELISA (DPI-ELISA) crude extracts of *E. coli* BL21 cells expressing these 6xHis-tagged WRKY candidates were added in two different protein concentrations (5 and 25 µg) to a streptavidin-coated ELISA plate covered with this biotinylated W-box2 (Figure 4B). Crude extracts of empty *E. coli* BL21 cells were used as a negative control. An increase in *E. coli* protein content in the binding reaction did not show any effect. In contrast, all binding reactions with WRKY expressing crude extracts revealed an increasing binding with increasing protein concentrations indicating a concentration-dependent binding for all tested factors except WRKY29. WRKY60 was not tested in the DPI-ELISA. In addition, a transient co-transformation assay was performed in Arabidopsis protoplasts to investigate the effect of these WRKY candidates on *WRKY53* regulation *in planta* (GUS-assay; Figure 4C). Therefore, *35S:WRKY* overexpression effector constructs of all candidates and a 2.759 kbp promoter:GUS construct of *WRKY53* were used. WRKY13 was not tested due to cloning problems. The empty overexpression effector construct was used as a

negative control. Western blots were done with separate transformation assays of Arabidopsis protoplasts to show the expression. Separate protoplasts had to be transformed because the protein concentration in the GUS-assay approaches was too low for detection. Unfortunately, WRKY38 could not be detected, so it does not seem to be expressed, which is also confirmed by the non-effect in the GUS-assay. Several WRKYs showed a good regulation effect on the reporter gene expression driven under the *WRKY53* promoter. This effect can be either positive or negative. The most prominent positive effect was seen by WRKY25; the most prominent negative effect was seen by WRKY18. Surprisingly, overexpression of WKY29 affected GUS expression in a positive manner, even though no binding was detected in the DPI-ELISA, which indicates that complex formation with other proteins might be responsible for this effect. WRKY70 has no effect in the GUS-assay despite good expression in the western blot and is therefore not analysed further. This example also shows that binding does not automatically lead to gene expression. For these reasons, I focused my further main work on WRKY18, as a negative regulator of *WRKY53* and on WRKY25, as a positive regulator of *WRKY53*.

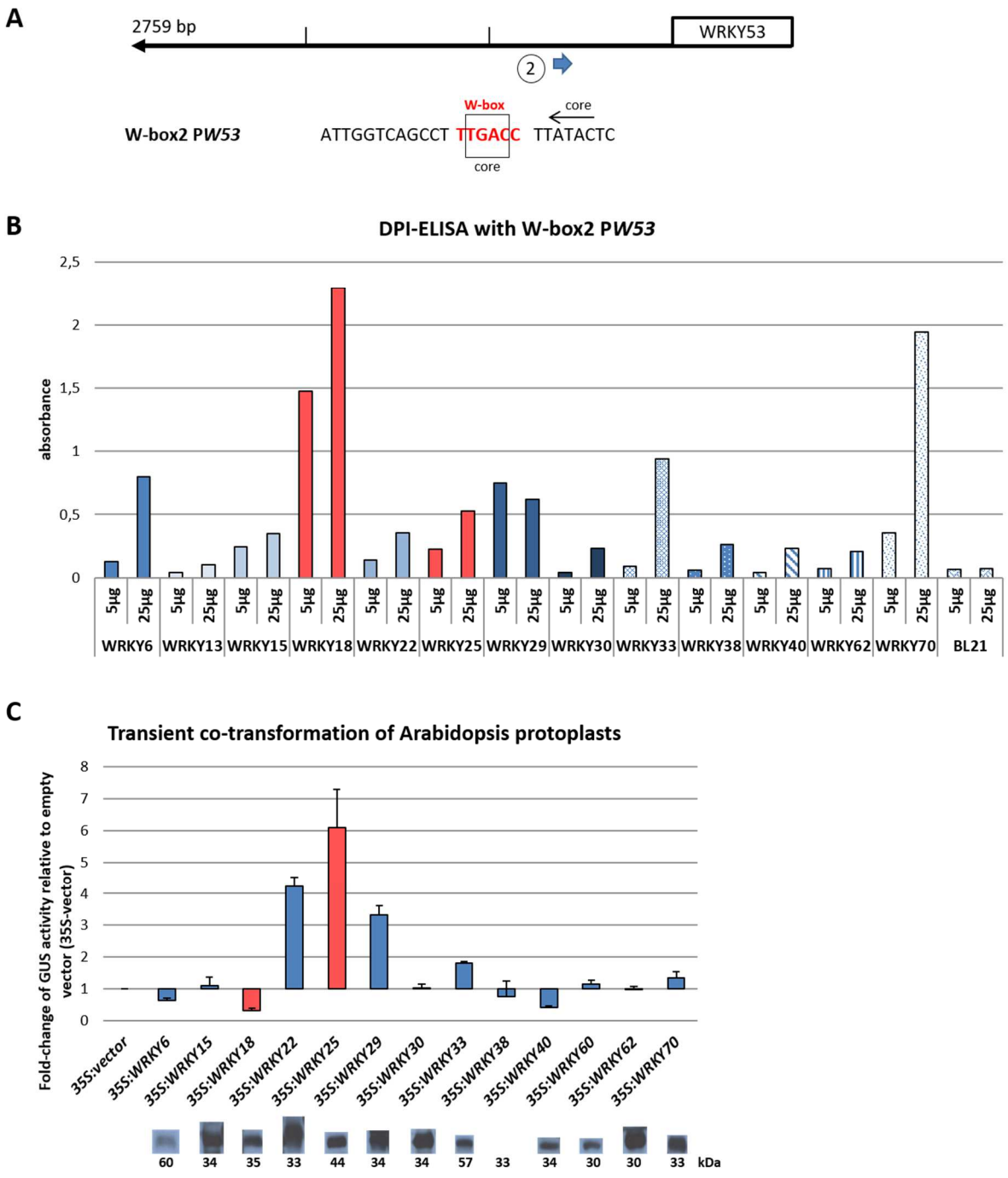

Figure 4: Possible upstream regulators of *WRKY53*. A. To characterize WRKY factors involved in the regulation of *WRKY53* expression, a selected *WRKY53* W-box with its native surrounding sequence was used (W-box2). B. Binding of the selected WRKY factors to this W-box2 of the promoter of *WRKY53* was tested by a DPI-ELISA (mean values of 2 technical replicates, n=1). C. A transient co-transformation assay in Arabidopsis protoplasts was performed to detect an effect in plant cells; a 2.759 kbp promoter fragment of *WRKY53* was used (mean values + SD, n=3). The expression strength was shown on a separate western blot.

The methods mentioned in this chapter, DPI-ELISA and GUS-assay are described in detail in the following articles:

Potschin, M., Schlienger, S., Bieker, S., Zentgraf, U. (2014). "Senescence networking: WRKY18 is an upstream regulator, a downstream target gene, and a protein interaction partner of WRKY53." Journal of Plant Growth Regulation **33**(1): 106.

Doll, J., **Muth, M.**, Riester, L., Nebel, S., Bresson, J., Lee, H-C., Zentgraf, U. (2020). "*Arabidopsis thaliana WRKY25* transcription factor mediates oxidative stress tolerance and regulates senescence in a redox-dependent manner." Frontiers in Plant Science **10**:1734.

4.3 Characterization of WRKY18

4.3.1 WRKY18 regulates *WRKY53*

In order to test whether WRKY18 can bind directly to the promoter of *WRKY53* and thus regulate it, we performed a DPI-ELISA with crude extracts of *E. coli* BL21 cells expressing 6xHis-tagged WRKY18 protein and different biotinylated W-boxes of the *WRKY53* promoter. WRKY18 showed a strong binding to all W-boxes; a weaker affinity was only seen for a cluster motif of three imperfect W-boxes (TGAC) in tandem.

To investigate which effect WRKY18 has on the expression of *WRKY53* we did transient co-transformation assays of Arabidopsis protoplasts with *WRKY53* promoter:GUS and *35S:WRKY18* overexpression constructs. Two different promoter constructs were used; a shorter fragment of 1.1 kbp and a longer one of 2.759 kbp. Overexpression of *WRKY18* led to a strong negative effect in reporter gene expression meaning a negative influence of WRKY18 on the expression of *WRKY53*, which was even more pronounced when the longer promoter fragment with more regulatory binding sites was used. This clearly shows that WRKY18 acts as a repressor on the expression of *WRKY53*.

4.3.2 WRKY18 regulates senescence

We did phenotypic analyses of *WRKY18* T-DNA insertion and *WRKY18* overexpression lines to test if these mutant and transgenic lines show a different senescence progress. Pictures were taken from rosette leaves, which were sorted according to their age. For statistical analyses, these leaves were also classified into groups of their respective colour: 'green', 'green/yellow', 'yellow' and 'brown/dry'.

Furthermore, Atleaf+ values of two defined leaves were measured indicating the chlorophyll content and expression of two senescence marker genes and *WRKY53* were analysed by qRT-PCR. Compared to wild type plants, *WRKY18* overexpressors showed a delayed senescence phenotype, which is reflected by the leaf colour distribution, the delayed loss of chlorophyll content and a delayed and lower expression of the marker genes. The *wrky18* mutant plants show the opposite phenotype, meaning that senescence is accelerated for all criteria in comparison to the wild type plants. The expression of *WRKY53* is in the *WRKY18* overexpressors reduced and in the *wrky18* mutants higher as in the wild type plants confirming again the negative effect of WRKY18 on *WRKY53*. Taken together, this demonstrates that WRKY18 is a negative regulator of *WRKY53* and a negative regulator of senescence. Therefore, it is possible that the senescence regulation at least partially proceeds via WRKY53. We could also demonstrate by qRT-PCR analyses that *WRKY18* expression increased with the progression of senescence, suggesting that WRKY18 might restrict *WRKY53* expression in later stages of senescence.

4.3.3 WRKY18 interacts with WRKY53

Because WRK18 can regulate the gene expression of *WRKY53*, it is also interesting to examine whether there is a possible protein-protein interaction increasing the regulation possibilities. Therefore, we used the split ubiquitin system in yeast, which is recommended for the study of interactions between transcription factors. WRKY18 and WRKY53 were found to interact and form heterodimers, but we were also able to show that homodimers can be formed for the respective WRKY. To verify these results *in planta*, we used the Foerster Resonance Energy transfer system with subsequent Fluorescence life time imaging microscopy (FRET-FLIM system) in transiently transformed Arabidopsis protoplasts and tobacco leaves. This clearly showed a protein-protein interaction between WRKY18 and WRKY53 and also between the respective WRKY. The transient co-transformation assay in Arabidopsis protoplasts with both WRKYs simultaneously resulted in a different, non-additive result compared to each WRKY construct transformed alone. This shows that the formed heterodimers have a different spectrum of activity than the individual WRKYs, which in turn increases the possibilities of the regulatory WRKY network.

All results addressing the *WRKY53* and senescence regulation by WRKY18 are described in detail in the following publication:

Potschin, M., Schlienger, S., Bieker, S., Zentgraf, U. (2014). "Senescence networking: WRKY18 is an upstream regulator, a downstream target gene, and a protein interaction partner of WRKY53." Journal of Plant Growth Regulation **33**(1): 106.

4.4 Characterization of WRKY25

4.4.1 WRKY25 regulates *WRKY53*

To test whether WRKY25 can directly bind to the promoter of *WRKY53*, again DPI-ELISAs with *E. coli* crude extracts containing 6xHis-tagged WRKY25 protein and different biotinylated W-boxes of the *WRKY53* promoter were performed. There are clear binding preferences of WRKY25 to specific W-boxes, indicating a direct and targeted binding to the promoter of *WRKY53*.

Several transcription factors, such as the WRKY factors, contain cysteine residues in their DNA-binding domain that are redox-sensitive via their thiol group. By modification, such as oxidation of these cysteine residues, the activity can be changed positively or negatively, and so WRKY factors are good candidates for gene expression regulation according to the intracellular redox state (Arrigo, 1999). WRKY25 even has two DNA-binding WRKY domains (C2H2) and thus seems to be a well-suited redox-sensitive transcription factor. In order to analyse this we developed a Redox-DPI-ELISAs, in which the *E. coli* protein crude extract was previously reduced or oxidized by the addition of Dithiothreitol (DTT) or hydrogen peroxide (H_2O_2) and then titrated back again to show reversibility of the effect. WRKY25 showed a clear redox-effect with a significantly stronger binding under reducing conditions and a significantly lower binding under oxidizing conditions in comparison to non-redox-treated WRKY25 binding. Therefore, WRKY25 binds directly to the promoter of *WRKY53* and the binding activity depends on the intracellular redox state and can be modulated by it.

To examine the effect of WRKY25 on *WRKY53* expression, transient co-transformations of Arabidopsis protoplasts with a *WRKY53* promoter:GUS construct and a *35S:WRKY25* overexpression effector construct were performed. WRKY25 significantly regulates gene expression of *WRKY53* in a positive way. Since the Redox-DPI-ELISAs showed a significant decrease in binding activity of WRKY25 to certain W-boxes of the *WRKY53* promoter after H_2O_2 treatment, and H_2O_2 is an important

signal molecule in senescence, it is interesting to know whether H_2O_2 or an oxidized cell status can change gene expression regulation. For that we repeated the transient co-transformation assays with Arabidopsis protoplasts that were treated with 3-Amino-1,2,4-triazole (3'-AT) to inhibit catalases and maintain H_2O_2 generation at a consistently higher but still physiological level. The expression of the reporter gene under the *WRKY53* promoter decreases significantly for the effector WRKY25 when the H_2O_2 content in Arabidopsis protoplasts is higher after treatment with 3'-AT. Therefore, we can assume that WRKY25 is a redox-sensitive transcription factor which can act as an activator on the *WRKY53* expression under reducing conditions in vivo. This is also demonstrated in *wrky25* Arabidopsis mutants treated with H_2O_2 and screened for their *WRKY53* expression. The *WRKY53* induction is in this *wrky25* background clearly dampened compared to wild type plants. Hence, WRKY25 is involved in H_2O_2 response of *WRKY53*. In addition, we performed transient co-transformation assays with WRKY25 and MEKK1 as effectors simultaneously to see if phosphorylation can alter gene expression regulation. This was the case, and the expression of *WRKY53* increased compared to assays with single transformed WRKY25. Thus, *WRKY53* expression regulation by WRKY25 is influenced by various post-translational modifications that may lead to both positive and negative regulation.

4.4.2 WRKY25 regulates senescence

To test the involvement of WRKY25 in the regulation of senescence *in planta*, we characterized a *WRKY25* T-DNA insertion line, *WRKY25* overexpression lines and a *wrky25*/*wrky53* double-knock-out mutant. During early development of the plants the leaves were colour-coded with coloured threads according to their age, and classified into 4 groups according to their leaf colour ('green', 'green/yellow', 'yellow' and 'brown/dry') to statistically assure the senescence phenotype. The Fv/Fm values reflecting the photosynthetic activity of the plants were measured using a Pulse-Amplitude-Modulation (PAM) method and the expression of the senescence marker genes *CHLOROPHYLL A/B BINDING PROTEIN 1* (*CAB1*), *SENESCENCE-ASSOCIATED GENE 12* (*SAG12*) and the NAC transcription factor *ANAC092* was analysed by qRT-PCR. However, these analyses did not match the expectations on the senescence process for a positive regulator of *WRKY53* expression. In comparison to the wild type plants, *WRKY25* overexpressors showed a significantly delayed senescence phenotype, accompanied by a delay in the loss of the Fv/Fm ratio and a lower expression of *ANAC092* and *SAG12* as senescence up-regulated marker genes

in later plant stages. The senescence down-regulated marker gene *CAB1* is expressed more strongly in the *WRKY25* overexpressors matching the delayed senescence phenotype. Consistently, an accelerated senescence phenotype, Fv/Fm ratio decline, higher *ANAC092* and *SAG12* expression, and lower *CAB1* expression at later plant stages were, observed in *wrky25* KO plants. However, expression of *WRKY53* was lower in both, the *WRKY25* transgenic and *wrky25* mutant lines compared to wild type during the progression of senescence indicating a more complex regulation between WRKY25 and WRKY53 *in planta*.

4.4.3 WRKY25 mediates hydrogen peroxide tolerance

In order to investigate whether WRKY25 also participates in the signalling pathway of hydrogen peroxide (H_2O_2) in other development stages and in stress response, we performed a germination experiment, in which the *WRKY25* transgenic and mutant seeds germinated on plates containing 10 mM H_2O_2 in comparison to the wild type seeds. The *WRKY25* overexpressors germinated significantly better on these H_2O_2 plates, whereby the *wrky25* mutant line as well as the *wrky25*/*wrky53* double mutant line germinated significantly worse compared to wild type plants. Therefore, WRKY25 seems to mediate a higher tolerance against H_2O_2. To verify this higher tolerance, leaf discs of 6-week-old *WRKY25* transgenic and mutant lines and wild type plants were incubated in H_2O_2-solution for 2 hours and remaining H_2O_2 was measured using peroxide strips. Compared to wild type, the overexpressors scavenged more H_2O_2 and showed higher antioxidative capacity, while the mutant lines scavenged less, thus supporting the results of the germination assays. We also measured the intracellular H_2O_2 content in these *WRKY25* transgenic and mutant lines and wild type plants in one defined leaf of 8-week-old plants. In these more senescent plants, less intracellular H_2O_2 was measured for the overexpressors and more for the mutant lines in comparison to the wild type. Therefore, WRKY25 appears to participate in scavenging processes of H_2O_2. In addition, *WRKY25* is induced in wild type plants by H_2O_2 as many senescence-associated genes respond to elevated levels of H_2O_2, including especially NAC and WRKY transcription factors (Wu et al., 2012; Balazadeh et al., 2011; Xie et al., 2014; Chen et al., 2010). Taken together, WRKY25 mediates higher H_2O_2 tolerance by probably lowering intracellular H_2O_2 levels. This may also explain the negative senescence regulation of WRKY25, because H_2O_2 is a trigger of senescence.

All results addressing the *WRKY53* and senescence regulation by WRKY25 are described in detail in the following manuscript:

Doll, J., **Muth, M.**, Riester, L., Nebel, S., Bresson, J., Lee, H-C., Zentgraf, U. (2020). "*Arabidopsis thaliana WRKY25* transcription factor mediates oxidative stress tolerance and regulates senescence in a redox-dependent manner." Frontiers in Plant Science **10**:1734.

A set of tools to study hydrogen peroxide (H_2O_2) levels is described in detail in the following methods article:

Bieker, S., **Potschin, M.**, Zentgraf, U. (2018). "Study of Hydrogen Peroxide as a Senescence-Inducing Signal." Plant Senescence, Springer**:** 173-193.

4.5 Characterization of REVOLUTA

REVOLUTA (REV) is a class III homeodomain leucine zipper (HD-ZIPIII) transcription factor which was characterized by us as a new regulator in controlling the onset of senescence. REV can bind directly to the promoter of *WRKY53* and positively regulates its expression. Similar to WRKY25, it functions as a redox-sensitive transcription factor and participates in the response of *WRKY53* after hydrogen peroxide (H_2O_2) treatment. For this reason, I was involved in the characterization of this redox-regulation by REVOLUTA.

4.5.1 REVOLUTA is a positive regulator of *WRKY53* and of senescence

REV has been described in regulating polarity-associated growth processes in embryos, leaves, stems, vasculature and roots (Carlsbecker et al., 2010; McConnell et al., 2001; Smith and Long, 2010). During early leaf development, REV participates in the definition of the dorsoventral leaf axis by defining the domain that later develops into the upper side of the leaf (Byrne, 2006). Using a genome-wide chromatin-immunoprecipitation sequencing approach (ChIP-Seq) binding regions for REV across the Arabidopsis genome, including binding sites to the promoter of *WRKY53*, were found (Brandt et al., 2012). Quantitative ChIP-PCRs verified the binding of REV to the ChIP-Seq identified region of the promoter of *WRKY53*. Transient co-transformation assays of Arabidopsis protoplasts with *WRKY53* promoter:GUS and *35S:REVd* overexpression constructs revealed an induction in reporter gene expression, meaning a positive influence of REV on the expression of *WRKY53*. *REVOLUTA* is controlled by microRNAs at the post-transcriptional level (Rhoades et al., 2002); therefore, a

dominant microRNA-resistant version of *REV* (*REVd*) was used. Expression of *WRKY53* is lower in plants bearing loss-of-function mutation in *REV*, confirming that REV is a positive regulator of *WRKY53*. In addition, these *rev* mutant plants are delayed in senescence, suggesting a positive regulation of REV in senescence. Taken together, this demonstrates that REV is a positive regulator of *WRKY53*, a positive regulator of senescence, and that it combines early and late leaf development through involvement in leaf growth processes and senescence.

4.5.2 REVOLUTA is required for high induction of *WRKY53* to oxidative stress

WRKY53 expression is highly upregulated by hydrogen peroxide (H_2O_2; Miao et al., 2004) and the expression pattern during development and senescence clearly follows the intracellular hydrogen peroxide levels observed during bolting and onset of senescence. Since REV is a new upstream regulator of *WRKY53* and has a domain (PAS-domain) that is thought to detect changes in the redox state of the cell, we investigated whether REV is required for the induction of *WRKY53* in response to oxidative stress. Therefore, wild type plants and *rev* mutant plants were sprayed with H_2O_2 and the response of *WRKY53* was analysed by qRT-PCR. *Rev* mutants show much lower *WRKY53* induction compared to wild type, suggesting that REV activity is required for complete induction of *WRKY53* expression in response to H_2O_2.

4.5.3 REVOLUTA acts upstream of *WRKY53* and is a redox-sensitive transcription factor

The fact that REV is necessary to complete the induction of *WRKY53* expression after oxidative stress can be implemented either by upregulation of the REV transcript and protein amount or by a response of the REV protein to the altered redox conditions. To test whether the mRNA amount of REV increases, quantitative RT-PCRs with H_2O_2 treated plants were performed. The transcript amount of REV does not increase, but even decreases slightly, which excludes the possibility that the involvement of REV in the complete induction of WRKY53 under oxidative stress occurs via an increase in the mRNA of REV. Therefore, most likely the REV protein responds to the altered redox conditions. In addition, it is known 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). In order to test this for REV, we made Redox-DPI-ELISAs in which the *E. coli* protein crude extract was previously reduced or oxidized by the addition of Dithiothreitol (DTT) or hydrogen peroxide (H_2O_2). A possible altered binding

to the REV-binding site 1 (BS1) of the *WRKY53* promoter was tested in this assay. REV showed enhanced binding under reducing conditions, whereas under oxidizing conditions DNA-binding was reduced. Subsequently, we performed *in vitro* gel retardation assays and used purified REV protein in the presence of reducing or oxidizing conditions, and could confirm the result of the Redox-DPI-ELISA. These results contradict the finding that upregulation of *WRKY53* under oxidative conditions requires REV and is driven by the increasing hydrogen peroxide levels during onset of senescence indicating a more complex regulatory mechanism. One possible explanation is that REV is a redox-sensitive transcription factor that regulates, among others, transcriptional repressors. Reduced DNA binding of REV to these genes under oxidative conditions leads to weaker expression and thus to a reduction in repressive activity on their targets. Taken together the results show that REV is a redox-sensitive transcription factor and its binding activity to the promoter of *WRKY53* can be modulated by the intracellular redox state.

All results addressing the *WRKY53* and senescence regulation by REVOLUTA are described in detail in the following publication:

Xie, Y., Huhn, K., Brandt, R., **Potschin, M.**, Bieker, S., Straub, D., Doll, J., Drechsler, T., Zentgraf, U., Wenkel, S. (2014). "REVOLUTA and WRKY53 connect early and late leaf development in Arabidopsis." Development **141**(24): 4772-4783.

4.6 Conclusion

This work shows three new upstream regulators of *WRKY53* which are also involved in the senescence process. The network around WRKY53 appears to be extremely complex because there are many levels of regulation, involving: transcriptional cross-regulations, auto regulatory feedback loops, post-translational modifications like oxidation and protein-protein interactions. All this integrates more signals into the network around WRKY53. However, the WRKY network is only a small part of the entire senescence network and a better understanding of this single network will help to understand the whole network in the future.

A schematic model brings together the new insights we have gained from the results of this work (Figure 5). WRKY18 and WRKY25 are negative regulators of senescence; REV and WRKY53 are positive senescence regulators. WRKY18, WRKY25, and WRKY53 are part of the WRKY network with a strong mutual regulation. WRKY18

regulates its own expression and that of *WRKY53* negatively, WRKY25 regulates *WRKY53* positively and its own expression negatively. For WRKY53, we were able to show overall positive transcriptional regulatory activity, also a positive auto regulatory feedback loop. REV regulates *WRKY53* positively. In addition, we are beginning to understand more about the importance of hydrogen peroxide (H_2O_2) in the senescence process. For senescence signalling of H_2O_2 the subcellular compartment of its production appears to play a role. Thus, the cytoplasmic H_2O_2 seems to be more effective in the induction of senescence than the peroxisomal or mitochondrial H_2O_2 (Bieker et al., 2012; Zentgraf et al., 2012). Besides, an immense rearrangement of the transcriptome takes place at the beginning of senescence, which reflects the importance of the transcription factors. ROS such as H_2O_2 are one way of maintaining directly an altered activity of the transcription factors and thus changing the entire transcriptome. Many senescence-associated transcription factors, like WRKY18, WRKY25, WRKY53 and REV transcriptionally respond to elevated levels of H_2O_2. For WRKY25 and REV we were able to show that their activity depends on the intracellular redox state. Additionally, REV and WRKY25 are required for full *WRKY53* response to H_2O_2 and WRKY25 seems to be part of the H_2O_2 scavenging signalling as shown by us. A redox-sensitive action has also been shown for transcription factors of other families, like for HSFA8, a heat stress transcription factor (HSF). HSFA8 is translocated from the cytosol to the nucleus after treatment of protoplasts with H_2O_2 (Giesguth et al., 2015). Furthermore, GBF1-interacting protein 1 (GIP1) is involved in the formation of DNA-protein complexes of G-group bZIPs in its reduced form, whereas oxidized GIP1 emerges from these complexes and resumes its chaperone function (Shaikhali et al., 2015). In general, redox changes can lead to altered DNA-binding with a possible change in transactivation activity, or to altered intracellular localization, or to altered protein-protein interactions or to proteolytic degradation (He et al., 2018). This may be an interesting point for further investigation in order to better understand this complex network regulation.

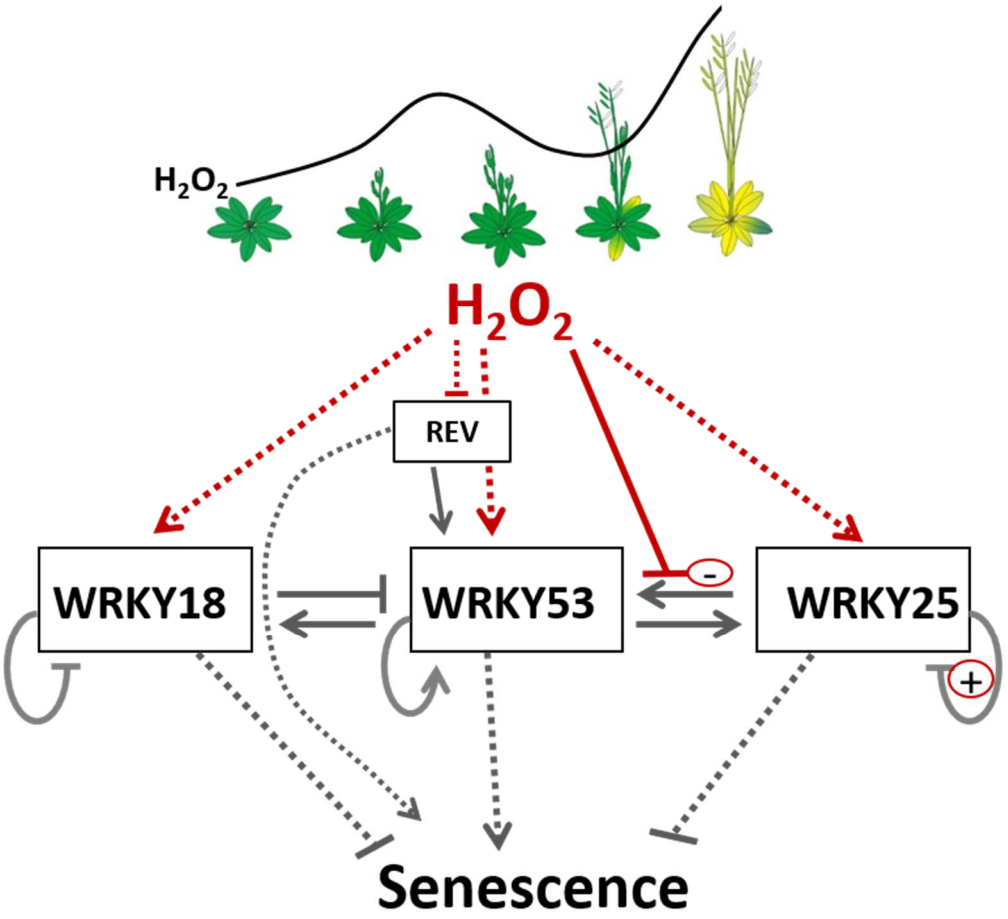

Figure 5: A model summarising the results of this work. Solid lines show direct interactions, dotted lines interactions that may be direct or indirect. All WRKYs and REV participate in the senescence process. There is a mutual regulation for the WRKYs, whereby at present for REV only a participation in the *WRKY53* regulation could be shown. Hydrogen peroxide participates as an important signal molecule for all WRKYs and REV, highlighted by the red colour; its two concentration peaks are shown above.

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6 Publications

6.1 Potschin, M., Schlienger, S., Bieker, S., Zentgraf, U. (2014). "Senescence networking: WRKY18 is an upstream regulator, a downstream target gene, and a protein interaction partner of WRKY53." Journal of Plant Growth Regulation 33(1): 106.

Personal contributions:

In this work, I performed most of the experiments. The Yeast-split-ubiquitin assays for the protein-protein interactions were carried out by Silke Schlienger as part of her diploma thesis and supervised by me. The FRET-FLIM analyses as verification for these Yeast-split-ubiquitin assays were done together with Stefan Bieker. Here, the sample preparation was carried out by me, the microscopic measurements together with Stefan Bieker. Experimental design of the paper was done together with Ulrike Zentgraf and writing of the manuscript was realised by Ulrike Zentgraf.

6.2 Doll, J.*, Muth, M.*, Riester, L., Nebel, S., Bresson, J., Lee, H-C., Zentgraf, U. (2020). "*Arabidopsis thaliana WRKY25* transcription factor mediates oxidative stress tolerance and regulates senescence in a redox-dependent manner." Frontiers in Plant Science 10:1734.

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Personal contributions:

In the present work, Jasmin Doll and I contributed equally to this work. All ELISAs and GUS-Assays were carried out by me, except for the GUS-Assays co-transformed with 35S:*MEKK1*, these were done by Lena Riester. The senescence phenotypes with the corresponding qRT-PCRs were mainly performed by Jasmin Doll and partly by me, however the ones concerning MEKK1 were done by Lena Riester. The experiments relating to H_2O_2 tolerance and gene expression under oxidizing conditions were carried out by Jasmin Doll. Statistics for all experiments were done by Jasmin Doll, Justine Bresson and Lena Riester. Experimental design was done by Ulrike Zentgraf, Jasmin Doll and me. A first draft of the first version of the manuscript was written by

myself, writing of the second version of the manuscript was realised by Ulrike Zentgraf.

6.3 Xie, Y., Huhn, K., Brandt, R., Potschin, M., Bieker, S., Straub, D., Doll, J., Drechsler, T., Zentgraf, U., Wenkel, S. (2014). "REVOLUTA and WRKY53 connect early and late leaf development in Arabidopsis." Development 141(24): 4772-4783.

Personal contributions:
In this article, I performed the Redox-DPI-ELISA to test whether *WRKY53* is redox-regulated by REVOLUTA.

6.4 Llorca, C. M.*, Potschin, M.*, Zentgraf, U. (2014). "bZIPs and WRKYs: two large transcription factor families executing two different functional strategies." Frontiers in Plant Science 5: 169.

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Personal contributions:
Carles Marco Llorca and I contributed equally to this work and the section about the WRKYs was written by me. The manuscript was prepared and discussed with Ulrike Zentgraf and Carles Marco Llorca.

6.5 Bieker, S., Potschin, M., Zentgraf, U. (2018). "Study of Hydrogen Peroxide as a Senescence-Inducing Signal." Plant Senescence, Springer: 173-193.

Personal contributions:
In this article, I have performed measurements of lipid peroxidation and chlorophyll content of *wrky53* plants in comparison to wild type plants.

6.6 Attachment of articles

1. **Potschin, M.**, Schlienger, S., Bieker, S., Zentgraf, U. (2014). "Senescence networking: WRKY18 is an upstream regulator, a downstream target gene, and a protein interaction partner of WRKY53." Journal of Plant Growth Regulation **33**(1): 106.

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Senescence Networking: WRKY18 is an Upstream Regulator, a Downstream Target Gene, and a Protein Interaction Partner of WRKY53

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Abstract Transcriptional reprogramming is a central feature of senescence regulation, implying an essential role for transcription factors. A regulatory function has already been attributed to different members of the plant-specific NAC and WRKY families in *Arabidopsis* but also in other plant species. WRKY53 is one important senescence regulator of the *Arabidopsis* WRKY family that is tightly regulated on different levels. In this study we show that WRKY18, which was formerly characterized as a downstream target of WRKY53 in the WRKY network, also regulates the expression of *WRKY53*. WRKY18 is able to bind directly to different W-boxes in the *WRKY53* promoter region and to repress expression of a *WRKY53* promoter-driven reporter gene in a transient transformation system using *Arabidopsis* protoplasts. Consistent with its repressing function on *WRKY53* as a positive senescence regulator, *WRKY18* overexpression led to delayed senescence, whereas *wrky18* mutant plants exhibited a clearly accelerated senescence. In addition, a direct interaction between WRKY53 and WRKY18 proteins could be detected in yeast using the split ubiquitin system and *in planta* in transiently transformed tobacco epidermal cells via FRET-FLIM. In contrast to WRKY18/18 homodimers, WRKY18/53 heterodimers positively influenced *WRKY53* promoter-driven reporter gene expression but appear to act only on a shorter 1.1 kbp promoter fragment but not on a 2.8 kbp longer fragment, indicating a more complex protein-protein-DNA interaction on the longer *WRKY53* promoter, most likely also triggered by the accessibility of the promoter on the chromatin level.

Keywords WRKY transcription factors · Senescence · DPI-ELISA · Feedback regulation · Yeast split ubiquitin · FRET-FLIM

Introduction

Senescence is associated with massive changes in the transcriptome, implying an important role for transcription factors (Buchanan-Wollaston and others 2005; Breeze and others 2011). The two plant-specific transcription factor families WRKY and NAC are both overrepresented in the senescence transcriptome of *Arabidopsis*, and several members of both families have already been characterized as playing important roles in senescence regulation, not only in *Arabidopsis* but also in other plant species (Guo and others 2004; Uauy and others 2006; Ulker and others 2007; Balazadeh and others 2010, 2011; Besseau and others 2012; Gregersen and others 2013). The *Arabidopsis* WRKY transcription factor family consists of at least 75 members playing diverse biological roles in plant growth, development, and responses to biotic and abiotic stress. The name is derived from the almost invariant WRKYGQK sequence at the N-terminus, which is followed by a more variable zinc-finger motif. The WRKY proteins have been grouped into three subgroups according to their structural features (Eulgem and others 2000; Rushton and others 2010); nevertheless, almost all analyzed WRKY proteins recognize the TTGACC/T W-box sequence. W-boxes are found in many promoters of senescence- and pathogen-associated genes (SAGs and PR

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genes), but also in almost all *WRKY* promoters, indicating that a WRKY transcriptional network exists. Besides regulating transcription of each other, WRKY factors are also able to form heterodimers leading to a change in DNA-binding specificity (Xu and others 2006). In addition, many other proteins have been shown to physically interact with WRKY proteins, influencing their activity and stability (see Chi and others 2013 for review). A firm link between plant-specific NAC and WRKY proteins was deduced from low-resolution X-ray structures and small-angle X-ray scattering on complexes in the presence of DNA in which both use a β-strand motif for DNA binding (Welner and others 2012). Moreover, family members of both groups have been shown to react on elevated levels of reactive oxygen species, especially hydrogen peroxide, and are even involved in regulating the intracellular levels of these molecules (Miao and others 2004; Balazadeh and others 2011; Besseau and others 2012; Wu and others 2012).

Within the WRKY family, several factors have already been associated with senescence, namely, WRKY6 (Robatzek and Somssich 2002), WRKY70 (Ülker and others 2007; Besseau and others 2012), WRKY54 (Besseau and others 2012), WRKY22 (Zhou and others 2011), and WRKY53 (Hinderhofer and Zentgraf 2001; Miao and others 2004). Senescence-specific regulation of WRKY53 has already been analyzed in detail. The *WRKY53* gene shows a very interesting expression pattern, with a switch from leaf-age-dependent to a plant-age-dependent expression during bolting and flowering (Hinderhofer and Zentgraf 2001). Expression is most likely switched by increasing hydrogen peroxide levels at this time point (Miao and others 2004; Bieker and others 2012). Furthermore, epigenetic changes can also be observed at the promoter of *WRKY53* during leaf senescence induction in which histone modifications H3K4me2 and H3K4me3 are specifically enriched at the *WRKY53* promoter region (Ay and others 2009; Brusslan and others 2012), whereas DNA methylation remains low and unchanged (Zentgraf and others 2010). A transcriptional activator with homology to a HPT kinase (AD protein) was characterized to be one of the upstream regulatory proteins of WRKY53 (Miao and others 2008). Moreover, a mitogen-activated protein kinase kinase kinase (MEKK1) directly binds to the *WRKY53* promoter region, which is responsible for the switch from leaf-age- to plant-age-dependent expression (Miao and others 2007). However, MEKK1 does not directly activate transcription but acts most likely through phosphorylation of other promoter-associated proteins. One of these proteins could be WRKY53 itself sitting at its own promoter since, at least in vitro, MEKK1 is able to directly phosphorylate WRKY53 and thereby increase its DNA-binding activity (Miao and others 2004, 2007). In contrast, the AD protein could not be phosphorylated by MEKK1 (Miao and others 2008). The DNA-binding activity of WRKY53 is also triggered by the interaction with ESP/ESR, but in this case interaction leads to inhibition of DNA binding (Miao and Zentgraf 2007). In addition, WRKY53 protein levels are controlled by degradation through the HECT-domain ubiquitin ligase UPL5 (Miao and Zentgraf 2010).

In this study we analyzed which WRKY factors interact directly with the *WRKY53* promoter and what impact these factors have on the expression of *WRKY53*. One of the strongest interactions was detected between the *WRKY53* promoter and WRKY18, which was formerly characterized to be a downstream target of WRKY53 (Miao and others 2004). In a transient transformation assay of *Arabidopsis* protoplasts, WRKY18 could be characterized as a negative regulator of the *WRKY53* promoter-driven reporter gene expression. In consistence, a *WRKY18* T-DNA insertion line exhibited accelerated senescence, whereas a *WRKY18* overexpressing line showed a delay. Because both proteins can bind to *WRKY53* promoter elements, we analyzed whether the proteins can also form heterodimers. Protein-protein interaction between WRKY18 and WRKY53 was observed in yeast using the split ubiquitin system and in transiently transformed tobacco leaves using FRET-FLIM. Cotransformation of *WRKY18* and *WRKY53* overexpression constructs in *Arabidopsis* protoplasts together with a *WRKY53* promoter-driven reporter gene revealed that heterodimers resulted in a different outcome, as expected if the single effects of both factors were simply combined.

Material and Methods

Plant Material

Arabidopsis thaliana plants were grown in a climatic chamber at 20 °C under long-day conditions (16 h of light) with only moderate light intensity (60–100 μmol s^{-1} m^{-2}) to slow down development for better phenotyping. Under these conditions, the plants developed bolts and flowers within 5–6 weeks. During growth and development, the positions of individual leaves within the rosette were color-coded with different colored threads so that even at very late stages of development individual leaves could be analyzed according to their age (Hinderhofer and Zentgraf 2001). Plants were harvested in a weekly rhythm and samples were always taken at the same time in the morning to avoid circadian effects. T-DNA insertion lines of *WRKY18* (SALK_093916C) were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). Homozygous plants were characterized by PCR using gene-specific and T-DNA left border primers. Plants overexpressing *WRKY18* were transformed by a floral dip of Col-0 plants into *Agrobacterium tumefaciens* cultures carrying a

35S:*WRKY18* construct and subsequent selection of overexpressing plants by BASTA selection and qRT-PCR.

Senescence Phenotyping

Chlorophyll content was estimated using an atLeaf+ chlorophyll meter. Each leaf was measured in triplicate and values were averaged. For the evaluation of leaf senescence phenotypes, rosettes were scanned upside down for better visualization of the older leaves and leaves were sorted according to their age using a color code. In addition, leaves of at least five plants were categorized into four groups according to their leaf color: (1) "green"; (2) "yellow-green," leaves starting to get yellow from the tip; (3) "yellow," completely yellow leaves; and (4) "brown/dry," dry and/or brown leaves. Furthermore, expression of the senescence-associated marker genes *SAG12* (At5g45890), encoding a cysteine protease, and *SAG13* (At2g29350), encoding a short-chain alcohol dehydrogenase, was analyzed by qRT-PCR and normalized to the expression of the *ACTIN2* gene (At3g18780). In addition, expression of *WRKY53* (At4g23810) and *WRKY18* (At4g31800) were analyzed in the same way.

Protein Expression and Extraction for DPI-ELISA

The coding sequences of *WRKY18* and *WRKY53* and several other *WRKY*s were cloned into the pETG-10A vector for expression of the proteins with N-terminal-fused 6 × His-tag. The *E. coli* strain BL21-SI was used for protein expression. The cells were grown in 10 ml of selective medium overnight and subsequently diluted 1:20 in a final volume of 100 ml in medium without antibiotics. Protein expression was induced after 1.5 h by the addition of 1 mM IPTG and the cells were grown overnight at 18 °C. After centrifugation (2,500 g, 20 min, 4 °C) and washing [10 mM Tris-HCl (pH 7.5) and 100 mM NaCl], the bacterial pellet was resuspended in protein extraction buffer [4 mM Hepes (pH 7.5), 100 mM KCl, 8 % (v/v) glycerol, 1× complete proteinase inhibitor without EDTA (Roche)] and protein extraction was performed by sonication under native conditions. The protein concentration of the crude extract was measured by Bradford assay (Bio-Rad).

DPI ELISA

The DNA-protein interaction assay was performed basically as described by Brand and others (2010). 5′-Biotinylated complementary oligonucleotides were annealed (final concentration 2 µM) to get double-stranded DNA fragments. The sequences of the individual fragments containing different W-boxes are listed in Fig. 1a. These double-stranded oligonucleotides were added to a streptavidin-coated ELISA plate (Nunc Immobilizer) for binding for 1 h at 37 °C. After blocking using blocking reagent (Roche) for 30 min, the blocking reagent was removed and crude extracts were added in different protein concentrations (5, 10, and 25 µg) and incubated for 1 h at room temperature. Subsequently, biotinylated DNA-protein complexes were washed two times for 10 min at room temperature (blocking solution, Qiagen) and incubated with anti-His-HRP conjugate antibodies (Qiagen) 1:1,500 diluted in blocking solution for 1 h at room temperature. After washing, interaction was detected by a peroxidase reaction with ortho-phenylenediamine [OPD tablets, Agilent Technologies (Dako)] for 15 min in darkness. After stopping the reaction with 0.5 M H_2SO_4 solution, positive interactions that resulted in a yellow color could be measured with an ELISA reader (TriStar LB 941 plate reader, Berthold).

Real-time PCR

mRNA extraction from pooled leaves (leaf No. 5) of five different plants per plant line and time point was performed using the chemagic mRNA Direct Kit (chemagen). Subsequent cDNA synthesis was done with qScript™ cDNA SuperMix Kit (Quanta BioSciences). For the qRT-PCR, the iQ™ SYBR® Green Supermix (Bio-Rad) was used following the manufacturer's protocol. *ACTIN2* was chosen as the reference gene for senescence because the variation of *ACTIN2* expression over different leaf and plant stages in *Arabidopsis* was very low in contrast to other housekeeping genes (Panchuk and others 2005). Expression of analyzed genes was normalized to *ACTIN2* expression according to Pfaffl (2001). Each value represents three technical replicates of a pool of five biological replicates (Table 1).

Protoplast Transformation

The transient expression assays were performed by transforming protoplasts derived from a cell culture of *Arabidopsis thaliana* var. Columbia 0. Cells were transformed with 5 µg of effector and reporter plasmid DNA each, roughly following the protocol of Negrutiu and others (1987) (for details see the protocol at http://www.zmbp.uni-tuebingen.de/CentralFacilities/transf/index.html). Protoplasts were used for GUS assays or FRET-FLIM analyses.

Tobacco Leaf Infiltration

Agrobacteria tumefaciens cells (strain GV3101 RK) were transformed with 35S constructs carrying the coding sequences of *WRKY18* and *WRKY53* fused N-terminal to *CFP* or *YFP* coding sequences. *Agrobacteria* were grown overnight at 28 °C in 5 ml of LB medium containing the

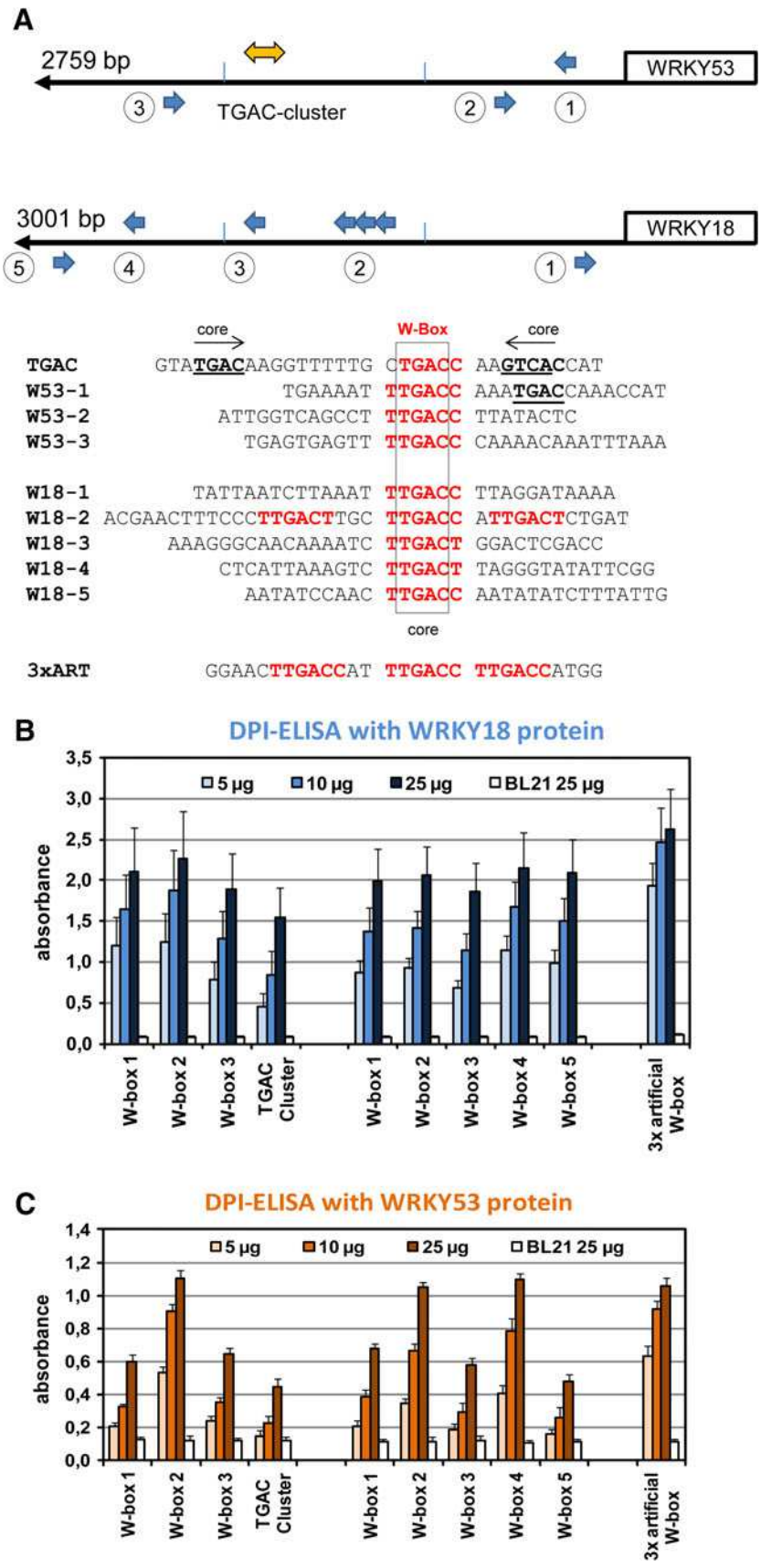


Fig. 1 **a** Schematic drawing of the W-boxes in the *WRKY53* and *WRKY18* promoter and sequence comparison of the DNA fragments used for DPI ELISAs. Perfect W-box motifs are highlighted in *red*; the core sequence TGAC is indicated in *bold* and *underlined*, and the direction of the motif is indicated by the *arrows*. An artificial sequence containing three perfect W-boxes was used as a positive control (3× ART). **b** Quantification of DPI-ELISAs performed with different amounts of crude extracts of *E. coli* BL21 cells expressing WRKY18 proteins combined with different biotinylated DNA fragments containing different W-boxes of the *WRKY53* and *WRKY18* promoter. **c** Quantification of DPI-ELISAs with different amounts of crude extracts of *E. coli* BL21 cells expressing WRKY53 proteins combined with different biotinylated DNA fragments containing different W-boxes of the *WRKY53* and *WRKY18* promoter. *Error bars* indicate standard deviation of three biological and two technical replicates

Table 1 Primers used for expression analyses by qRT-PCR

Gene	Forward primer	Reverse primer
WRKY53	5′-CAGACGGGGATGCTACGG-3′	5′-GGCGAGGCTAATGGTGGT-3′
SAG12	5′-GCTTTGCCGGTTTCTGTTG-3′	5′-GTTTCCCTTTCTTTATTTGTGTTG-3′
SAG13	5′- GTGCCAGAGACGAAACTC-3′	5′-GCTGTAAACTCTGTGGTC-3′
WRKY18	5′-TGGACGGTTCTTCGTTTCTCGAC-3′	5′-TCGTAACTCACTTGCGCTCTCG-3′
ACTIN2	5′-AAGCTCTCCTTTGTTGCTGTT-3′	5′-GACTTCTGGGCATCTGAATCT-3′

antibiotics of the strain and the plasmid. The preculture was transferred to 20 ml of LB medium supplemented with antibiotics for the plasmid only and grown further overnight at 28 °C. After centrifugation (4,000 rpm, 10 min) the pellet was resuspended in H_2O to an $OD_{600} = 0.8$. These cultures were combined to equal amounts with *Agrobacteria* cultures carrying p19 silencing inhibitor before infiltration. The *Agrobacteria* mixture was applied to the tobacco leaf using a syringe. After infiltration, plants were grown for 3–4 days before analyses of interaction using FRET-FLIM.

GUS Reporter Assay

Arabidopsis protoplasts were transformed as described above. A luciferase construct was cotransfected as an internal control. The protoplasts were incubated overnight in the dark and then used for GUS enzyme activity assays as described by Jefferson and others (1987). GUS fluorescence values were normalized to luciferase fluorescence to correct for transformation efficiency. A 1,099 bp fragment and a 2,759 bp fragment upstream of the *WRKY53* start codon were cloned into the binary vector pBGWFS7.0 and served as reporter construct. For *WRKY18*, a 1,500 bp sequence and a 3,001 bp sequence upstream of the start codon were used.

Protein-Protein Interaction via Yeast Split Ubiquitin

Protein-protein interaction was analyzed in yeast using the split-ubiquitin system of the DUALhunter kit following the manufacturer's protocol (Dualsystems Biotech, for details see http://www.dualsystems.com). The full-length cDNA of *WRKY53*, *WRKY18*, *WRKY6*, and *WRKY30* were cloned in-frame into bait vector (pDHB1) and prey vector (pPR3-N), respectively. All constructs were confirmed by sequencing. Empty vectors were used as negative controls. For the interaction analyses, the bait constructs were cotransformed with the prey constructs in the yeast strain NMY51 and grown at 30 °C for 4 days on SD media without Leu and Trp to select for transformation of both constructs. Subsequently, the yeast was transferred to quadruple dropout media without Leu, Trp, His, and Ade for analyses of protein-protein interaction. A Cub and a NubG construct were provided by the manufacturer as a negative control for the bait and prey, respectively.

Fluorescence Lifetime Imaging Microscopy

The full-length cDNAs of *WRKY18* and *WRKY53* were cloned into the pENSG-CFP:GW and pENSG-YFP:GW vectors for expression of the proteins with N-terminal-fused CFP or YFP (Wenkel and others 2006). Image and data acquisition was done with a Leica TCS SP8, combined with a PicoHarp 300 TCSPC Module and a Sepia Multichannel Picosecond Diode Laser (PDL 808-SC) (PicoQuant). Excitation was done with a 440-nm pulsed laser, with the intensity regulated via a Thorlabs Laser Combining Unit (PBH51502/SS/SPL-S6) and the emission recorded at 480 ± 25 nm. Analysis was performed using PicoQuant SymphoTime Software (ver. 5.3.2.2). The bi-exponential decay function was used for fluorescence decay analysis.

Results

To identify WRKY factors involved in the regulation of *WRKY53* expression, we cloned cDNAs of those WRKY factors that are expressed during onset of senescence according to genevestigator expression profiles (https://www.genevestigator.com/) into bacterial expression vectors fusing a 6xHis-tag to the WRKY proteins, namely, WRKY6, 13, 15, 18, 22, 25, 29, 30, 33, 38, 40 53, 60, 62, and 70. Crude extracts from *E. coli* cells expressing these proteins were used to perform DNA-protein interaction ELISAs (DPI-ELISAs) to screen for DNA binding to the *WRKY53* promoter. For WRKY18-6xHis, the strongest interaction with *cis* elements in the *WRKY53* promoter was observed; therefore, we concentrated our further analyses on the role of WRKY18 in *WRKY53* regulation.

Influence of *WRKY18* on *WRKY53* Expression

Several W-boxes or W-box-like *cis* elements can be detected in the upstream region of *WRKY53* as well as in

the upstream region of the *WRKY18* coding region (Fig. 1a). Five perfect W-box motifs of the *WRKY18* and three of the *WRKY53* promoter were used for DPI-ELISAs. In both promoters, motif clusters of at least three very closely linked motifs are present and were also used for DNA-binding studies in which the cluster in the *WRKY18* promoter consists of three perfect W-boxes and the cluster in the *WRKY53* promoter consists of only three core motifs (TGAC). Increasing amounts of crude extracts of *E. coli* BL21 cells (5, 10, and 25 µg) which were induced for expression of the 6× His-tagged versions of WRKY53 and WRKY18, respectively, were used for binding assays to the biotinylated double-stranded DNAs containing the different W-box motifs indicated in Fig. 1a. All W-box *cis* elements were bound in a concentration-dependent manner by both proteins. Both WRKY proteins showed the highest affinity to a 3× perfect but artificial W-box cluster (3× ART), which was used as positive control. Only very low background signals could be detected for extracts of empty BL21 cells, which were used as negative control. WRKY18 appears to have the same binding affinity to all perfect W-boxes in both promoters and a lower affinity to the imperfect TGAC cluster of the *WRKY53* promoter (Fig. 1b). In contrast, WRKY53 has a clear preference for W-box-2 of the *WRKY53* promoter and W-box-2 and -4 of the *WRKY18* promoter, whereas the affinity to the perfect W-box-5 of the *WRKY18* promoter is as low as it is to the imperfect TGAC cluster of the *WRKY53* promoter, indicating that WRKY53 appears to be more selective in DNA binding (Fig. 1c). However, no obvious further conservation in the surrounding DNA sequences of the preferred W-box motifs, which might be responsible for further selectivity, could be detected.

To investigate which effect *WRKY18* has on the expression of *WRKY53* and vice versa, transient cotransformation of *Arabidopsis* protoplasts with *WRKY53* and *WRKY18* promoter:*GUS* and 35S:*WRKY18* and 35S:*WRKY53* overexpression effector constructs was performed. *WRKY18* expression was activated by overexpression of *WRKY53* in protoplasts. This was already expected because in earlier experiments performed for *wrky53* and 35S:*WRKY53* plants (Miao and others 2004), expression of *WRKY18* was also activated by overexpression of *WRKY53* and reduced by the lack of a functional *WRKY53* protein in the *wrky53* mutant. In contrast, expression of *WRKY53* was inhibited by WRKY53 itself when only a short promoter fragment of 1 kb was used for the transient *GUS* expression experiments (Miao and others 2004). However, if a slightly longer fragment of 1.1 kb was used containing one additional W-box-like element, the negative effect was eliminated and was reversed into a slightly activating potential (Fig. 2). If the longer 2.8 kb fragment was used, an even more pronounced induction could be observed for the *WRKY53* promoter-

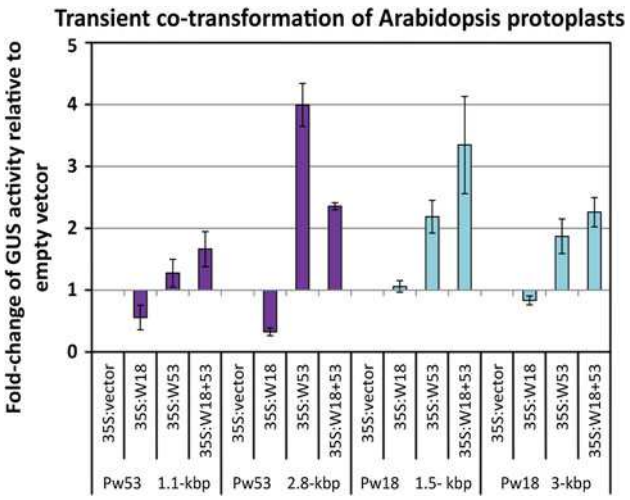


Fig. 2 *Arabidopsis* protoplasts were transiently transformed with 5 µg of effector and reporter plasmid DNA each, and a luciferase construct was cotransfected as an internal control. GUS fluorescence values were normalized to luciferase fluorescence to correct for transformation efficiency. A 1.1- and a 2.8-kb fragment of the *WRKY53* promoter and a 1.5 and a 3.0 kbp sequence of the *WRKY18* promoter fused to the *GUS* gene were used as reporter constructs. 35S:*WRKY18* and 35S:*WRKY53* constructs were used as effector plasmids. Values of empty vector construct were set to 1. *Error bars* indicate standard deviations of at least three biological and three technical replicates

driven reporter gene, suggesting that most likely complex protein–protein–DNA interactions are formed on the longer promoter, changing the impact of WRKY53 on its own promoter. This might also indicate that chromatin structure and accessibility of the promoter are key elements of *WRKY53* transcriptional regulation. Overexpression of *WRKY18* did not or only marginally influenced its own expression, even though WRKY18 could bind to all W-boxes of its own promoter, indicating that binding does not automatically result in effects on gene expression and that WRKY18 has no autoregulatory potential. *WRKY53* expression was clearly inhibited by overexpressed *WRKY18*, with a stronger effect on the longer 3.0 kb fragment, clearly suggesting that WRKY18 works as a repressor on the *WRKY53* expression (Fig. 2). In contrast to protoplasts transformed with a single WRKY expression construct, the effect of double transformation was not simply additive but different from a simple combination of the single effects. This is very obvious when single transformations and double transformation of the 1.1 kb promoter fragment of the *WRKY53* promoter are compared. *WRKY18* single transformation led to a reduction of reporter gene expression, whereas *WRKY53* single transformation led to a slight induction of reporter gene expression. Simultaneous expression of both constructs led to an induction of the reporter gene expression, which was higher than that by WRKY53 alone, even though WRKY18 would rather

contribute repression than activation. A very similar effect could be observed for the 1.5 and the 3.0 kb fragment of the *WRKY18* promoter in which WRKY18 alone had no or a slightly negative effect, but double transformation of both WRKY constructs induced reporter gene expression to a higher extent than *WRKY53* single transformation. This clearly indicates that WRKY18 and WRKY53 form heterodimers and that these heterodimers have a positive activation potential. However, on the 3.0 kb *WRKY53* promoter fragment, the effect of a double transformation appears to be only additive (Fig. 2), suggesting that heterodimers are not formed in all cases or that additional and higher-order complexes can be formed on the longer promoter fragment.

Phenotype of *WRKY18* T-DNA insertion and *WRKY18* overexpression lines

To verify the negative effect on *WRKY53* expression *in planta* and test for the relevance of these regulatory cues, we used a SALK T-DNA insertion line in the first exon of *WRKY18* (SALK_093916C) which was already characterized by Xu and others (2006). The homozygous insertion line was confirmed by PCR. In addition, *WRKY18*-overexpressing plants were produced by transformation of a 35S:*WRKY18* construct using *Agrobacteria tumefaciens* and floral dip. Overexpression and severe knockdown of the *WRKY18* gene was confirmed by qRT-PCR (Supplementary Fig. 1); the line showing the highest expression of *WRKY18* was grown side by side with the *wrky18* and wild-type (WT) plants under long-day conditions for detailed senescence phenotyping. Overall development of the plants was not impaired in both lines (Supplementary Fig. 2). However, the *wrky18* mutant showed slightly earlier flowering and the number of leaves was slightly but significantly lower (9.13; *t* test, $p = 0.0168$) than in WT (10.88) and overexpressing plants (10.79).

During early development of the plants, the leaves were color-coded with colored threads according to their age. Whole rosettes in different developmental stages (5- and 7-week-old plants) were scanned upside down to visualize the older leaves (Fig. 3a). In addition, the leaves were sorted with the help of the age-based color code (Fig. 3a). Furthermore, the leaves of at least five plants were categorized into four groups according to their leaf color (fully green, green/yellow, fully yellow, and brown/dry) to assure the senescence phenotype statistically (Fig. 3b). Chlorophyll contents of leaves 3 and 5 were measured by using an atLeaf+ chlorophyll meter (Fig. 4a), and expression of the senescence marker genes *SAG12* and *SAG13* as well as *WRKY53* itself as a marker gene was analyzed by qRT-PCR (Fig. 4b). Whereas in young plants no differences in expression of the senescence marker genes *SAG12* and *SAG13* could be observed between the lines, clear differences were detected in later stages. In comparison to the wild-type plants, *WRKY18*-overexpressing plants showed a delayed senescence phenotype accompanied by a delay in chlorophyll loss and delayed and lower expression of *SAG12*, *SAG13*, and *WRKY53*. In contrast, senescence, chlorophyll degradation, and *SAG* expression were accelerated in *wrky18* mutants, clearly indicating that WRKY18 is a negative regulator of *WRKY53* expression also *in planta* and has a clear impact on senescence regulation. qRT-PCR analyses of *WRKY53* expression in the *wrky18* and *WRKY18*-overexpressing plants also confirmed the negative effect of WRKY18 on *WRKY53* expression (Fig. 4B).

Protein-Protein Interaction between WRKY18 and WRKY53

Because both WRKY proteins bind equally well to the *WRKY53* promoter and to the *WRKY18* promoter and double transformation assays did not show simple additive effects, we analyzed whether these two proteins can physically interact using the yeast split ubiquitin system. The split ubiquitin system is advantageous for studying the interactions between transcription factors compared to the yeast-two-hybrid system based on the yeast GAL4 transcription factor because no deletion variants have to be used. In the yeast split ubiquitin system, homodimers of WRKY53 as well as of WRKY18 could be detected, but both WRKYs were also able to physically interact with each other forming heterodimers. Yeast cells were selected for transformation of both constructs by plating on double-selection medium SD-Leu-Trp. These yeast cells were then transferred on quadruple-dropout medium SD-Leu-Trp-His-Ade to test for the interaction. A clear interaction between WRKY53 and WRKY18 could be detected by the growth of the yeast on the quadruple-dropout medium and the white color, as weak interactions would lead to a pink color of the growing yeast cells (Fig. 5a). Protein complex formation between different WRKYs appears to be selective because WRKY6 did not interact with WRKY53. Besseau and others (2012) have already shown that WRKY53 also did not interact with WRKY70 and WRKY54. However, we clearly detected a homodimer formation of WRKY53 and of WRKY18 which was not observed previously (Besseau and others 2012). To verify these interactions *in planta*, we used a CFP/YFP Foerster resonance energy transfer (FRET) with subsequent fluorescence lifetime imaging microscopy (FLIM) analyses in transiently transformed tobacco leaves and *Arabidopsis* protoplasts. An interaction of two proteins is indicated by a reduction of the fluorescence lifetime of CFP after FRET from CFP to YFP. A strong interaction between WRKY53 and WRKY18 could be confirmed by FLIM in epidermal cells of tobacco leaves (Fig. 5b, c). Additional

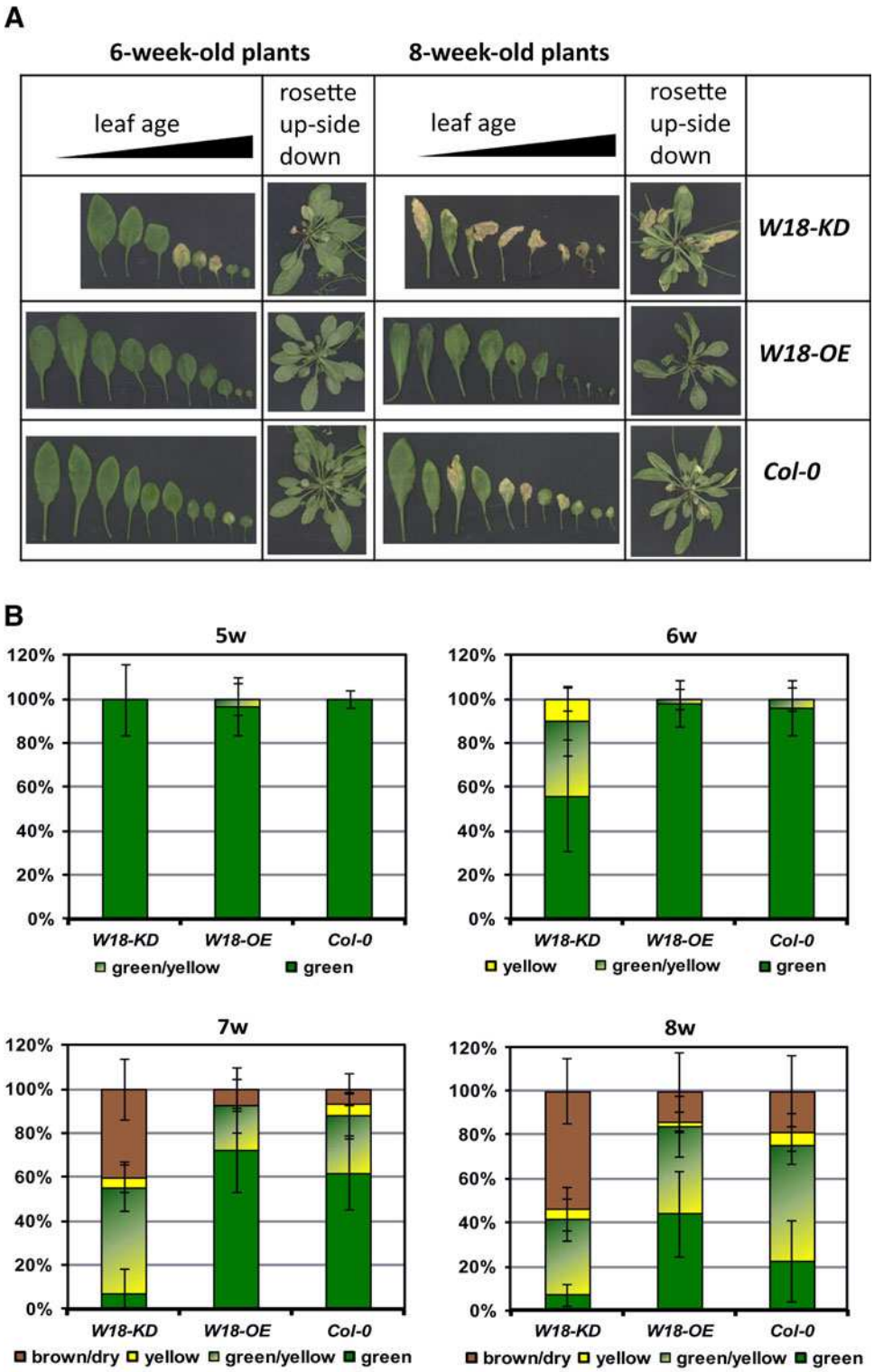


Fig. 3 Wild type (Col-0), *wrky18* mutant, and *WRKY18*-overexpressing plants were analyzed over development. **a** Rosette leaves of 6- and 8-week-old plants were sorted according to their age; whole rosettes were photographed from upside down to visualize also the older leaves. **b** For a quantitative evaluation of leaf senescence, plant leaves of at least five plants were categorized into four groups according to their leaf color: (1) "green"; (2) "yellow-green," that is, leaves starting to get yellow from the tip; (3) "yellow," completely yellow leaves; and (4) "brown/dry," dry and/or brown leaves. The percentages of each group with respect to total leaf numbers are presented. *Error bars* indicate standard deviation of at least five plants

confirmation of the homodimer formation of WRKY53 and WRKY18 could be achieved in tobacco (Fig. 5c) and in the *Arabidopsis* protoplast system (Supplementary Fig. 3). As already suggested by the results shown in Fig. 2, heterodimers are indeed formed *in planta* and they appear to have different effects on transcription than the homodimers. The complex cross-regulation between WRKY18 and WRKY53 is summarized in a model shown in Fig. 6.

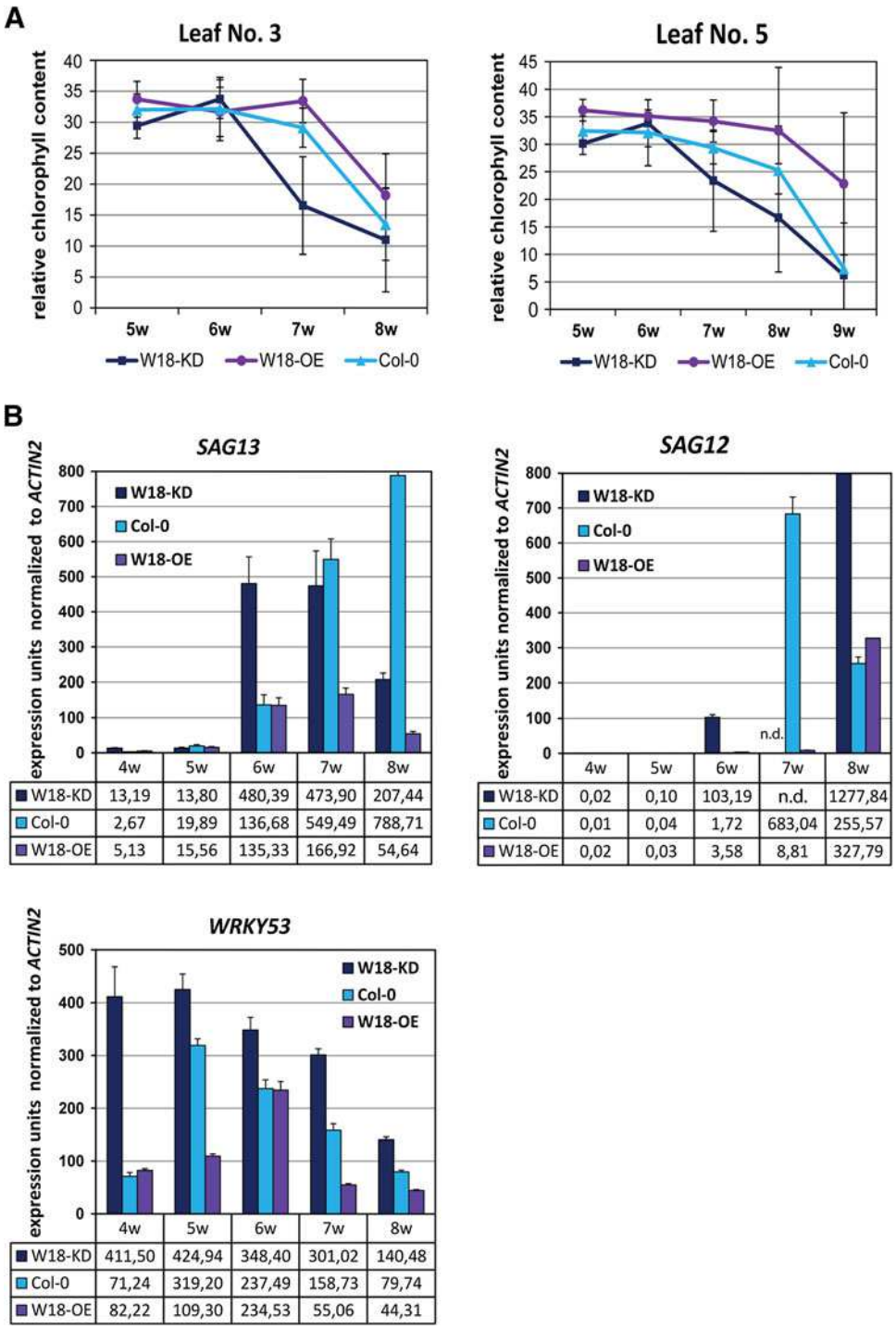
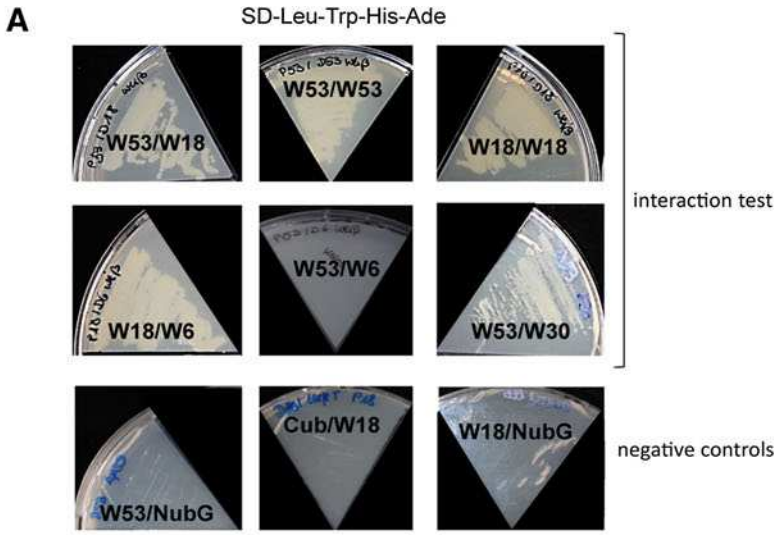


Fig. 4 **a** Chlorophyll values of leaves No. 3 and 5 were measured from 5- to 8-week-old plants of wild-type (Col-0), *wrky18* mutant, and *WRKY18*-overexpressers using an atLeaf+ chlorophyll meter. *Error bars* indicate standard deviation. **b** qRT-PCR expression analyses of the senescence marker genes *SAG12*, *SAG13*, and *WRKY53* normalized to *ACTIN2* according to Pfaffl (2001). Pools of leaf No. 5 of 4- to 8-week-old plants of five biological replicates were analyzed. *Error bars* indicate standard deviation of three technical replicates. *n.d.* not determined

Discussion

Senescence is characterized by a massive transcriptional reprogramming (Breeze and others 2011) to assure reorganization for reallocation of nutrients and minerals out of senescing tissue into developing parts of the plants such as fruits and seeds. This implies an important role for transcription factors, and NAC and WRKY transcription factors have a significant impact in *Arabidopsis* but also in other plant species. WRKY transcription factors can influence transcription of their target genes positively as well as negatively and are also able to regulate transcription of each other in a complex regulatory network. Besides senescence, WRKY transcription factors are involved mainly in plant responses to biotic and abiotic stress conditions and are often discussed as a node of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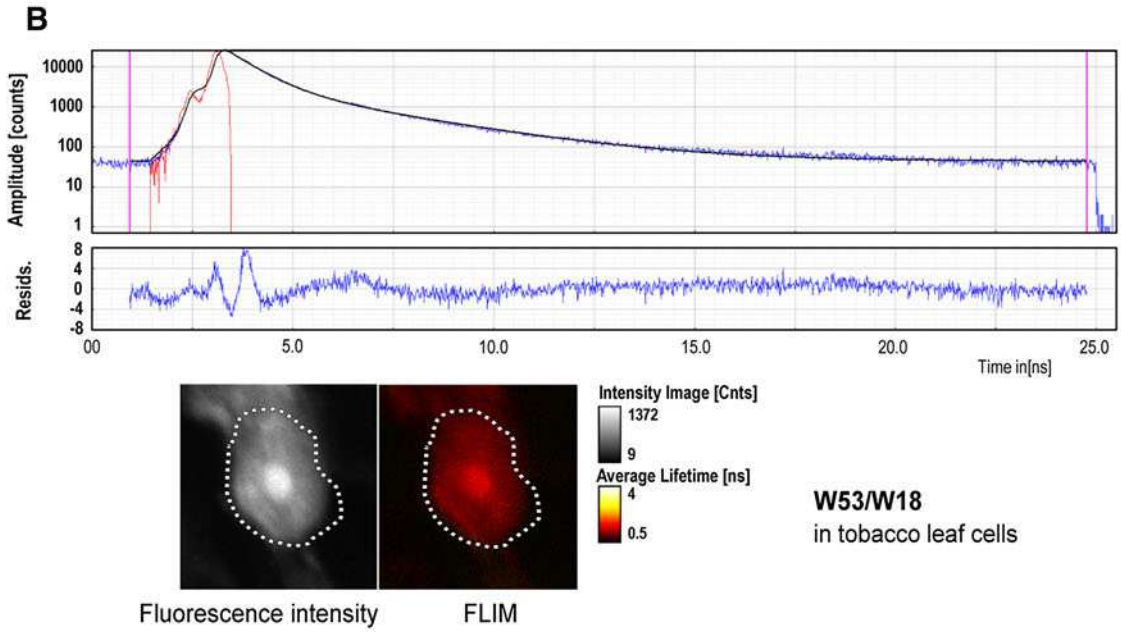
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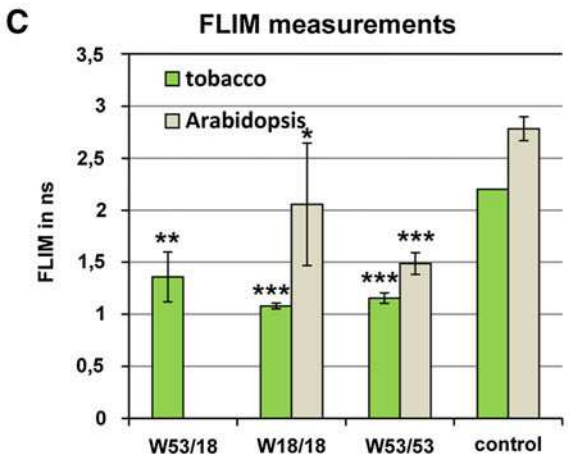
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◀ **Fig. 5** Protein–protein interaction between WRKY53 and WRKY18. **a** Yeast split ubiquitin system. Yeasts were cotransformed using prey-and-bait constructs of different WRKY factors indicated in the figure and were grown on selective medium SD-Leu-Trp to select for cotransformation. Subsequently, yeast cells were transferred to SD-Leu-Trp-His-Ade medium to analyze protein-protein interaction by growth of the yeasts. White colonies indicate strong interaction and pink colonies indicate weaker interaction. To show selectivity, WRKY6 was used interacting only with WRKY18 but not with WRKY53. Cub and NubG were provided by the manufacturer and were used as a negative control for the bait and prey, respectively. **b** Protein-protein interaction in transiently transformed tobacco epidermal leaf cells using FRET-FLIM analyses. In the upper graph, the *red line* describes the instrument response function, the *blue line* describes the actual decay, and the *black line* describes the bi-exponential fit. The *blue line* in the lower graph describes the residuals. Fluorescence lifetime is indicated by a color code reaching from *dark red* (very short decay times, ~0.5 ns) to yellow (long decay times, ~4.5 ns). The position of the nucleus is indicated by the dotted line. **c** Fluorescence lifetime was measured in *Arabidopsis* protoplasts and transiently transformed tobacco leaves for the WRKY53/53 homodimer, the WRKY18/18 homodimer, and the WRK53/18 heterodimer. Empty CFP and YFP vectors were used as control. *Error bars* indicate standard deviation of two to five replicates, $n = 2$–6; *n.d.* not determined. *t* tests have been performed and * indicated significance compared to control: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

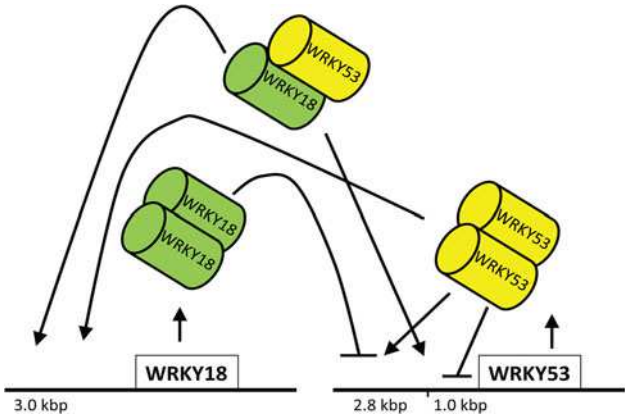

Fig. 6 Schematic drawing of the cross-regulation of WRKY18 and WRKY53

convergence between stress response and senescence. WRKY18 was initially characterized as being involved in the response of plants to pathogen attack because transgenic plants overexpressing *WRKY18* showed a marked increase in the expression of PR genes and resistance to the bacterial pathogen *Pseudomonas syringae* (Chen and Chen 2002). Potentiation of developmentally regulated defense responses by WRKY18 was not associated with enhanced biosynthesis of salicylic acid but required the disease resistance regulatory protein NPR1. In addition, WRKY18 together with WRKY40 negatively modulated the expression of positive regulators of defense such as CYP71A13, EDS1, and PAD4, but positively modulated the expression of some key JA-signaling genes by partly suppressing the expression of JAZ repressors (Pandey and others 2010). Besides the response to pathogen attack, WRKY18, 40, and 60 are involved in abiotic stress response to salt and osmotic stress and in ABA signaling. Both WRKY18 and WRKY40 are rapidly induced by ABA, and a delayed response of WRKY60 argues that *WRKY60* might be a direct target gene of WRKY18 and WRKY40 in ABA signaling. The requirement of both WRKY18 and WRKY40 for induction of WRKY60 suggests an involvement of the WRKY18/WRKY40 heterodimers that may recognize the W-boxes in the *WRKY60* promoter and activate the expression of the *WRKY70* gene (Chen and others 2010). Moreover, an indispensable role for WRKY18 in bacterial volatile responses was described (Wenke and others 2012). Wang and others (2006) took a genomics-directed approach and positioned the five group III WRKY factors 18, 53, 54, 58, and 60 in the complex transcriptional regulatory network of systemic acquired resistance (SAR). During SAR, salicylic acid (SA) accumulation triggers nuclear localization of the transcription factor NPR1, leading to the activation of WRKY transcription. *WRKY18*, *53*, *54*, *58*, and *60* are activated in this SA-mediated response pathway in which WRKY54 and 60 are also involved in a feedback loop in the regulation of SA production (Wang and others 2006). Remarkably, also in SAR, WRKY18 and WRKY53 act in the same regulatory cue.

WRKY53, a factor in group III of the WRKY family according to structural features, was characterized as a positive regulator of leaf senescence in *Arabidopsis* and appears to be very tightly regulated (Zentgraf and others 2010). Other group III factors like WRKY54 and WRKY70 cooperate as negative regulators of leaf senescence in *Arabidopsis* (Besseau and others 2012). Analyses of the SA-deficient *sid2* mutant revealed that expression of WRKY30, 53, 54, and 70 during senescence is partially SA-dependent. In addition to SA, WRKY30 and WRKY53 can be induced by H_2O_2 and both factors are tightly coexpressed during development. In contrast to *wrky53* mutant plants, miRNA-*WRKY30*-silenced plants displayed no significant senescence phenotype compared to wild-type plants (Miao and others 2004; Besseau and others 2012), indicating that WRKY30 does not directly regulate senescence. However, WRKY30 can form heterodimers with all other tested group III WRKYs, and one can speculate that WRKY30 acts as an integrator of WRKY function. In this study, we could show that not only group III factors are involved in cross-talk and feedback regulation of leaf senescence; the group IIa factor WRKY18 is also involved in the WRKY network regulation of senescence. *WRKY18* overexpression and knockdown clearly revealed an inhibitory function of WRKY18 on senescence. WRKY18 can

directly bind to different W-boxes in the *WRKY53* promoter so that senescence regulation is most likely mediated at least in part by *WRKY53* regulation. Comparison of the gene expression pattern according to the developmental map of the *Arabidopsis* eFP-Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) reveals that *WRKY18* is highly expressed in young leaves with a decrease toward old and senescence leaves, whereas *WRKY53* is expressed antagonistically, with its highest expression in senescence leaves and low expression in young leaves. Genevestigator profiles (https://www.genevestigator.com) and our own qRT-PCR analyses revealed (Supplementary Fig. 4) that *WRKY18* expression increased with progression of senescence, suggesting that increasing *WRKY18* expression might restrict *WRKY53* expression in later stages of senescence. Interestingly, WRKY53 itself induces the expression of WRKY18 so that WRKY18 can be positioned in an autoregulatory feedback loop of *WRKY53* expression. However, because there are many more proteins directly binding to the promoter of *WRKY53*, and, in addition, the length of the accessible promoter fragment also has to be taken into account, the regulation of WRKY53 appears to be much more complex.

Moreover, WRKY18 can form heterodimers with WRKY53, as demonstrated in yeast using the split ubiquitin system and *in planta* via transient expression in tobacco leaves with a subsequent FRET FLIM analysis. Homodimer and heterodimer formation between members of WRKY group IIa have already been described as mediated by leucine zipper motifs in the N-terminus of the proteins (Xu and others 2006). For these group IIa factors, heterodimer formation modulated the selectivity of DNA binding. Group III factors do not contain canonical leucine zipper motifs but nevertheless are able to interact with each other (Besseau and others 2012) and also across group borders, as shown in this study by the direct interaction of WRKY53 and WRKY18. Transient coexpression of both factors resulted in a different outcome as expected, if the effects of both factors were simply combined, indicating that heterodimers are most likely formed and responsible for these differences. Heterodimer formation in vivo was confirmed in yeast using the split ubiquitin system, and FRET FLIM analyses supported that heterodimers also occur *in planta*. The WRKY18/53 heterodimers appear to act only on a short version of the *WRKY53* promoter, suggesting that accessibility of the *cis* elements in the *WRKY53* promoter might determine whether heterodimers form. Changes in chromatin structure and histone modification have already been observed in the promoter of *WRKY53* (Ay and others 2009; Brusslan and others 2012) during plant development, disclosing a further level of regulation in the WRKY network. Moreover, WRKY factors not only interact with each other but also with many other regulatory proteins, integrating even more signals into the network. A very nice overview on protein interactions of WRKY factors has recently been published by Chi and others (2013). WRKY53 appears to be an important integration point between senescence, pathogen response, and SA and JA signaling. MEKK1 and ESP/ESR have already been characterized as mediating the cross-talk between senescence regulation and pathogen response via interaction with WRKY53 (Miao and others 2007; Miao and Zentgraf 2007). Cross-talk between WRKY18 and WRKY53 is another node of convergence between pathogen response and senescence.

In conclusion, the WRKY network regulation appears to be extremely complex, because in addition to transcriptional cross-regulation between different WRKYs and autoregulatory feedback loops, heterodimer formation modulates WRKY action (Fig. 6). Imaging of the dynamics of protein-protein interactions and competition between different interaction partners will be a future challenge to understand the complex regulatory WRKY network.

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Arabidopsis thaliana WRKY25 Transcription Factor Mediates Oxidative Stress Tolerance and Regulates Senescence in a Redox-Dependent Manner

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Senescence is the last developmental step in plant life and is accompanied by a massive change in gene expression implying a strong participation of transcriptional regulators. In the past decade, the WRKY53 transcription factor was disclosed to be a central node of a complex regulatory network of leaf senescence and to underlie a tight multi-layer control of expression, activity and protein stability. Here, we identify WRKY25 as a redox-sensitive up-stream regulatory factor of *WRKY53* expression. Under non-oxidizing conditions, WRKY25 binds to a specific W-box in the *WRKY53* promoter and acts as a positive regulator of *WRKY53* expression in a transient expression system using Arabidopsis protoplasts, whereas oxidizing conditions dampened the action of WRKY25. However, overexpression of *WRKY25* did not accelerate senescence but increased lifespan of Arabidopsis plants, whereas the knock-out of the gene resulted in the opposite phenotype, indicating a more complex regulatory function of WRKY25 within the WRKY subnetwork of senescence regulation. In addition, overexpression of WRKY25 mediated higher tolerance to oxidative stress and the intracellular H_2O_2 level is lower in *WRKY25* overexpressing plants and higher in *wrky25* mutants compared to wildtype plants suggesting that WRKY25 is also involved in controlling intracellular redox conditions. Consistently, *WRKY25* overexpressers had higher and *wrky* mutants lower H_2O_2 scavenging capacity. Like already shown for WRKY53, MEKK1 positively influenced the activation potential of WRKY25 on the *WRKY53* promoter. Taken together, WRKY53, WRKY25, MEKK1 and H_2O_2 interplay with each other in a complex network. As H_2O_2 signaling molecule participates in many stress responses, WRKK25 acts most likely as integrators of environmental signals into senescence regulation.

Keywords: Arabidopsis, transcription factor network, WRKY factors, oxidative stress tolerance, redox-dependent DNA-binding, leaf senescence

INTRODUCTION

Senescence is the last step during plant development and is genetically programmed to maximize the remobilization of nutrients out of the senescing tissue into developing parts of the plants before organs finally die. Before anthesis, sequential leaf senescence leads to the reallocation of mineral, nitrogen and carbon sources from older leaves to newly developing non-reproductive organs. After anthesis, monocarpic leaf senescence is launched and governs the nutrient repartitioning to the now developing reproductive organs and, therefore, has a critical impact on yield quality and quantity. Induction and progression of leaf senescence is mainly achieved by switching-on genes involved in degradation and mobilization of macromolecules and turning-off genes related to photosynthesis. A temporal transcript profiling, using microarrays with high-resolution covering 22 time points of a defined leaf of *Arabidopsis thaliana* during onset and progression of leaf senescence, revealed a distinct chronology of events (Breeze et al., 2011). Remarkably, the first processes to be activated are autophagy and transport followed by reactions to reactive oxygen species (ROS) and subsequently to abscisic acid (ABA) and jasmonic acid (JA). This clearly indicates that ROS, ABA and JA are important early signals in leaf senescence. In consistence, intracellular hydrogen peroxide contents increase during bolting and flowering of Arabidopsis plants when monocarpic senescence is induced (Zimmermann et al., 2006) while decreasing hydrogen peroxide levels lead to a delay of the onset of leaf senescence (Bieker et al., 2012).

These massive changes in the transcriptome suggest a central role for transcriptional regulators. The two transcription factor families of WRKY and NAM-, ATAF-, and CUC-like (NAC) factors, which largely expanded in the plant kingdom, are overrepresented in the senescence transcriptome of Arabidopsis (Guo et al., 2004) and appear to be ideal candidates for regulatory functions. Several members of both families play important roles in senescence, not only in Arabidopsis but also in other plant species (Miao et al., 2004; Uauy et al., 2006; Ülker et al., 2007; Kim et al., 2009; Breeze et al., 2011; Yang et al., 2011; Besseau et al., 2012; Wu et al., 2012; Gregersen et al., 2013).

The WRKY transcription factor family of *A. thaliana* consists of 75 members, subdivided into three different groups according to their protein motifs and domains (Eulgem et al., 2000; Rushton et al., 2010). Many WKRY factors are activated after pathogen attack but also in response to abiotic stress (for review see Birkenbihl et al., 2017; Jiang et al., 2017). Moreover, members of all three groups are involved in senescence regulation and many of these react to ROS, SA and JA signals indicating a cross-talk between stress responses and senescence. Besides this cross-talk to stress responses, the *WRKY53* upstream regulator REVOLUTA mediates a redox-related communication between early leaf patterning and senescence as REVOLUTA is involved in both processes (Xie et al., 2014; Kim et al., 2017).

Interestingly, almost all members of the WRKY family contain one or more W-boxes (the consensus binding motif TTGACC/T of all WRKY factors) in their promoters, pointing to a WRKY transcriptional network (Dong et al., 2003; Llorca et al., 2014). Even though all WRKYs bind to these consensus sequences, there appears to be a selectivity of specific factors for specific boxes most likely due to the surrounding sequences (Rushton et al., 2010; Brand et al., 2013; Potschin et al., 2014). However, besides regulating transcription of each other, WRKY factors are also able to form heterodimers, leading to a change in DNA-binding specificity (Xu et al., 2006). In addition, many other proteins interact physically with WRKY proteins influencing their activity and stability (for review see Chi et al., 2013). One central node in the WRKY network regulating early senescence is WRKY53. WRKY53 underlies a tight regulation governed by multi-layer mechanisms to control expression, activity and protein stability. When leaf senescence is induced, the *WRKY53* gene locus is activated by the histone modifications H3K4me2 and H3K4me3 (Ay et al., 2009; Brusslan et al., 2012), whereas DNA methylation remains unchanged and overall very low (Zentgraf et al., 2010). At least 12, most likely even more, proteins are able to bind to the promoter of *WRKY53* (GATA4, AD-Protein, WRKY53 itself, several other WRKYs, MEKK1, REVOLUTA, WHIRLY1) and influence the expression of *WRKY53* (Miao et al., 2004; Miao et al., 2007; Miao et al., 2008; Potschin et al., 2014; Xie et al., 2014; Ren et al., 2017, unpublished results). All these factors are involved in senescence regulation but it is still unclear whether they all bind at exactly the same time, whether they compete with each other, or whether they form higher order complexes. Except for the WRKYs that bind to the W-boxes in the *WRKY53* promoter, all other proteins have different binding motifs so that in principle a simultaneous interaction would be possible. The *WRKY53* promoter contains at least three W-boxes, which show preferential binding activities for different WRKY factors but competition would be also a mean of regulation. Moreover, WRKYs can also form heterodimers, which makes the situation even more complicated. However, all these aspects need further investigations. For MEKK1, it has already been shown that it can interact with WRKY53 and AD-Protein on the protein level (Miao et al., 2007; Miao et al., 2008). Whether WRKY53 or other WRKYs compete with AD-protein for MEKK1 interaction or whether they form higher order complexes is currently analyzed in more detail. These findings have been compiled in a model (Zentgraf et al., 2010) und smaller subnetworks have already schematically drawn for some candidates like WRKY18, REVOLUTA or WHIRLY1 (Potschin et al., 2014; Xie et al., 2014; Ren et al., 2017).

Moreover, the WRKY53 protein also directly interacts with a histone deacetylase 9 (HDA9) to recruit POWERDRESS and HDA9 to W-box containing promoter regions to remove H3 acetylation marks and thereby suppress the expression of key negative senescence regulators (Chen et al., 2016). This clearly suggests that WRKY53 itself is also involved in changing epigenetic marks of senescence regulators in a feedback loop. Phosphorylation by the MAP kinase kinase kinase MEKK1 or the interaction with the epithiospecifier ESP/ESR directly influences the DNA-binding activity of WRKY53 (Miao et al., 2007; Miao and Zentgraf, 2007). On top of that, the E3 ubiquitin

ligase UPL5 tightly controls the protein amount of WRKY53 (Miao and Zentgraf, 2010). The complexity of the WRKY network is illustrated by the fact that one and the same WRKY factor, namely WRKY18, acts as upstream regulator, downstream target and protein interaction partner of WRKY53 (Potschin et al., 2014).

In order to unravel the molecular mechanisms of the senescence-regulating WRKY network in more detail, we screened the W-boxes of the *WRKY53* promoter for DNA-protein interactions with other leaf senescence-associated WRKY proteins and tested their impact on *WRKY53* expression using a transient expression system in Arabidopsis protoplasts. Here we used WRKYs which are expressed in leaves during onset and progression of senescence and which belong to the three different subgroups of the WRKY family, namely WRKY18 (group IIa), WRKY25 (group I) and WRKY53 itself (group III). Out of the 15 WKRYs analyzed by an ELISA-based DNA-protein interaction assay and reporter gene expression assays, WRKY18 had a very strong binding affinity to all W-boxes of the *WRKY53* promoter but a very low selectivity. Moreover, WRKY18 was characterized to be the strongest negative regulator of *WRKY53* expression (Potschin et al., 2014). Besides WRKY18, WRKY25 was one of the strongest interaction partners of the *WRKY53* promoter but in this case turned out to be a strong positive regulator of *WRKY53* expression. Therefore, we wanted to analyze the interplay between WRKY53 and WRKY25 in more detail. Here we could show that DNA-binding as well as transcriptional activation potential of WRKY25 is dependent on the redox conditions. Intracellular hydrogen peroxide concentrations are altered in plants with altered *WRKY25* expression and the *WRKY25* overexpressing plants are more tolerant against oxidative stress. WRKY25 appears to foster the activation of the H_2O_2-mediated expression of the transcription factors *WRKY18* but dampens the H_2O_2-response of *WRKY53*, *ZAT12*, and *ANAC092* in mature leaves. However, contradicting its positive effect on *WRKY53* expression and the senescence phenotype of the *WRKY53* overexpressing plants, *WRKY25* overexpressing plants exhibited a delayed senescence phenotype, whereas *wrky25* mutant plants showed slightly accelerated senescence. This clearly points to a more complex regulatory network. Moreover, the influence of MEKK1 as modulator of WRKY53 activity on the action of WRKY25 was tested.

RESULTS

WRKY25 Binds Directly to the Promoter of *WRKY53 in* a Redox-Sensitive Manner

Out of the 15 WKRYs analyzed by an ELISA-based DNA-protein interaction assay, WRKY18 (group IIa) and WRKY25 (group I) had a very strong binding affinity. In contrast to WRKY18, which strongly binds to all W-boxes in the *WRKY53* promoter (Potschin et al., 2014), WRKY25 also had a strong binding activity but selectively bound to W-box1, to a much lesser extend to W-box2 and 3, the TGAC cluster and an artificial 3× W-box (**Figures 1A, B**). Binding was completely abolished when W-box1 was mutated or an unrelated G-box motive was coupled to the ELISA plates. All binding reactions increased with protein concentrations and no binding could be detected with crude extracts of *E. coli* cells expressing no recombinant protein. Both proteins were present approximately to the same extent in *E. coli* crude extracts (**Figure S1**). As many WRKY factors signal back to their own promoters in positive or negative feedback loops, we also tested whether WRKY25 can bind to the W-boxes in its own promoter. Here, WRKY25 was able to bind to W-box1 and 4, whereas W-box2 and 3 exhibited lower binding affinities. *Vice versa*, WRKY53 also bound preferentially to W-box 1 of the *WRKY25* promoter but to W-box2 of its own promoter, as already shown before (**Figure 1C**, Potschin et al., 2014). This indicates that according to DNA-binding, there is a cross-regulation between both genes and both genes are regulated by feedback mechanisms.

We already know for a long time that *WRKY53* expression can be induced by H_2O_2 treatment (Miao et al., 2004; Xie et al., 2014). As the WRKY25 protein contains two potentially redox-sensitive zinc-finger DNA-binding domains, it is an excellent candidate for direct redox regulation (Arrigo, 1999). Therefore, we wanted to test whether the WRKY25 DNA-binding reaction is sensitive to reducing or oxidizing agents and analyzed the ability of WRKY25 to bind to W-box1 of the *WRKY53* promoter and W-box1 of the *WRKY25* promoter under different redox conditions. Whereas reducing conditions (DTT addition) clearly and significantly increased DNA-binding ability to both W-boxes, oxidizing conditions (H_2O_2 addition) significantly reduced the binding activity in comparison to standard binding conditions (**Figure 2**). In order to test whether this redox-dependent binding can be driven back and forth when redox conditions change, we added increasing amounts of H_2O_2 to the DTT pre-treated binding reactions and *vice versa*. Both redox-related changes in DNA-binding activity of WRKY25 were reversible indicating that WRKY25 can directly adapt its DNA-binding activity to the redox status of the cell. However, not all WRKYs show this redox-sensitivity, e.g. WRKY18 appears to be insensitive, whereas WRKY53 DNA-binding seems to be diminished under oxidizing and reducing conditions, but this reduction was not statistically significant (**Figure S2**).

WRKY25 Acts as Positive Regulator of *WRKY53* Expression Under Non-Oxidizing Conditions

To investigate, how WRKY25 affects the expression of *WRKY53* and *vice versa*, we performed a transient co-transformation of *WRKY53* or *WRKY25* promoter:*GUS* constructs with 35S:*WRKY25* and 35S:*WRKY53* effector constructs, respectively, using an Arabidopsis protoplast system (**Figure 3A**). The protoplast system was used to confirm the identified DNA-Protein interactions of the *in vitro* assay also *in vivo*. However, it is clear that the protoplast system is still an artificial system not taking into account development cues, but it can provide inside into the possible basic regulatory mechanisms. Using this *in vivo* system, the WRKY25 effector significantly up-regulated promoter *WRKY53*-driven *GUS* expression. In contrast, it

down-regulated *GUS* expression driven by its own promoter pointing to a negative feedback regulation. The WRKY53 effector slightly activated reporter gene expression driven by its own promoter as already described before (Potschin et al., 2014). Surprisingly, WRKY53 had only low effects (1.4-fold) on reporter gene expression driven by the promoter of *WRKY25* (**Figure 3A**), even though WRKY53 is able to bind strongly to the W-boxes of this promoter (**Figure 1C**) indicating that strong binding does not necessarily mean that gene expression is highly affected. If both effector constructs were co-expressed, additive effects were detected leaving the question open whether or not heterodimers are formed.

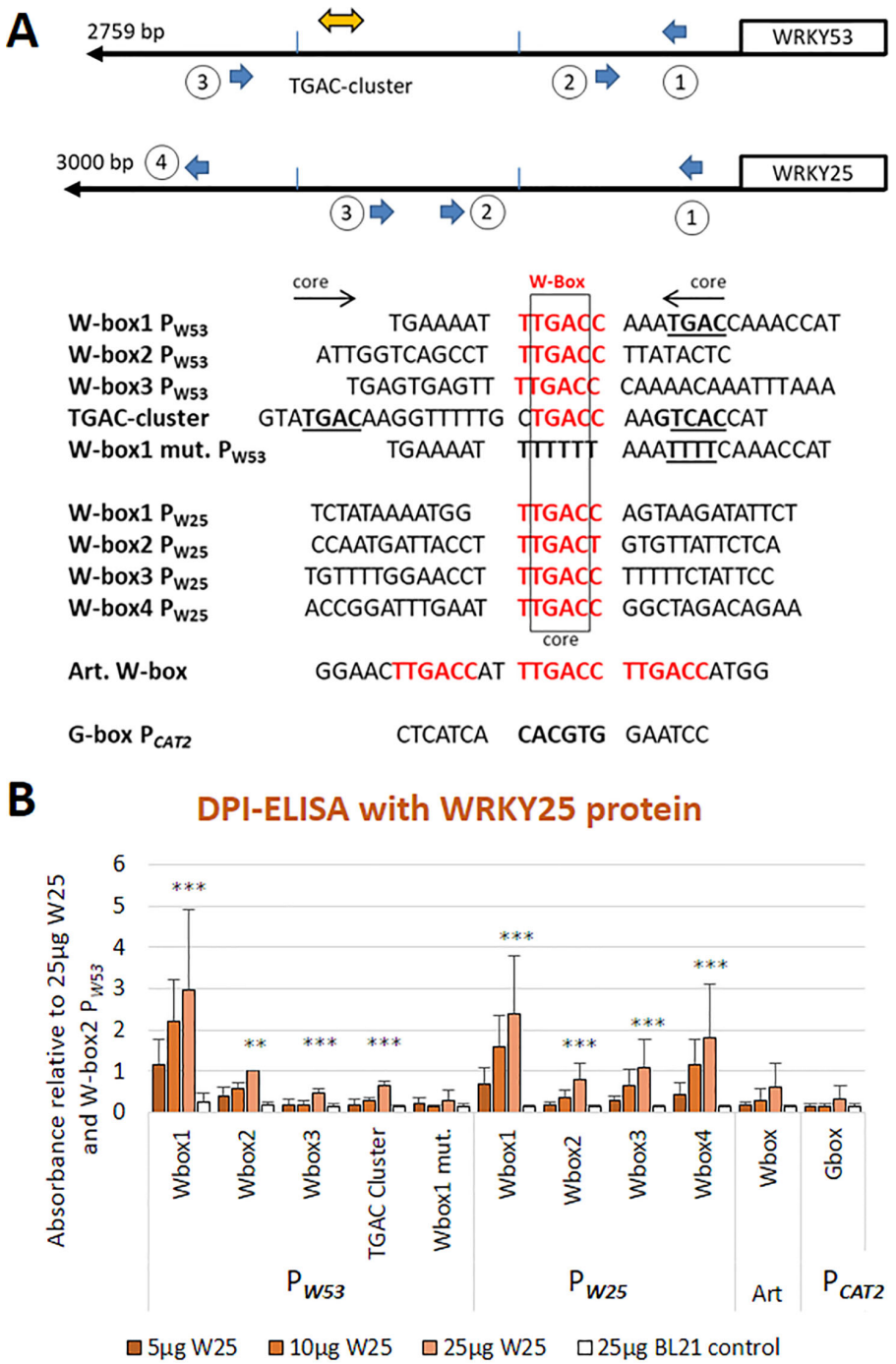

FIGURE 1 | Continued

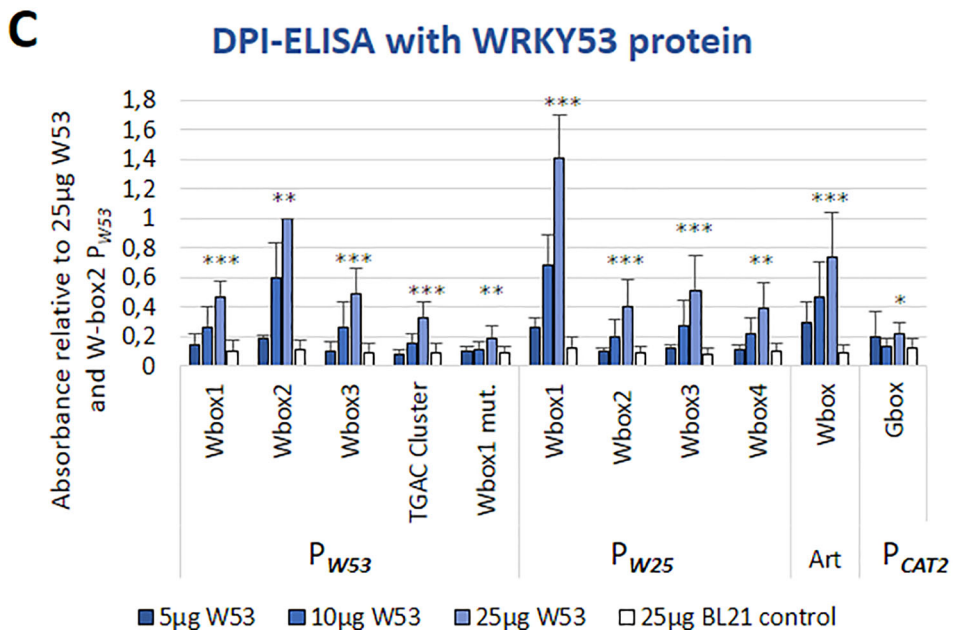

FIGURE 1 | WRKY25 is able to bind directly to the *WRKY53* promoter. **(A)** Schematic drawing of positions and sequences of the W-boxes in the *WRKY53* and *WRKY25* promoters used for DPI-ELISAs; perfect W-box motifs are highlighted in red; the TGAC core sequence is indicated in bold and underlined; the direction of the motif is indicated by the arrows. An artificial sequence containing three perfect W-boxes was used as a positive control (Art. W-box), a G-box of the *CATALASE2* promoter was used as a negative control. **(B)** DPI-ELISA with different amounts of crude extracts of *E. coli* BL21 cells expressing WRKY25 proteins and different biotinylated DNA fragments. **(C)** DPI-ELISA with different amounts of crude extracts of *E. coli* BL21 cells expressing WRKY53 proteins and different biotinylated DNA fragments. Absorbance values are indicated relative to values of 25 µg WRKY25 or WRKY53 to W-box2 P_{W53}, respectively, (mean values ± SD, n = 3-4). Kruskal–Wallis-test was performed for statistically significant differences of all values compared to 25 µg BL21 control (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

As DNA-binding of WRKY25 was redox-sensitive, we wanted to find out, whether also target gene expression is affected by the redox conditions. Since we wanted to change the redox conditions within a physiological range, we did not treat protoplasts directly with high amounts of H_2O_2. Instead, we developed a transient expression system using Arabidopsis protoplast in the presence of 3-Amino-Triazol (3′-AT), which inhibits catalase function, and would therefore provoke physiological changes in intracellular H_2O_2 levels. Inhibition of catalase activity was almost complete and leads to increasing concentrations of H_2O_2 in the cells, but had no effect on the GUS activity measurement (**Figure S3**). Using this assay, WKRY25 effector proteins were significantly less efficient under oxidizing conditions, most likely due to lower DNA-binding affinity. WRKY53 effector proteins appeared also to be less efficient, but the effect was only significant for the WRKY25 promoter, not for its own. The effects were still significant when a combination of both effectors constructs was used (**Figure 3B**).

MEKK1 Increases the Effect of WRKY25 Proteins on Promoter of *WRKY53* Driven Gene Expression

As expression of *WRKY53* is enhanced by a direct binding of MEKK1 to the promoter region of *WRKY53* and a protein–protein interaction between WRKY53 and MEKK1 leads to phosphorylation of WRKY53 (Miao et al., 2007), we tested whether WRKY25 activity can also be enhanced by adding a 35S:*MEKK1* construct as additional effector in a protoplast co-transformation assay. Indeed, the presence of the MEKK1 protein significantly increased *WRKY53* promoter-driven reporter gene expression by WRKY25 to approximately the same extent as MEKK1 presence exhibits on WRKY53 activity itself (**Figure 3C**). Thus, MEKK1 interplay with WRKY factors is not restricted to WRKY53, but appears to be a more general phenomenon. First evidence for a direct protein-protein interaction between several WRKY factors and MEKK1 was obtained in a Yeast-Split-Ubiquitin system, in which many, but not all tested WRKYs could interact with MEKK1 (data not shown). WRKY18, which acted as a repressor on promoter *WRKY53*-driven reporter gene expression (Potschin et al., 2014), even changed its activity in the presence of MEKK1 from a repressor to an activator (**Figure S4A**). Moreover, we tested the role of MEKK1 in senescence regulation. As *MEKK1* knock-out plants die before they develop the first true leaves, we used an estradiol-inducible $amiRNA_{MEKK1}$ line to knock-down MEKK1 by treatment with 3 µM ß-estradiol or mock every 7 days starting on day 25 after germination. In this system, knock-down of MEKK1 can be controlled by GFP expression, which is under the control of the same amiRNA (Li et al., 2013). Here we could show that conditional knock-down of *MEKK1* in plants exhibit an accelerated senescence phenotype (**Figures S4B–D**). Taken together, MEKK1 appears to act as negative regulator of senescence at least in part by modulating the activity of different WRKY factors. However, whether the interaction with WRKY25

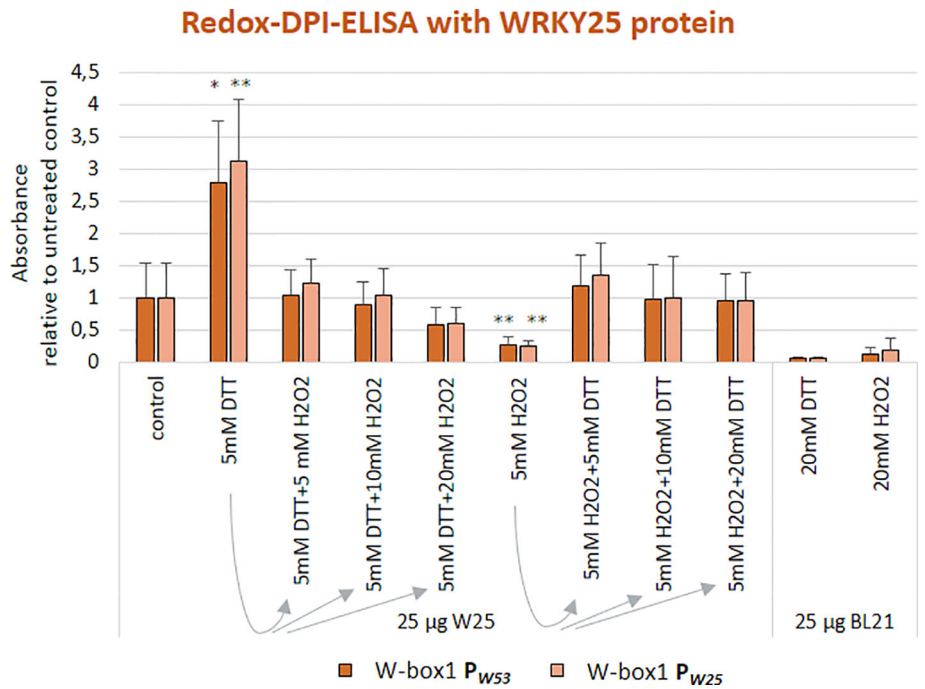

FIGURE 2 | WRKY25 binding to the *WRKY53* promoter is redox-dependent. Redox-DPI-ELISA with 25 µg of crude extracts of *E. coli* BL21 cells expressing WRKY25 proteins and the 5'biotinylated annealed oligonucleotides W-box1 P_{W53} and W-box1 P_{W25}. Protein extracts were reduced or oxidized by addition of either DTT or H_2O_2 to examine a redox-dependent binding of WRKY25. A fraction of the DTT-reduced proteins was re-oxidized by addition of increasing H_2O_2 concentrations to prove the reversibility of the redox effect. The same procedure was applied to the H_2O_2-oxidized proteins using increasing amounts of DTT. Absorbance values are indicated relative to control without treatment (mean values ± SD, n = 4). Kruskal–Wallis-test was performed for statistically significant differences of all values compared to control (*$P \leq 0.05$, **$P \leq 0.01$).

or WRKY18 is direct as it is for WRKY53, or is mediated by the classical MAPK pathway, still has to be elucidated.

WRKY25 Is Involved in Senescence Regulation

To evaluate the participation of WRKY25 in senescence regulation, we analyzed plants with a T-DNA insertion in the *WRKY25* gene lacking a functional WRKY25 protein and *WRKY25* overexpressing plants. A T-DNA insertion in the last of five exons of *WRKY25* (SAIL_529_B11) was confirmed by PCR and expression of *WRKY25* was analyzed by qRT-PCR (**Figure S5B**). Moreover, for overexpression of *WRKY25,* we first transformed plants using a 35S:*WRKY25* construct. However, qRT-PCR revealed that *WRKY25* was not overexpressed; in contrast, the endogenous gene expression was severely silenced throughout plant development (**Figure S5A**) so that we used this line as knock-down line (35S:*WRKY25si*) to confirm the results of the *wrky25* mutant plants. In a second attempt, we used the *UBIQITIN10* promoter for more moderate overexpression and we created two independent plant lines overexpressing *WRKY25* to different extents with different transgene expression levels (**Figure S5B**). In addition, double-knock-out mutants were created by crossing the single mutant lines *wrky25* (SAIL_529_B11) and *wrky53* (SALK_034157; Miao et al., 2004) with each other. F2 progenies were screened for homozygous double-knock-out plants. In order to compare leaves of the same position within the rosette for senescence symptoms, leaves were color-coded during development (Bresson et al., 2018). Altered *WRKY25* expression had almost no effect on the speed of the general development of the plants (**Figure S6**). Bolts appeared at approximately week 5 in all lines, first flowers at approximately week 6 and first siliques also developed synchronously. However, leaf size slightly increased in the overexpression lines whereas leaves of the *wrky25* mutant, the *wrky25/wrky53* double-knock-out plants and the *WRKY25* silenced line were slightly smaller. To evaluate senescence in detail, we sorted the rosette leaves of all lines by the color code according to their age to compare the respective leaves with each other. A typical example of rosette leaves of 8-week-old plants is shown in **Figure 4A**. However, as there are always differences between individual plants of one line, a statistical analysis of at least six plants was done by grouping the leaves into four categories by an automated colorimetric assay (ACA; Bresson et al., 2018) according to their leaf color (green; green/yellow; fully yellow and brown/dry) from weeks 5 to 8 (**Figure 4B**). The photosynthetic status of the plants was analyzed using a Pulse-Amplitude-Modulation (PAM) method (**Figure 4C**). Amongst the chlorophyll fluorescence imaging parameters, the Fv/Fm ratio is reflecting the maximal quantum yield of PSII photochemistry. Moreover, the expression of the senescence marker genes *CHLOROPHYLL A/B BINDING*

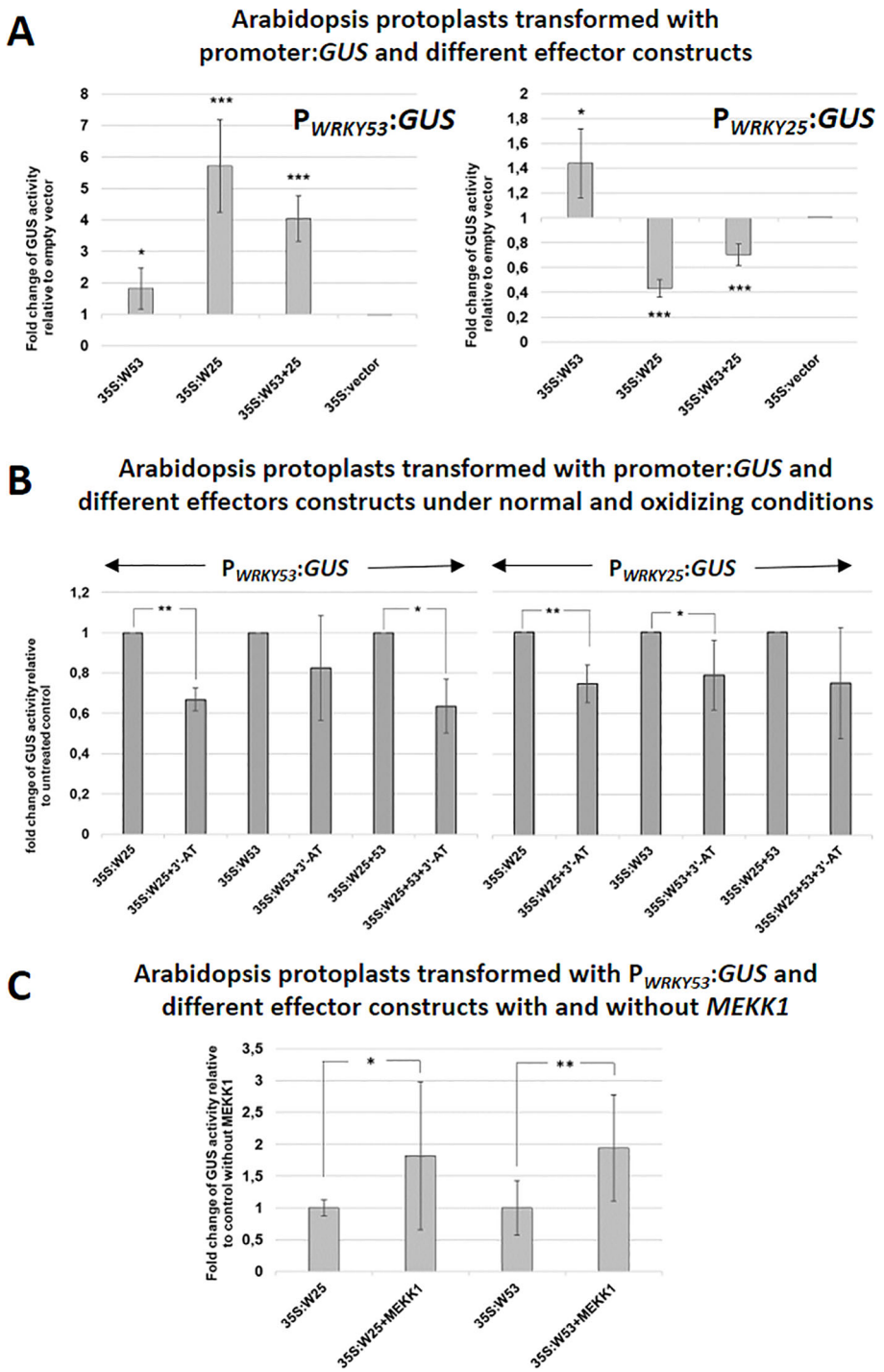

FIGURE 3 | WRKY25 positively regulates *WRKY53* under non-oxidizing conditions. **(A)** Arabidopsis protoplasts were transiently transformed with 5 µg of effector-, 5 µg of reporter-plasmid DNA and 0.1 µg of a luciferase construct for normalization. A 2.8-kbp-fragment of the *WRKY53* promoter and a 3.0-kbp-sequence of the *WRKY25* promoter fused to the *GUS* gene were used as reporter constructs. 35S:*WRKY25* and 35S:*WRKY53* constructs were used as effector plasmids. GUS activity was measured on the next day. The values are presented relative to the empty vector control (mean values ± SD, n = 6 independent transfromations). One sample t-test was performed, (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$) **(B)** GUS assays were performed with protoplasts, which were simultaneously incubated overnight with 10 mM 3'-AT to inhibit catalase activities leading to higher H_2O_2 level. The values are presented relative to the untreated control transformations (mean values ± SD, n = 3–7 independent transformations). One sample t-test was performed, (*$P \leq 0.05$, **$P \leq 0.01$). **(C)** Co-transformation assays with 35S:*MEKK1* were performed. The values are presented relative to transformation without MEKK1 (mean values ± SD, n = 4–5 independent transformations). Kruskal–Wallis-test was performed, (*$P \leq 0.05$, **$P \leq 0.01$).

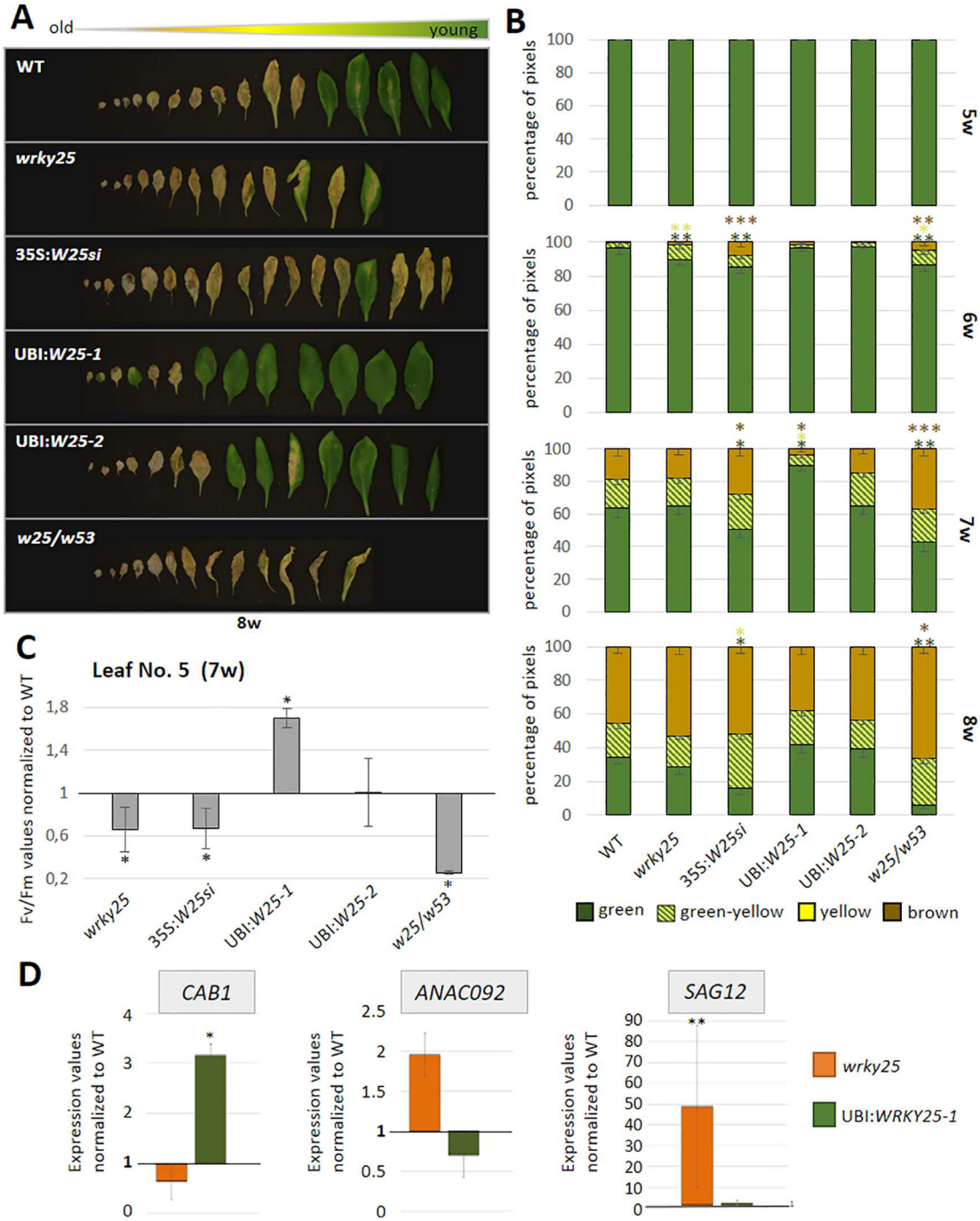

FIGURE 4 | Senescence phenotypes of *WRKY25* transgenic and mutant lines. **(A)** Col-0 wildtype (WT), *wrky25* mutant (*wrky25*), *WRKY25* overexpressing (UBI:*W25-1* and UBI:*W25-2*), *WRKY25* silenced (35S:*W25si*) and *wrky25-wrky53* double-knock-out (*w25/w53*) plants were analyzed over development. A photograph of rosette leaves of 8-week-old plants sorted according to their age is shown. **(B)** Quantitative evaluation of leaf senescence by categorizing individual leaves of at least six plants into four groups according to their color: green, green leaves starting to get yellow (green-yellow), completely yellow leaves (yellow) and dead and/or brown leaves (brown/dry). The percentage of each group with respect to total leaf numbers are presented (mean values ± SE, n = 6). **(C)** Fv/Fm values were measured with PAM for leaves of position 5 of 7-week-old plants (mean values ± SE, n = 6). One sample t-test was performed for statistical differences of all values compared to Col-0 (*$P \leq 0.05$) **(D)** Expression of the senescence associated marker genes *ANAC092*, *CAB1, SAG12* were analyzed by qRT-PCR and normalized to the expression of the *ACTIN2* gene. *SAG12* in 6-week and *ANAC092* and *CAB1* in 7-week-old plants. Shown are *wrky25* and UBI:*W25-1* plants normalized to Col-0 (mean values ± SE, n = 3). Kruskal–Wallis-test was performed for statistically significant differences (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

PROTEIN 1 (*CAB1*) being downregulated, the NAC transcription factor *ANAC092*, and *SENESCENCE-ASSOCIATED GENE 12* (*SAG12)* being upregulated were analyzed by qRT-PCR (**Figure 4D**). In comparison to Col-0 wildtype plants, *WRKY25* overexpressing plants showed significantly delayed visible senescence symptoms, which was in consistence with a delay in the decrease of the Fv/Fm ratio measured in leaf No. 5 and leaf No. 10 (**Figure 4C**, **Figure S7**). Furthermore, a higher expression of *CAB1* in 7-week-old plants as well as a lower expression of *ANAC092* and *SAG12* in 6-week-old plants of *WRKY25* overexpressing line compared to wildtype confirmed a delayed senescence phenotype. In contrast, senescence and loss of photosynthetic activity was accelerated in the *wrky25* mutant plants and the 35S:*WRKY25si* line (**Figures 4A–C**). Higher up-regulation of *ANAC092* and *SAG12* expression in 6-week-old plants and lower *CAB1* expression in 7-week-old plants in *wrky25* mutant line clearly indicate an accelerated senescence phenotype (**Figure 4D**). Remarkably, the expression of *WRKY53* was lower in the *WRKY25* overexpressing as well as in the *wrky25* mutant lines in comparison to WT (**Figure 5**), suggesting a more complex regulation of WRKY53 expression during development. Moreover, the expression of two tested WRKY genes (*WRKY18* and *WRKY40*) was antagonistic in *WRKY25* overexpressing and mutant plants, but only at week 5; at later stages also these two WRKY genes were down-regulated in both lines. We have chosen WRKY40 as it is also expressed in senescent leaf tissue. WRKY40 was also shown to regulate *WRKY53* expression in a negative way, but to a lesser extent. Moreover, it is the closest relative of WRKY18 also belonging to group IIa so that we can see whether regulatory processes are group specific which appears to be the case. This clearly indicates that no simple regulatory circuits are in place between these WRKY proteins and genes. WRKY25 as well as WRKY53 and WRKY18 appear to be part of a WRKY subnetwork, which is embedded in the overall complex senescence regulatory network. Interfering on the expression of one WRKY gene can lead to an imbalance in the subnetwork, which might explain that mutant and overexpressing plants showed the same effects on the expression of specific WRKYs. Taken together, WRKY25 appears to be part of the WRKY subnetwork and a redox-sensitive negative regulator of senescence.

WRKY25 Mediates Tolerance Against Oxidative Stress

As WRKY25 action appears to be redox-sensitive, we wanted to analyze whether WRKY25 is also involved in the response to oxidative stress or plays a role in the signaling of H_2O_2 *in planta*. Therefore, we germinated seeds of WT, *wrky25* mutant, *wrky53* mutant, 35S:*WRKY25si*, the *WRKY25* overexpressing lines as well as the double mutant *wrky25/wrky53* on plates containing 10 mM H_2O_2 (**Figure 6A**). After 7 to 10 days, the percentages of green seedlings per total seedling numbers were counted. The experiment was repeated six times and the outcome of these series were summarized in a heat map showing the tolerance against H_2O_2 (**Figure 6B**). The germination rate on the control plates without H_2O_2 was almost 100% for all plant lines used. The 35S:*WRKY25si* and the *WRKY25* overexpressing lines *(*UBI:*W25-1* and UBI:*W25-2*) germinated much better on H_2O_2 plates in comparison to WT as well as the *wrky53* single mutant. In contrast, the *wrky25* as well as the *wrky25/wrky53* mutant seeds germinated significantly worse compared to WT. Therefore, WRKY25 seems to mediate a higher tolerance against H_2O_2. Even though gene silencing was clearly shown from the late seedling stage until the end of leaf development (**Figure S5**), the *wrky25* mutant and the 35S:*WRKY25si* knock-down line behave different in this experiment. This behavior can only be explained by the assumption that during very early stages of germination, the 35S:*WRKY25* line overexpressed the transgene but gene silencing was not yet established at this very early time points. This could indeed be confirmed by expression analyses of *WRKY25* in the 35S:*WRKY25si* line using qRT-PCR in very early germination states (4 and 7 day old seedlings, **Figure S5A**).

In order to test whether this tolerance is due to higher antioxidative capacities in these lines, we measured intracellular H_2O_2 contents of leaf No. 8 in 8-week-old plants of these lines (**Figure 6C**). Less intracellular H_2O_2 was measured in the overexpressing lines, while more H_2O_2 appears to be present in the mutants and the silenced line in comparison to WT (**Figure 6C**). Moreover, the H_2O_2 scavenging capacity of leaf discs of the different lines was tested by incubating these discs for 2 h in H_2O_2 solution and measure the remaining H_2O_2 using peroxide strips (**Figure 6D**). As expected, the antioxidative capacity of the *WRKY25* overexpressing lines was slightly higher, whereas scavenging in the mutant and silencing lines was lower. Taken together, WRKY25 does not only mediate a higher tolerance against oxidative stress but is also involved in the regulation of intracellular H_2O_2 levels, at least in later developmental stages. This might also contribute to the negative effect of WRKY25 on senescence since H_2O_2 acts as signaling molecule to induce senescence and, most likely, also participates in membrane deterioration and lipid peroxidation processes in later stages (Chia et al., 1981). The conclusions on the role of WRKY25 in senescence-related redox signal transduction is further supported by a dark-induced senescence experiment including *wrky25*, *catalase2* (*cat2*) and *wrky25/cat2* double-knock-out plants (**Figure S8**). As expected, *cat2* and *wrky25* had lower H_2O_2 scavenging capacity than wildtype plants resulting in a higher H_2O_2 content in the mutant lines (**Figures S8B, C**). Remarkably, *wrky25/cat2* double mutants showed an additive effect indicating that higher H_2O_2 content in *wrky25* mutant plants is not due to lower catalase activity. This was also visualized by the CAT-activity staining of a native PAGE, in which CAT2 activity of wildtype and *wrky25* mutant plants appear to be very similar (**Figure S8A**). Moreover, dark-induced senescence was more pronounced in *wrky25* or *cat2* mutant compared to wildtype leaves and was enhanced in the *wrky25/cat2* double mutant, correlating with their intracellular H_2O_2 contents (**Figures S8B, D**).

WRKY25 Enhances *WRKY53* Response to Oxidizing Conditions

Because *WRKY53* is strongly up-regulated after treatment with H_2O_2 in Arabidopsis (Miao et al., 2004; Xie et al., 2014), WRKY25 DNA-binding is redox-sensitive (**Figure 2A**) and its positive effect on *WRKY53* expression is diminished under

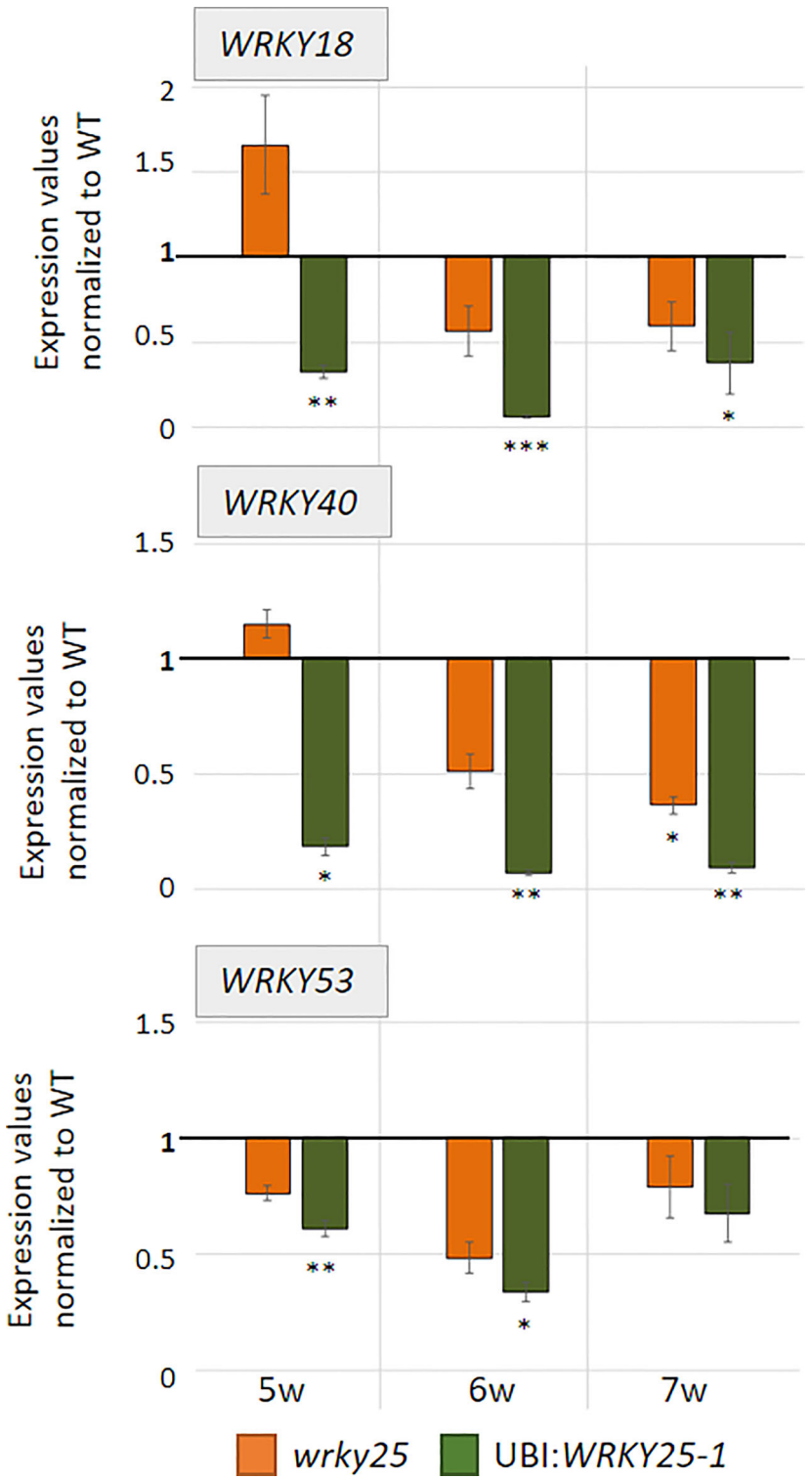

FIGURE 5 | WRKY genes expression analyses. Expression of different WRKY genes (*WRKY53*, *WRKY18*, *WRKY40*) were analyzed in Col-0 (WT), *wrky25* mutant and *WRKY25* overexpression line (UBI : *WRKY25-1)* by qRT-PCR and normalized to the expression of the *ACTIN2* gene. Three pools were analyzed; one pool consists of leaf No. 6 and 7 of two different plants. In week 7, only two pools of the *35S:W25si* plant line and of the *wrky25* line were analyzed but here with six technical replicates. Expression values were normalized to Col-0 and Col-0 was set to 1 (mean values, n = 3, ± SE). Kruskal–Wallis-test was performed for statistically significant differences of all value compared to Col-0 (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

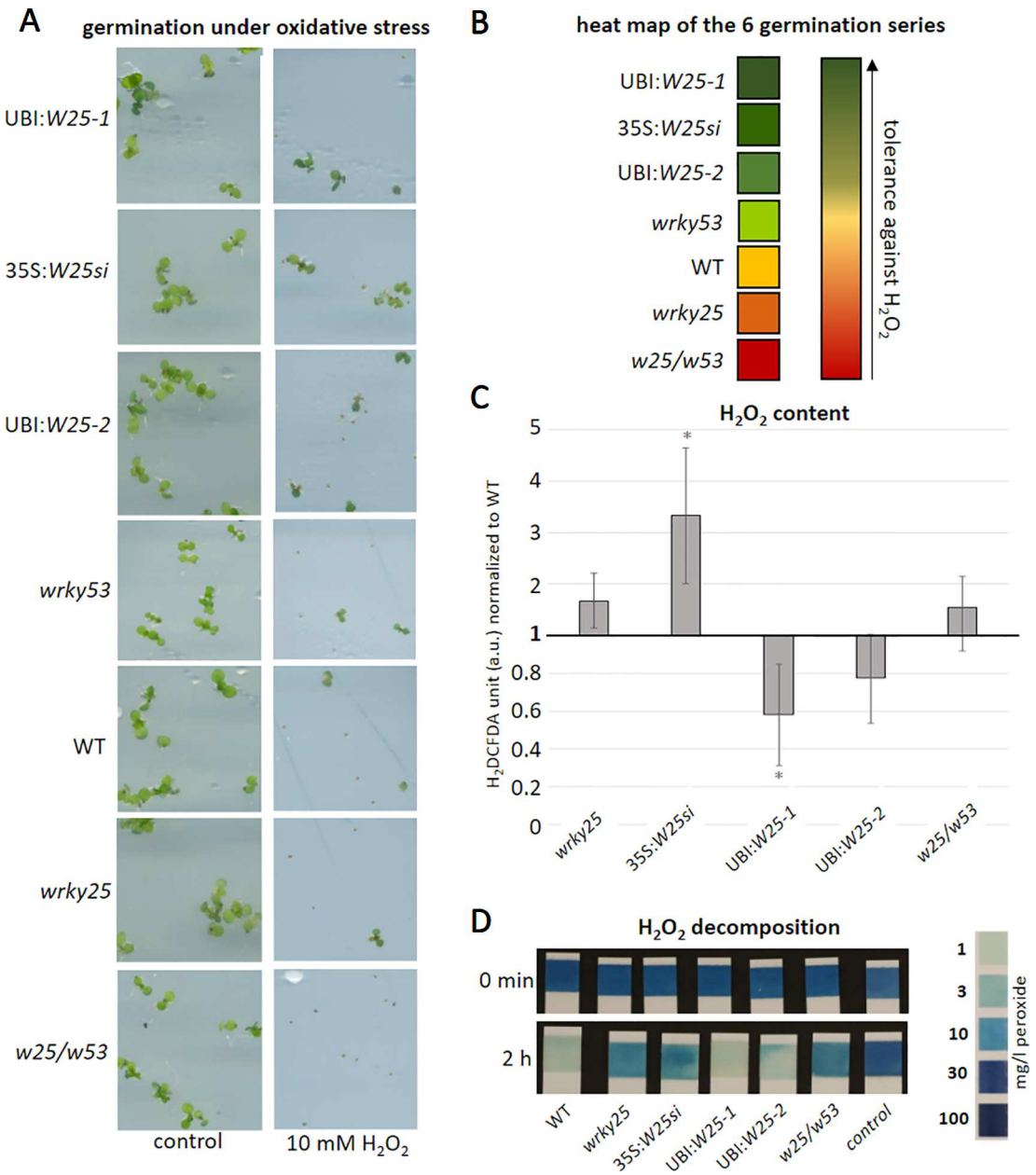

FIGURE 6 | WRKY25 mediates H_2O_2 tolerance. **(A–B)** Col-0 (WT), *wrky25* mutant, *wrky53* mutant, *WRKY25* overexpressing (*UBI:W25-1; UBI:W25-2*) and *WRKY25* silenced (*35S:W25si*) as well as the double-knock-out plants (*w25/w53*) were sown on ½ MS plates with and without 10 mM H_2O_2. A minimum of 30 seeds were put onto the plates and the experiment repeated 6 times (n = 6) **(A)** shows a representative sector of the plates with and without H_2O_2 in media. **(B)** summarizes the six independent experiments in a heat map. Dark green means the most tolerant against H_2O_2, dark red the most sensitive towards H_2O_2. **(C)** H_2O_2 content was measured over plant development using H_2DCFDA fluorescence; leaves No. 8 of 8-week-old plants are shown. Fluorescence is indicated in arbitrary units (a.u.), normalized to leaf weight and expressed relative to WT (± SE, n = 4). One Sample t-test was performed for statistical differences of all values compared to WT (*$P \leq 0.1$) **(D)** Leaf discs of leaf No. 5 of 6-week-old plants were incubated in a 30 mg/l H_2O_2 solution. As control H_2O_2 solution without leaf discs was measured. At timepoint 0 min and 2 h the decomposition of H_2O_2 was determined using commercially available peroxide sticks, color scale for H_2O_2 content is provided on the right.

oxidizing conditions (**Figure 3B**), we wanted to find out, whether WRKY25 is required for the induction of *WRKY53* expression after H_2O_2 treatment. Therefore, leaves of *wrky25* and *wrky53* single as well as double mutants and WT plants were detached and incubated in 10 mM H_2O_2. The expression of *WRKY53* and several other H_2O_2-responsive genes (*WRKY25*, *ANAC092, WRKY18* and *ZAT12*) was determined after 0 min, 30 min, 1 h and 3 h using qRT-PCR (**Figure 7**). All tested genes were responsive to H_2O_2 in wildtype. *WRKY53* expression increased most prominently in 7-week-old plants after 1 h of H_2O_2

treatment. This response is clearly dampened in *wrky25* mutant leaves. In contrast, the *WRKY25* mRNA level highly increased in leaves of young 5-week-old wildtype plants 1 h after H_2O_2 treatment and responsiveness becomes lower with age. Again, this response is diminished in *wrky53* mutant leaves in all tested developmental stages indicating that WRKY25 is involved in H_2O_2 response of *WRKY53* and *vice versa*. *ANAC092* responded most prominent also in 7-week-old leaves, similar to *WRKY53*. This response is also suppressed in the *wrky25* and in the *wrky53* leaves, and even more in the double mutant suggesting that both factors are involved in the H_2O_2 responsiveness of *ANAC092*. The same held true for *ZAT12* expression, here a higher basal expression could be observed in 7-week-old leaves of *wrky53* so that the H_2O_2 treatment did not lead to a further induction. In contrast, induction of *WRKY18* expression by H_2O_2 was much more pronounced in 5-week-old *wrky25*, *wrky53* and the double mutant compared to wildtype leaves, whereas the response was attenuated in older stages in all mutant lines. This supports the idea of a variable function of WRKY53 and WRKY25 on the *WRKY18* promoter: in early developmental stages, they act as repressors, in later stages as activators. Taken together, WRKY25 as well as WRKY53 are involved in H_2O_2 induction of variable genes including each other and, depending on the developmental stage of the plants; they can have opposing effects on the same gene promoters, again indicating a very complex regulatory interaction.

DISCUSSION

ROS, especially hydrogen peroxide, act as signaling molecules during senescence and/or stress responses. However, how this signal is perceived and transmitted into senescence onset and progression or stress response activation is still far from being understood. One of the central features of senescence is a massive change in the transcriptome, in which photosynthesis related genes are shut down and genes related to degradation and remobilization processes are turned on. Therefore, transcription factors would be ideal candidates to take up ROS signals directly. Indeed, for some transcription factors of different families such as class I TCP factors (Viola et al., 2013), HSF8 (Giesgut et al., 2015) or the bZIP factor GBF1 (Shaikhali et al., 2012), a redox-sensitive action has already been disclosed. Moreover, the plant specific protein GIP1 enhances DNA-binding activity of GBF3 and reduces DNA-binding activity of other members of the G-group bZIP factors in Arabidopsis, namely bZIP16, bZIP68, and GBF1, under non-reducing conditions through direct physical interaction. Whereas reduced GIP1 predominantly exists in a monomeric form and is involved in formation of DNA–protein complexes of G-group bZIPs, oxidized GIP1 is released from these complexes and instead performs chaperone function (Shaikhali, 2015). Due to space limitation, not all examples can be mentioned here, but taken together redox conditions can influence gene expression through the action of transcription factors in several ways: changing DNA-binding activity or activation potential or intracellular localization or interaction with specific partners or proteolytic degradation or a combination of those. He et al. (2018) just recently reviewed this topic very nicely. Here, we could show that WRKY25 DNA-binding activity is redox-sensitive, and that these redox-sensitive changes in activity are reversible as a function of the redox conditions (**Figure 2**). Not all WRKY factors show these features, as e.g. WRKY18 DNA-binding activity appears not to be redox-sensitive at all and WRKY53 DNA-binding activity was only very slightly influenced by changes in the redox environment (**Figure S2**). Redox sensitivity often relies on the alteration of the redox state of certain Cys residues. In WRKY25 belonging to the group I WRKY factors, two DNA-binding domains including $CX_{4-5}CX_{22-23}HXH$ zinc fingers are present. Moreover, an additional Cys can be found very closely to the N terminus, which cannot be detected in WRKY53 or WRKY18 proteins and could therefore be involved in redox sensitivity. Whereas WRKY53 has no other Cys besides the Cys of the zinc finger, WRKY18 has three additional Cys residues but is not redox sensitive at all (**Figure S9**). Hence, we speculate that either the two zinc fingers are necessary to confer redox sensitivity or an additional Cys has to be at a certain position within the protein to contribute to redox sensitivity. However, this will be subject of further investigations. Currently, we are mutating the additional Cys in WRKY25 to see whether this residue is involved or responsible for the redox sensitivity of WRKY25. Moreover, we will include an additional Cys at the N terminus of WRKY18 to see whether we can render WRKY18 redox sensitive. In contrast to WRKY18, which strongly binds to all W-boxes of the *WRKY53* promoter (Potschin et al., 2014), WRKY25 binds selectively to a specific W-box in the promoter of *WRKY53* and can positively influence its expression (**Figures 1** and **3**). Under oxidizing conditions, activation of *WRKY53* expression by WRKY25 is dampened (**Figure 3**). Even though binding and transactivation is lower under oxidizing conditions, WRKY25 is still involved in the response of the WRKY53 promoter to oxidizing conditions *in planta*, as H_2O_2 response of *WRKY53* was much lower in the *wrky25* mutants compared to WT plants, especially in later stages (**Figure 7**). At the first glance, this appears to be a contradiction, but as WRKY25 is also involved in down-regulation of H_2O_2 contents and negatively regulates its own expression, two negative feedback loops are at work. This indicates that WRKY25 function might be to prevent an overshooting of the reaction to H_2O_2. In addition, not only *WRKY53* response to oxidative stress appears to be attenuated by WRKY25 but also *ZAT12* and *ANAC092* response. In contrast, *WRKY18* reaction appears to be enhanced, but only in young plants (**Figure 7**). As already mentioned before, *WRKY25* expression is induced by H_2O_2, whereas WRKY25 at the same time reduces intracellular H_2O_2 contents, especially in later stages of senescence, as lower or higher H_2O_2 levels were measured in *WRKY25* overexpressing plants and *wrky25* mutant or knock-down lines, respectively (**Figures 6** and **7**). High H_2O_2 contents in later stages of senescence are most likely involved in membrane deterioration and lipid peroxidation processes as part of the senescence degradation processes

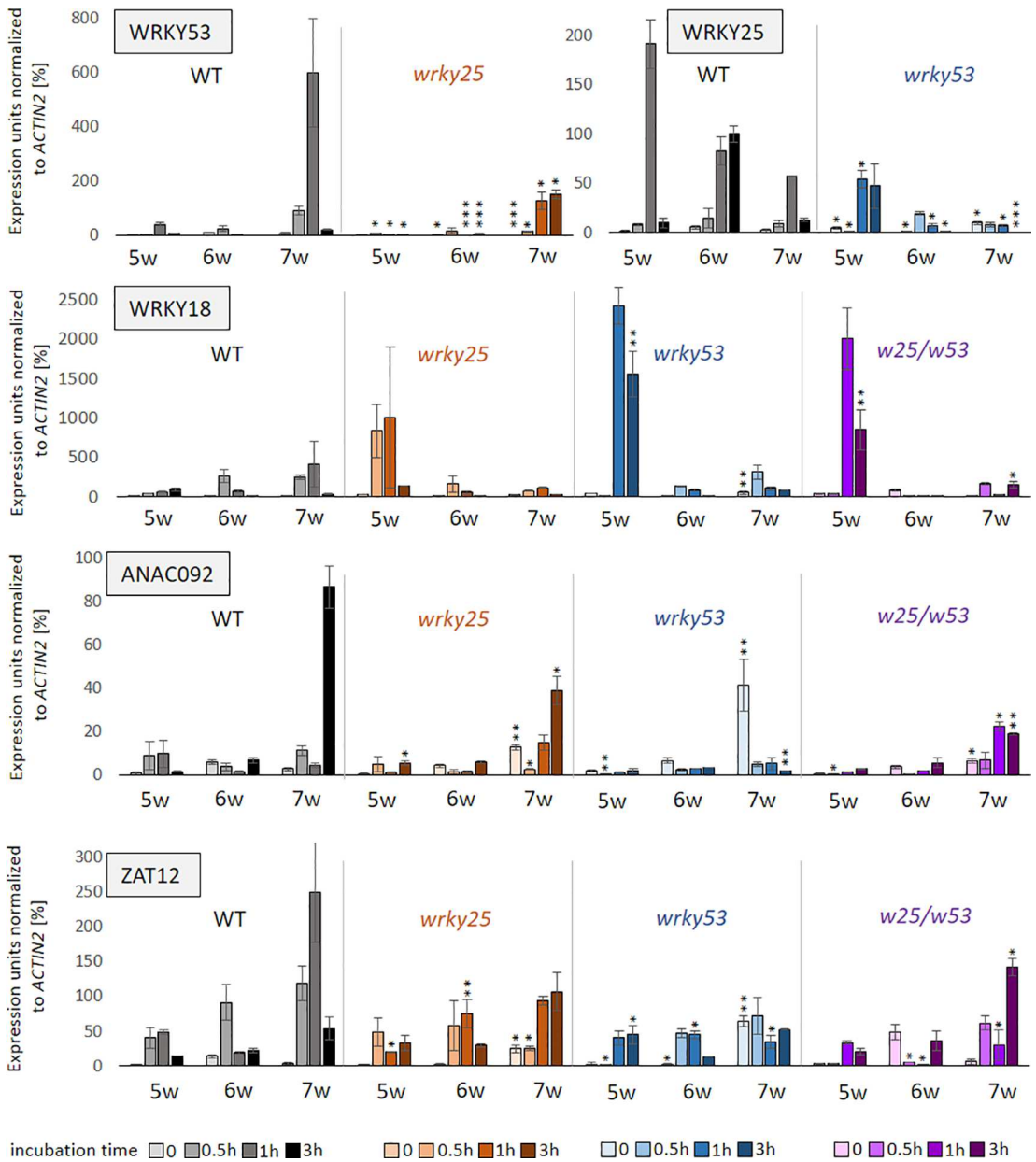

FIGURE 7 | Influence of oxidizing conditions on gene expression. Leaves of position 10 were harvested of six plants of Col-0 (WT), *wrky25* (orange) and *wrky53* (blue) single and double (purple) mutant plants, respectively. Leaves of 5, 6 and 7 week-old-plants were incubated in 10 mM H_2O_2 for 0–3 h. After incubation, three leaves were pooled and expression of *WRKY18, WRKY25*, *WRKY53*, *ZAT12* and *ANAC092* was determined by qRT-PCR in these two pools. Expression values normalized to *ACTIN2* expression are presented (mean values ± SD, n = 2, one biological replicate consists already of a pool of three plants for each timepoint). Kruskal–Wallis-test was performed for statistically significant differences of all values at each timepoint compared to Col-0 (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

(Chia et al., 1981). This is in line with the senescence acceleration or delay of the *WRKY25* overexpressing plants and *wrky25* mutant or knock-down lines, respectively (**Figure 4**, **Figure S8**).

A simple gene for gene relationship between *WRKY25* and *WRKY53* would suggest opposite phenotypes. As *WRKY53* has been characterized as positive regulator of leaf senescence (Miao et al., 2004), overexpression of *WRKY25* should lead to increased *WRKY53* levels and to the same senescence phenotype as *WRKY53* overexpression. *Vice versa*, knock-down or mutation of *WRKY25* should exhibit the same phenotype as knock-down or mutation of *WRKY53*. However, the senescence phenotype of *WRKY25* overexpressing plants and *wrky25* mutant or knock-down lines was found to be exactly opposite to the expected phenotype of a positive *WRKY53* regulator. This can be explained by the fact that WRKY25 and WRKY53 are not acting in a simple signal transduction pathway but in a

complex regulatory network between many members of the WRKY transcription factor family showing multilayer feedback regulations. In the same line of evidence, WRKY18 was characterized to be a negative up-stream regulator as well as a down-stream target and a protein interaction partner of WRKY53 (Potschin et al., 2014). Here, we could show that WRKY25 is also a redox sensitive up-stream regulator and down-stream target gene of WRKY53 (**Figures 3** and **7**). Moreover, WRKY25 appears to be involved in the H_2O_2 response of WRKY18 and WRKY53 expression but in opposite directions and at different times (**Figure 7**). In addition, MEKK1 action brings in a further layer of complexity. Co-expression of MEKK1 led to a reversal of WRKY18 action on *WRKY53* expression, since a 35S:*MEKK1* construct as co-effector to 35S:*WRKY18* reversed the repressor function of WRKY18 on the *WRKY53* promoter to an activator (**Figure S4A**). In contrast, the activator function of WRKY25 on the expression of the *WRKY53* promoter is enhanced approx. 2-fold by the addition of 35S:*MEKK1* as co-effector construct (**Figure 3C**). Whether this is due to a direct phosphorylation of WRKY25 by MEKK1, taking the same short cut as already shown for WRKY53 (Miao et al., 2007), or through classical MAPK signal transduction will be subject of further investigations. Noteworthy, WRKY25 and WRKY33 interact with many VQ proteins (Cheng et al., 2012), one of which is MKS1 (MAP KINASE SUBSTRATE 1), a substrate of MAPK4 (Andreasson et al., 2005). For WRKY33 it was shown that it exists in nuclear complexes with MPK4 and MKS1. Upon activation of MPK4 *via* MEKK1 and MKK1/2 signaling, MKS1 is phosphorylated by MPK4 and WRKY33 is released from MPK4 interaction and activates its downstream genes such as *PAD3* encoding an enzyme required for antimicrobial camalexin production (Qiu et al., 2008). Moreover, WRKY25 negatively regulates SA-mediated defense responses against *Pseudomonas syringae* (Zheng et al., 2007) and MPK4 is a repressor of SA-dependent defense responses (Petersen et al., 2000). Furthermore, MEKK1 kinase activity and protein stability is regulated by H_2O_2 in a proteasome-dependent manner and *mekk1* heterozygous mutants were compromised in ROS-induced MPK4 activation. Like WRKY25, MEKK1 regulates accumulation of intracellular H_2O_2 and alters expression of genes related to ROS signaling and homeostasis such as *ZAT12* (Nakagami et al., 2006). Like *WRKY25* and *WRKY53*, *MEKK1* expression is up-regulated by H_2O_2 treatment and mRNA levels start to increase with onset of senescence in parallel to *WRKY53* (Miao et al., 2007). Therefore, the influence of MEKK1 on the transactivation activity of WRKY25 provides another link to redox signaling. Moreover, we could show by conditional knock-down of *MEKK1* in plants that MEKK1 is part of the complex senescence regulation (**Figure S4**).

Expression of *WRKY25* is not only induced by oxidative stress but also during heat or salt stress. Moreover, *WRKY25* overexpressing plants were not only more tolerant to oxidative stress (**Figure 6**) but also to salt stress (Jiang and Deyholos, 2009) as well as to high temperatures (Li et al., 2011). During heat stress, WRKY25, WRKY26, and WRKY33 were positively cross-regulated, which confirms the complexity of the WRKY network (Li et al., 2011). Remarkably, ROS levels increase during salt and heat stress pointing to the possibility that *WRKY25* induction under salt and heat stress is mediated by oxidizing conditions. Many WRKY factors including WRKY25 and WRKY53 are up-regulated more than 5-fold in various plant lines with altered intracellular levels of specific ROS (Gadjev et al., 2006). In the same line of evidence, expression of *WRKY18*, *WRKY25* and *WRKY53* was also increased in *cat1,2,3* triple mutant plants (Su et al., 2018). Moreover, not only *WRKY25* gene expression and its DNA-binding activity are altered by higher ROS levels but WRKY25 is also involved in the regulation of the intracellular H_2O_2 content, especially in later stages of development (**Figure 6**) creating a feedback loop.

A further level of complexity is installed by epigenetic control of the WRKY gene expression. JMJ27, a jumonji-family demethylases, removes repressive H3K9me2 and H3K9me1 marks and thereby activates transcription. ChIP analysis revealed that the chromatin at the *WRKY25* promoter was hyper-methylated in *jmj27* mutants indicating that JMJ27 regulates *WRKY25* expression at least in part by directly controlling methylation levels of H3K9 histones (Dutta et al., 2017). *WRKY53* expression is also regulated by epigenetic changes in histone methylation (Ay et al., 2009). Moreover, the WRKY53 protein was detected in a complex with histone deacetylase 9 (HDA9) and POWERDRESS to recruit this complex to W-box containing promoter regions of key negative senescence regulators to remove H3 acetylation marks (Chen et al., 2016). Therefore, WRKY53 expression is regulated by epigenetic changes on its own promoter but the WRKY53 protein is also involved in changing epigenetic marks on other promoters.

We have summarized our data in a model, which describes a small subnetwork between WRKY18, WRKY25 and WRK53 and the role of H_2O_2 in this subnetwork at the onset of senescence (**Figure 8**). Several feedback loops are installed to control an overshooting of the system and to supply a high plasticity, which is needed to constantly integrate all kinds of incoming intracellular and environmental signals. The complex interactions within this subnetwork of just three WRKY factors illustrates the high complexity of the whole WRKY network, which is not only regulated by H_2O_2 as signaling molecule but also highly controlled by salicylic and jasmonic acid. Moreover, the WRKY network is just a subsection of the higher order regulatory network of leaf senescence. Nevertheless, understanding the regulation of single components or subnetworks will in the long run help to decipher the different mechanisms acting in the whole network and contribute to modeling approaches.

MATERIALS AND METHODS

Protein Expression and Extraction for DPI-ELISA

For protein expression of WRKY25 and WRKY53 in the *E. coli* strain BL21-SI, the coding sequences of *WRKY25* (1,182 bp) and

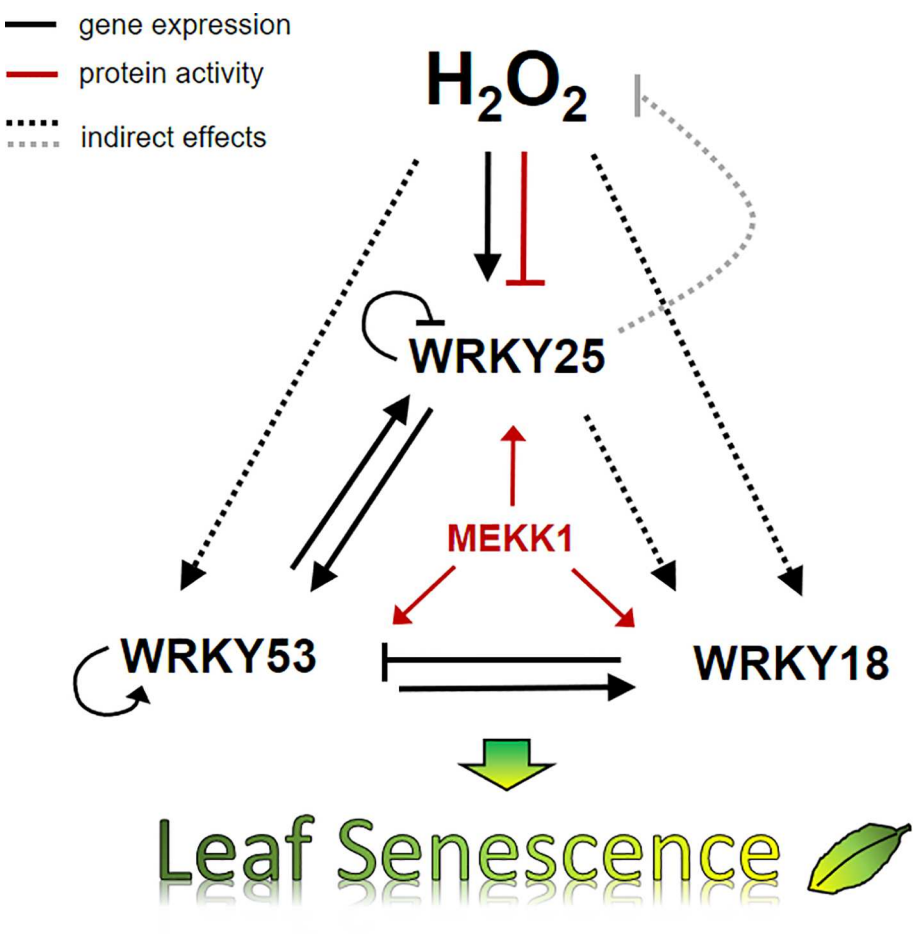

FIGURE 8 | Model of H_2O_2 and the WRKY18-53-25 subnetwork. A model summarizing the impact of H_2O_2 and WRKY25 on senescence in 7 week-old plants is presented. Solid lines show direct interactions whereas dotted lines show interaction, which may be direct or indirect. Black arrows describe the effects on gene expression, red arrows effects on protein activity, and the grey line effects on the intracellular hydrogen peroxide level. The expression of all three WRKY genes of the small WRKY53-25-18 subnetwork are controlled by hydrogen peroxide contents and hydrogen peroxide has a direct negative effect on the binding activity of WRKY25 to DNA. All three genes are under feedback control of their own gene products. In addition, MEKK1 increases the activity of all three WRKY factors. Moreover, WRKY25 can form heterodimers with WRKY53 and the heterodimer has a lower transactivation activity compared to the WRKY25 homodimer. This interplay determines in the end whether leaf senescence is accelerated or delayed.

WRKY53 (975 bp) were cloned into the vector pETG-10A to be coupled with an N-terminal fused 6×His-tag. The *E. coli* cells were grown in 10 ml selective medium overnight. One hundred milliliter LB-medium were inoculated with 3 ml of this pre-culture and, after shaking for 1.5 h at 37°C, a final concentration of 1 mM IPTG was added for induction of protein expression. After 1 h of shaking at 18°C, cells were harvested (2,500 g, 20 min, 4°C) and suspended in protein extraction buffer (4 mM Hepes pH 7.5, 100 mM KCl, 8% (v/v) glycerol, 1× complete proteinase inhibitor (*Roche*) without EDTA). Proteins were extracted by sonication to keep native conditions. The protein concentrations of the crude extracts were detected by Bradford assays (Bradford, 1976; *Bio-Rad*).

DPI-ELISA

The ELISA-based DNA–protein interaction assay was performed as described by Potschin et al. (2014). In brief, the 5' biotinylated double-stranded oligonucleotides were added to streptavidin-coated ELISA plates (*Thermo Scientific*). After blocking the plate with blocking solution *(Roche, blocking reagent for ELISA)*, crude extracts were diluted with protein dilution buffer (4 mM Hepes pH 7.5, 100 mM KCl, 8% (v/v) glycerol) and increasing protein concentrations (5, 10, 25 µg) were added to the DNA bound to the plates. The plates were incubated 1 h with mild shaking so that the biotinylated DNA–protein complexes were formed. Subsequently, wells were washed at least twice (*Qiagen blocking buffer*, *Anti-His-HRP conjugate kit*) and incubated for another hour with Anti His-HRP conjugate antibodies (*Qiagen*) diluted 1:1,500. After washing several times, positive interactions were detected by a peroxidase reaction with ortho-phenylenediamine (OPD-tablets, *Thermo Scientific*). The yellow color was measured using a plate-reader (*TECAN*, Safire XFluor4). For Redox-DPI-ELISAs, 25 µg of the protein crude extracts were used. Reduction or oxidation of the protein extracts was performed by adding

either DTT or H_2O_2 (final concentration 5 mM). In order to show reversibility of the redox effects, a fraction of the DTT-reduced proteins was oxidized again by addition of increasing amounts of H_2O_2 (final concentration 5, 10 and 20 mM). Similarly, the oxidized proteins were reduced again by adding increasing amounts of DTT. After these redox-treatments, a DPI-ELISA was performed as described above. To conserve the redox-state of the proteins bound to the biotinylated DNA, the same DTT or H_2O_2 concentrations were added to the washing buffer and the antibody solution. The antibody reaction was not altered by these treatments.

Protoplast Preparation and Transformation

Protoplasts derived from a cell culture of *A. thaliana* var. Columbia 0 were prepared following a standard protocol (for details see http://www.zmbp.uni-tuebingen.de/c-facilit/plant-transformation.html). These protoplasts were then treated with PEG1500 and transiently transformed with different constructs following the protocol published in Mehlhorn et al. (2018). Effector and reporter constructs were co-transfected with a luciferase construct for GUS reporter assays (for details see *GUS Reporter Assay*).

GUS Reporter Assay

Arabidopsis protoplasts were transformed using 5 µg of effector and 5 µg of reporter plasmid DNA. As an internal transformation control, a luciferase construct (pBT8-35SLUCm3) was co-transfected. After incubation overnight in the dark, GUS activity assays were performed with the protoplast as described by Jefferson et al. (1987). To correct for transformation efficiency, GUS activity was normalized to luciferase fluorescence. As effector constructs, the coding sequences of *WRKY25* (1182 bp), *WRKY53* (975 bp) and *MEKK1* (1827 bp) cloned into the vector pJAN33 were used. As reporter construct, a 3,000-bp-fragment upstream of the *WRKY25* start codon and a 2,759-bp-sequence upstream of the start codon of *WRKY53* was cloned into the binary vector pBGWFS7.0. The 3'-AT (3-Amino-1,2,4-triazole) GUS assays were performed as described above except that 10 mM 3'-AT or the same volume of water was added before overnight incubation.

Plant Material and Cultivation

All experiments were performed with *A. thaliana* Ecotype Columbia 0 (Col-0). Plants were grown on standard soil under long day conditions (16 h of light) with only moderate light intensity (60–100 µmol s^{-1} m^{-2}) in a climatic chamber at 20°C (day) and 18°C (night). Bolts and flowers developed within approx. 4–5 weeks. Individual leaf positions within the rosettes were coded with different colored threads, so that individual leaves could be analyzed according to their age even at very late stages of development (Hinderhofer and Zentgraf, 2001; Bresson et al., 2018). Numbering started with No. 1 for the first true leaf without taking the cotelydones into account. Plant material was harvested always at the same time to avoid circadian effects. The Nottingham Arabidopsis Stock Centre (NASC) kindly provided the T-DNA insertion line of *WRKY25* (SAIL_529_B11; previously characterized in Jiang and Deyholos, 2009), of *WRKY53* (SALK_034157, previously characterized in Miao et al., 2004), and *CAT2* (SALK_057998, previously described in Queval et al., 2007). Using PCR, homozygous plants were characterized with different combinations of gene specific and T-DNA left border primers. Double-knock-out mutants (*wrky25/wrky53*) were generated by crossing *wrky25* and *wrky53* mutants. F2 progenies were selected for homozygous double-knock-out plants by PCR. Dr. Changle Ma, Shangdong Normal University, China (Su et al., 2018), kindly provided seeds of the homozygous *wrky25/cat2* double-knock-out plants. The *WRKY25* overexpressing plants were transformed by floral dip of Col-0 wildtype plants into *Agrobacterium tumefaciens* cultures in two different attempts. First, a 35S:*WRKY25* construct was transformed leading to plants in which the transgene induced gene silencing (plant line 35S:*W25si*; pB7RWG2) and, therefore, was used as a *knock-down* line. Second, a *UBQ10:WRKY25* construct was transformed (plant line UBI:*W25-1* and UBI:*W25-2*; pUBN-GFP-Dest) and overexpression was confirmed by qRT-PCR. For the germination experiments, seeds of different plant lines were sterilized by sodium hypochlorite and plated on ½ MS-plates (1 L: 2.15 g MS micro and macro elements (Duchefa), 15 g sucrose, pH 5.7–5.8, 8 g agar).

Senescence Phenotyping

For the evaluation of leaf senescence phenotypes, rosette leaves were aligned according to the age of the leaves with the help of a color code and a variety of parameters indicating the state of senescence were measured (Bresson et al., 2018). Leaves of six plants per timepoint were analyzed. At position 5 and 10, Fv/Fm values were determined using the Imaging-Pulse-Amplitude-Modulation (PAM) method, indicating the activity of the photosystem II (PSII) (Chlorophyll fluorometer Maxi version; ver. 2-46i, Walz GmbH, Effeltrich, Germany). Leaves were photographed according to their age and by an automated colorimetric assay (ACA) pixelwise grouped into four categories: green leaves (green), leaves starting to get yellow (green-yellow), completely yellow leaves (yellow) and brown and/or dead leaves (brown/dead). (ACA; Bresson et al., 2018; http://www.zmbp.uni-tuebingen.de/gen-genetics/research-groups/zentgraf/resources.html)

In addition, RNA was extracted from leaves No. 6 and 7 and qRT-PCR analyses were performed for the senescence-associated marker genes *ANAC092* (At5g39610) encoding a NAC-domain transcription factor, *CAB1* (At1g29930) encoding a subunit of light-harvesting complex II, *SAG12* (At5g45890) encoding a cysteine protease and different WRKY genes (*WRKY53* (At4g23810), *WRKY18* (At4g31800), *WRKY40* (At1g80840)). Expression was normalized to *ACTIN2* (At3g18780).

Quantitative RT-PCR

Total RNA was extracted with the RNeasy Plant Mini Kit (*Qiagen*). Subsequent cDNA synthesis was performed with RevertAid Reverse Transcriptase (*Thermo Scientific*. For the qRT-PCR, KAPA SYBR® Fast Biorad iCycler (*KAPA Biosystems*) master mix was used following the manufacturer´s protocol. Expression of analyzed genes was normalized to

ACTIN2. In order to keep the results comparable to former results, we only used *ACTIN2* as reference gene since *ACTIN2* has been proven to be very stably expressed all over leaf development (Panchuck et al., 2005). Relative quantification to *ACTIN2* was calculated with the ΔΔCT-method according to Pfaffl (2001). Primers and Atg numbers are indicated in **Table S1**.

H_2O_2 Measurement and Treatments

For oxidative stress treatment during germination, 10 mM H_2O_2 was added to the 1/2 MS agar (1 L: 2.15 g MS micro and macro elements (*Duchefa*), 15 g sucrose, pH 5.7–5.8, 8 g agar) and seeds were spread on plates with and without H_2O_2. After 3 days in darkness, the plates were incubated in light in the climate chambers and the number of green seedlings was counted after 7–10 days. This experiment was repeated six times, plates were photographed, green seedlings counted and summarized according to their tolerance against H_2O_2 in a heat map.

For intracellular H_2O_2 measurement, carboxy-H_2DCFDA (2',7'-Dichlordihydrofluorescein-diacetat) was chosen, which is able to passively diffuse across cellular membranes as non-polar dye. After deacetylation by an intracellular esterase, the molecule gets polar and is trapped inside the cells. The deacetylated carboxy-H_2DCFDA can then be oxidized by H_2O_2 and converted to the highly fluorescent di-chlorofluorescein (DCF). Therefore, only intracellular H_2O_2 is measured. Leaves of position 8 of 4- to 8-week-old plants were harvested and incubated for exactly 45 min in carboxy-H_2DCFDA working-solution (200 µg in 40 ml MS-Medium pH 5.7–5.8). Subsequently, the leaves were rinsed with water and frozen in liquid nitrogen. After homogenization in 500 µl 40 mM Tris pH 7.0, the samples were centrifuged at 4°C for 15 min. Fluorescence of the supernatant was measured in a *Berthold* TriStar LB941 plate reader (480 nm excitation, 525 nm emission).

For testing the response to H_2O_2 treatment, leaves of position 8 of 5, 6 and 7-week-old plants were incubated for 0, 30 min, 1 h and 3 h in 10 mM H_2O_2 including 0.1% Tween. After incubation time, leaves were washed in water and immediately frozen in liquid nitrogen. Gene expression was determined by qRT-PCR.

The decomposition of H_2O_2 was examined using commercially available peroxide strips (Dosatest peroxide test strips 100, VWR Chemicals). Therefore, leaf discs were excelled of leaves of position 5 of 6- and 7-week-old plants and were incubated into a 30 mg/l H_2O_2 solution. Strips were submerged for 1 s into the solution immediately after placing the leaf disc into the solution (timepoint 0 min) and again after 2 h. The amount of peroxide can be read out by the given control color scale. The weaker the blue color the less peroxide is present in the solution.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

Conceptualization was done by UZ, MM and JD. Methodology was developed by MM and JD. Experiments were performed by JD, MM, LR, SN and H-CL. Data and formal analysis was done by JD, JB and LR. The original draft was written by UZ. Reviewing and editing was done by JD, LR, MM and UZ. Funding acquisition: UZ. Supervision: UZ.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01734/full#supplementary-material

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RESEARCH ARTICLE

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; Ülker 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;

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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-ZIPIIIs 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-ZIPIIIs 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 Wenkel, 2011; Wenkel 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. A

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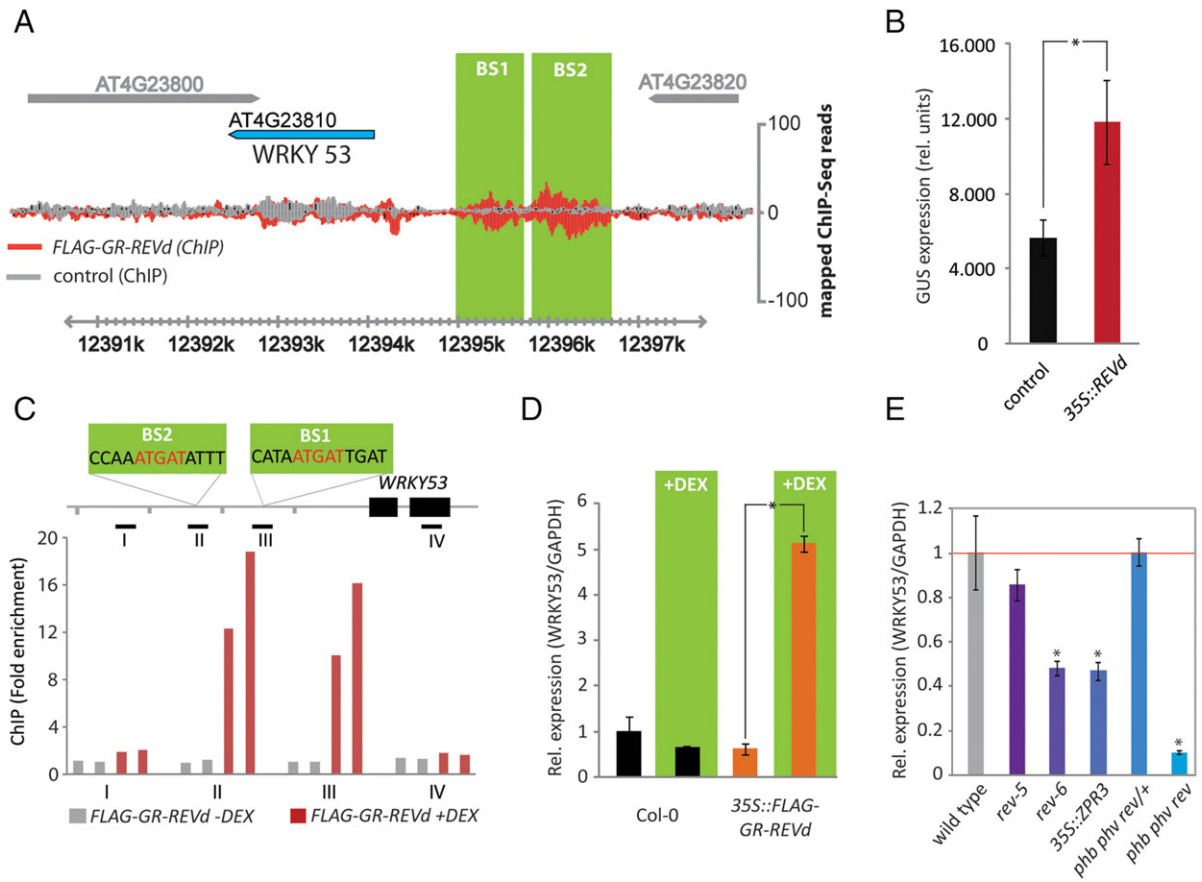

Fig. 1. REVOLUTA binds to the *WRKY53* promoter and is a direct and positive regulator of *WRKY53* expression. (A) ChIP-Seq results for the binding of REV to the *WRKY53* promoter. Two binding sites (BS) were identified, located −1.3 kb and −2.1 kb upstream of the transcriptional start site. Traces in gray are sequence reads derived from sequencing ChIP DNA from Col-0 wild-type plants; red plots ChIP DNA from dexamethasone-induced *35S::FLAG-GR-REVd* transgenic plants. (B) Transient expression assay in *Arabidopsis* protoplasts. A plasmid with a 2.8 kb *WRKY53* promoter fragment fused to the *GUS* gene was transformed along with a second plasmid containing a *CaMV35S*-promoter (control) or the *CaMV35S*-promoter driving expression of *REVd*. GUS activity was determined ~15 h after transformation. Data are mean±s.d. **P*<0.05. (C) Chromatin-immunoprecipitation qPCR experiments with two biological replicates for *35S::FLAG-GR-REVd* without DEX (gray bars) and *35S::FLAG-GR-REVd* with DEX (red bars) plants testing four positions in the *WRKY53* promoter. Y-axis shows the fold enrichment normalized to the non-induced IPs. Gene map above the chart shows the localization of the REV-binding site identified by ChIP-Seq and the regions that were tested. Distance between two marks along the chromosomes represents 1.0 kb. (D) Real-time quantitative PCR experiments showing expression changes of *WRKY53* in Col-0 (black) and *35S::GR-REVd* (orange) in response to 60 min DEX induction in the presence of the protein biosynthesis inhibitor cycloheximide (CHX). Data are mean±s.d. **P*<0.05. (E) Expression of *WRKY53* was analyzed in different *rev* mutant plants (*rev-5*, *rev-6*, *phb phv rev/+* and *phb phv rev*) and in plants with reduced activity of HD-ZIPIII proteins (*35S::ZPR3*). The bars indicate expression levels relative to wild type, including standard errors of the mean of three individual biological experiments. **P*<0.05.

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 (*rev5*, *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_2O_2 application in Col-0 wild-type plants. These changes of *WRKY53* mRNA levels were significantly dampened in *rev* mutant plants (*rev-5*) and *35S::miR165a*, 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). To assess which externally applied hydrogen peroxide concentration is able to elicit redox changes that would occur under natural conditions, we measured intracellular hydrogen peroxide levels after applying heat stress and compared them with the intracellular levels reached after external application of H_2O_2 by spraying. To be sure that only intracellular H_2O_2 is measured, we used non-fluorescent H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate), which is converted to the highly fluorescent 2′,7′-dichlorofluorescein upon cleavage of the acetate groups by intracellular esterases and

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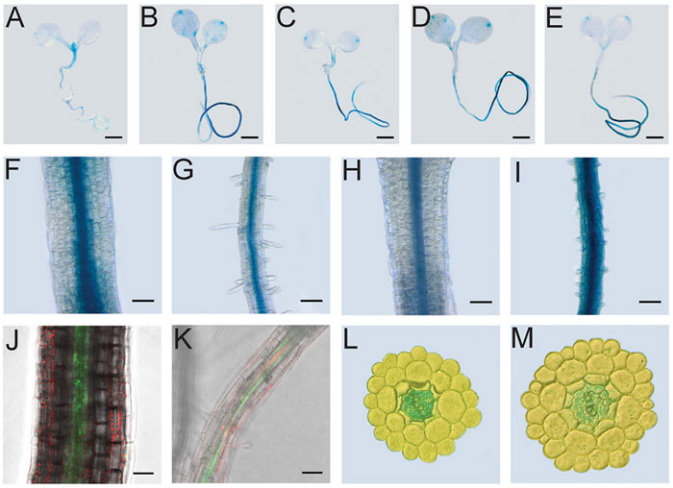

Fig. 2. Expression analysis of *REV* and *WRKY53*. (A-I) Spatial patterns of expression of *REV* (A,F,G) and *WRKY53* (B-E,H,I) in 8-day-old *Arabidopsis* seedlings. GUS staining of *REV::GUS* (A), *WRKY53::GUS* (B) in the Col-0 ecotype and *WRKY53::GUS, rev5* (C), *WRKY53::GUS, 35S::miR165* (D), *WRKY53::GUS, 35S::ZPR3* (E) seedlings. Scale bars: 1 mm. (F-I) Hypocotyls (F,H) and roots (G,I). (J,K) The pattern of GFP accumulation in the hypocotyl (J) and root (K) vascular tissue of 8-day-old plants carrying the *REV::REV-GFP* transgene. Scale bars: 50 µm. (L,M) Cross-sections of roots of 10-day-old seedlings reveal *REV* (L) and *WRKY53* (M) expression in the vascular cylinder.

subsequent oxidation. The increase in intracellular H_2O_2 was similar 1 h after heat treatment and 1 h after spraying 0.1% H_2O_2 but dropped more rapidly in the H_2O_2-treated samples. This indicates that external application of 0.1% H_2O_2 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 H_2O_2 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 H_2O_2) 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

		Microarray	ChIP-Seq				
AGI	Name	Fold change	q_rank	Enrichment	Distance	Location	DESG*
AT2G41940	ZFP8	2.0	469	7.5	1691	Down	Yes
AT5G47370	HAT2	3.1	253	8.0	1548	Up	No
AT2G39705	DVL11/RTFL8	2.8	1626	7.6	2509	Down	No
AT5G06710	HAT14	2.7	272	9.3	5364	Up	No
AT5G47180	Plant VAMP protein	1.7	35	15.2	168	Up	Yes
AT5G19590	DUF538 protein	1.3	465	9.4	2810	Up	Yes
AT4G18700	CIPK12	3.0	169	13.2	282	Down	No
AT4G27730	OPT6	2.7	30	16.1	1305	Up	No
AT4G03510	RMA1	7.0	33	14.9	1989	Up	No
AT1G17970	RING/U-Box protein	5.1	1173	9.7	8	Up	Yes
AT5G14730	DUF1645	5.8	726	6.6	2299	Up	No
AT2G45450	ZPR1	13.1	400	9.2		5′UTR	No
AT5G05690	CPD	1.9	202	8.0	4847	Up	No
AT1G74940	DUF581	2.2	106	17.5	81	Up	Yes
AT3G60390	HAT3	2.9	115	8.7	5597	Up	Yes
AT4G23810	WRKY53	3.9	450	8.7	2132	Up	Yes‡
AT5G16030	Unknown protein	2.8	789	9.6	2193	Up	Yes
AT1G49200	RING/U-Box protein	14.4	18	14.2	187	Up	No
AT2G02080	IDD4	0.5	528	8.2	8334	Down	No
AT3G13810	IDD11	0.4	1643	6.7	1422	Down	Yes

*Differentially expressed genes during senescence (Breeze et al., 2011).
‡Senescence-associated gene not included in the Breeze et al. (2011) analysis.

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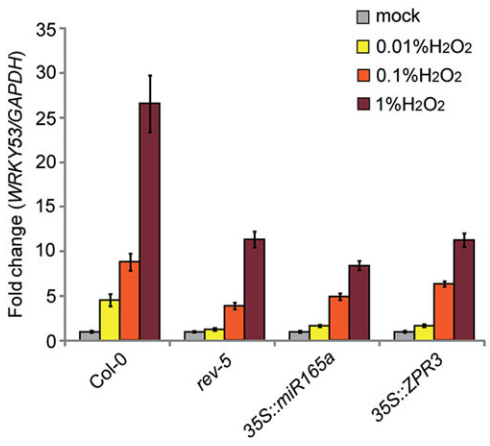

Fig. 3 HD-ZIPIII activity is required for H_2O_2-mediated upregulation of *WRKY53*. Real-time qPCR experiment showing *WRKY53* induction in response to hydrogen peroxide treatment in wild-type and *rev* mutant plants. Three-week-old plants were treated with different concentrations of H_2O_2 [0% (mock; gray bars), 0.01% (yellow bars), 0.1% (orange bars) and 1% (red bars)] for 40 min. Data are representative relative expression changes (fold change) of the mean of four technical replicates±s.d. Similar expression changes have been observed in at least two independent biological experiments.

are due to an influence of *E. coli* proteins on the activity of REV. To exclude such effects, we purified GST-REV protein from *E. coli* and performed *in vitro* gel retardation assays in the presence of reducing agents (DTT) and oxidizing agents (H_2O_2) (Fig. 4C). These gel-shift experiments largely confirm the results obtained by redox-DPI-ELISA and confirm that REV activity can be modulated by the intracellular redox state.

To validate redox-sensitive DNA binding *in planta*, we treated *35S::FLAG-GR-REVd* transgenic plants with either a mock substrate (0.1% ethanol), dexamethasone (DEX) or DEX+0.1% H_2O_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-ZIPIIIs, 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

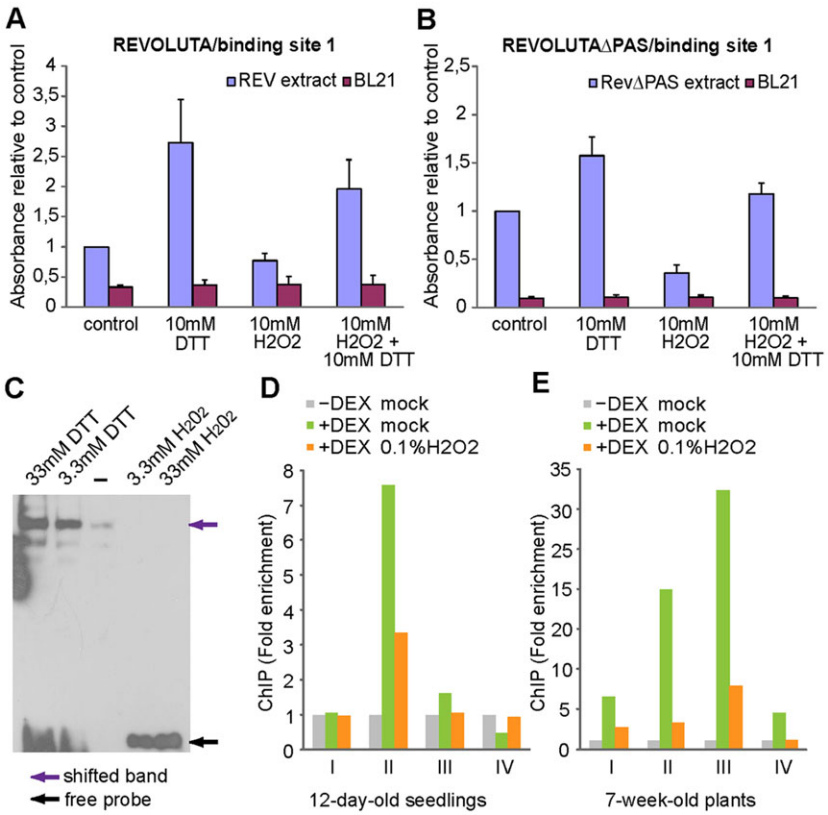

Fig. 4. Redox-mediated regulation of REVOLUTA-DNA-binding capability and influence of the PAS domain. (A,B) Redox-DPI-ELISAs. The DNA-protein interaction assays were performed by using 5′ biotinylated complementary annealed oligonucleotides coupled to a streptavidin-coated ELISA plate. Crude *E. coli* extracts (25 µg) expressing recombinant REV or REVΔPAS were pre-incubated with different concentrations of DTT and H_2O_2 to examine a redox state-dependent binding of REV. In order to test the reversibility of the redox effect, high concentrations of H_2O_2 were added first and then oxidizing conditions were reversed by addition of DTT. After binding, biotinylated DNA-protein complexes were detected using anti His-HRP conjugated antibodies. Results for REV binding site 1 of the *WRKY53* promoter are shown. *E. coli BL21* cells transformed with the empty vector were used as background control. (C) Non-radioactive electrophoretic mobility shift assays. Purified GST-REV protein was incubated with a biotinylated oligonucleotide containing the HB9-binding motif (Wenkel et al., 2007) in the presence of different redox conditions. After gel electrophoresis and subsequent blotting, the biotinylated DNA probe was detected with a HRP-strepatividin substrate. (D,E) Chromatin-immunoprecipitation qPCR assays of *35S::FLAG-GR-REVd* plants. Twelve-day-old seedlings (D) and 7-week-old transgenic plants (E) were treated with mock substrate (0.1% ethanol), DEX or 0.1% H_2O_2 and DEX. H_2O_2 was given 15 min prior to 45 min of DEX induction. Fold enrichment for the same primer sets as in Fig. 1 is shown.

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mediated by deregulation of *WRKY53* expression through HD-ZIPIIIs but also suggests that additional HD-ZIPIIIs 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*

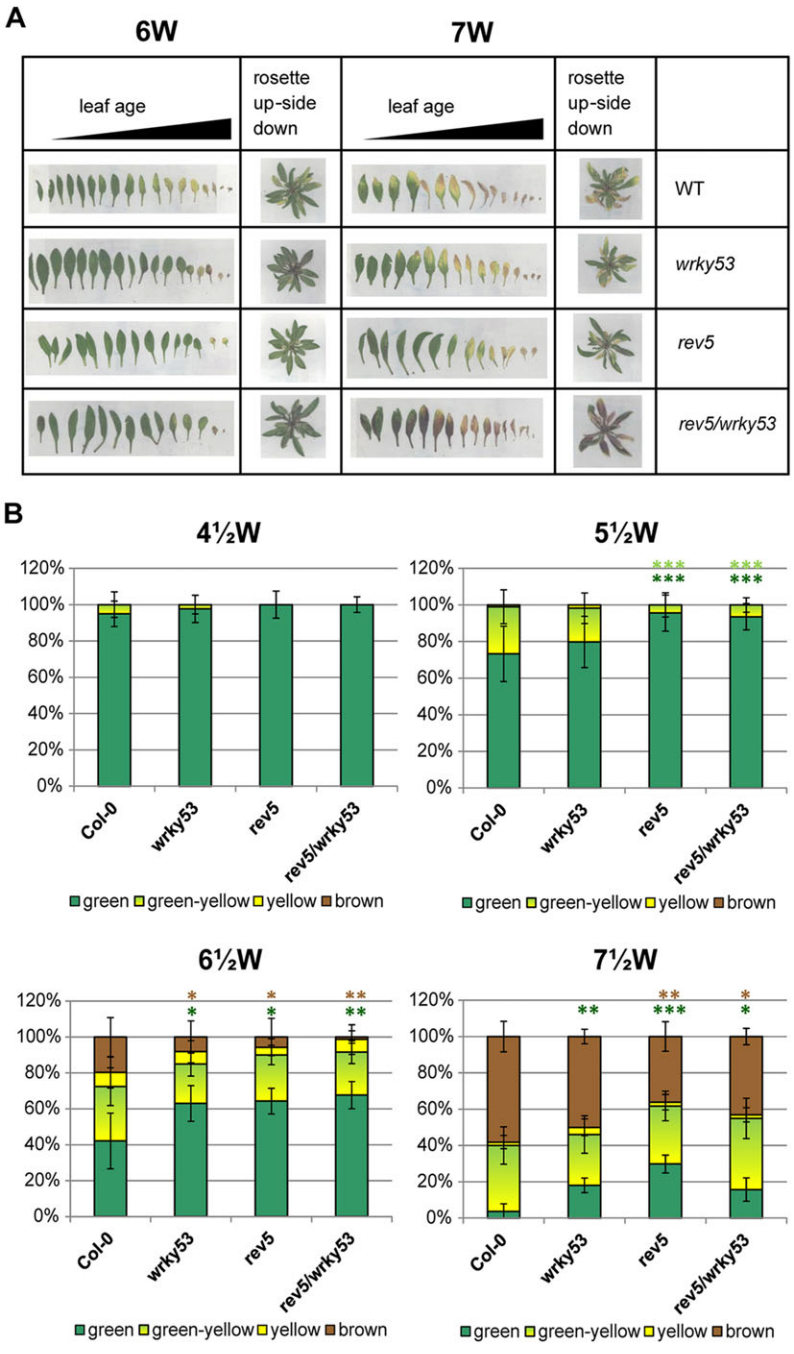

Fig. 5. Genetic interaction of *REV* with *WRKY53*. (A) Rosette leaves of 6- and 7-week-old representative plants were sorted according to their age; whole rosettes were also photographed upside down to visualize the older leaves. (B) For a quantitative evaluation of leaf senescence, plants were harvested 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.

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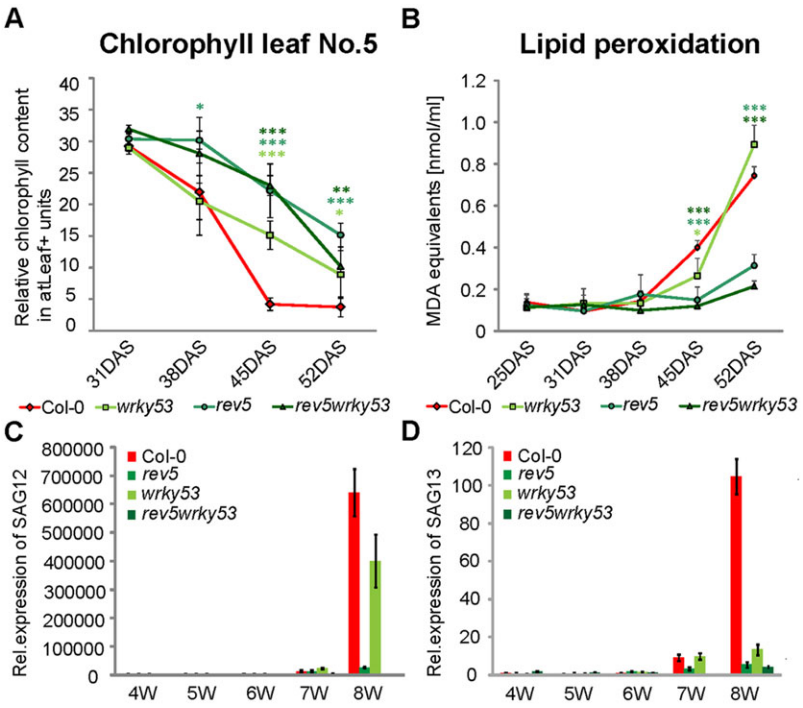

Fig. 6. Molecular senescence parameters. (A) Chlorophyll contents of number 5 leaves from *Arabidopsis* Col-0, *wrky53*, *rev5* and *rev5wrky53* plants. Left axis indicates atLeaf+ values. Plant age is indicated in days after seeding (DAS). (B) Lipid peroxidation in Col-0, *wrky53*, *rev5* and *rev5wrky53* 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.

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

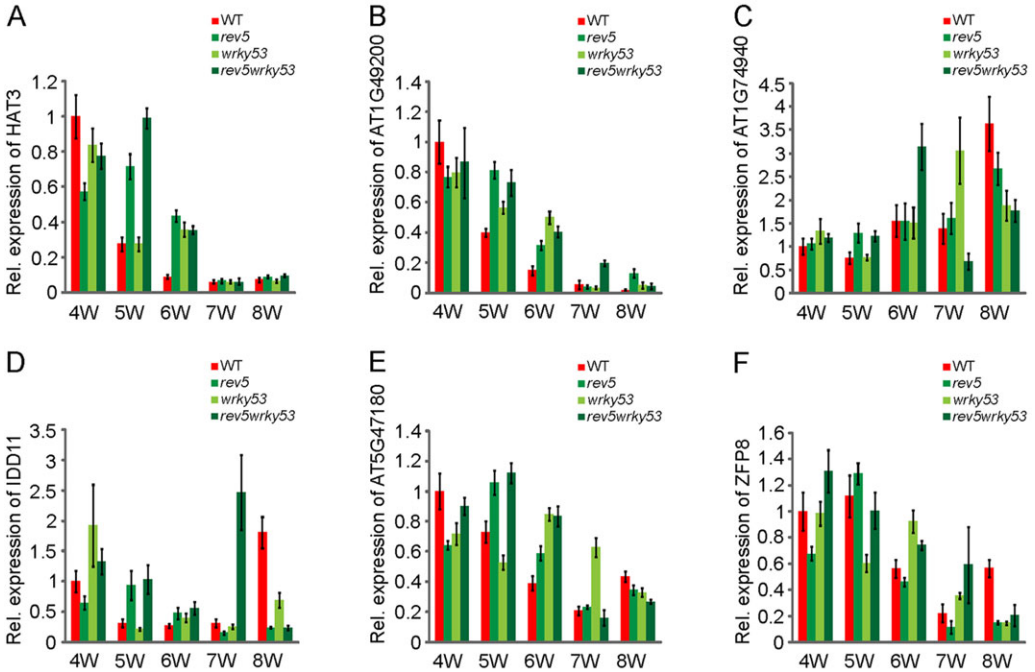

Fig. 7. qRT-PCR of other REV target genes differentially expressed during senescence. Quantitative real-time PCR profiling of putative REV target genes at late developmental stages in wild-type and mutant plants (4-, 5-, 6-, 7- and 8-week-old plants). (A-F) Expression changes over time of *HAT3*, *AT1G49200*, *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.

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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-ZIPIIIs 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.

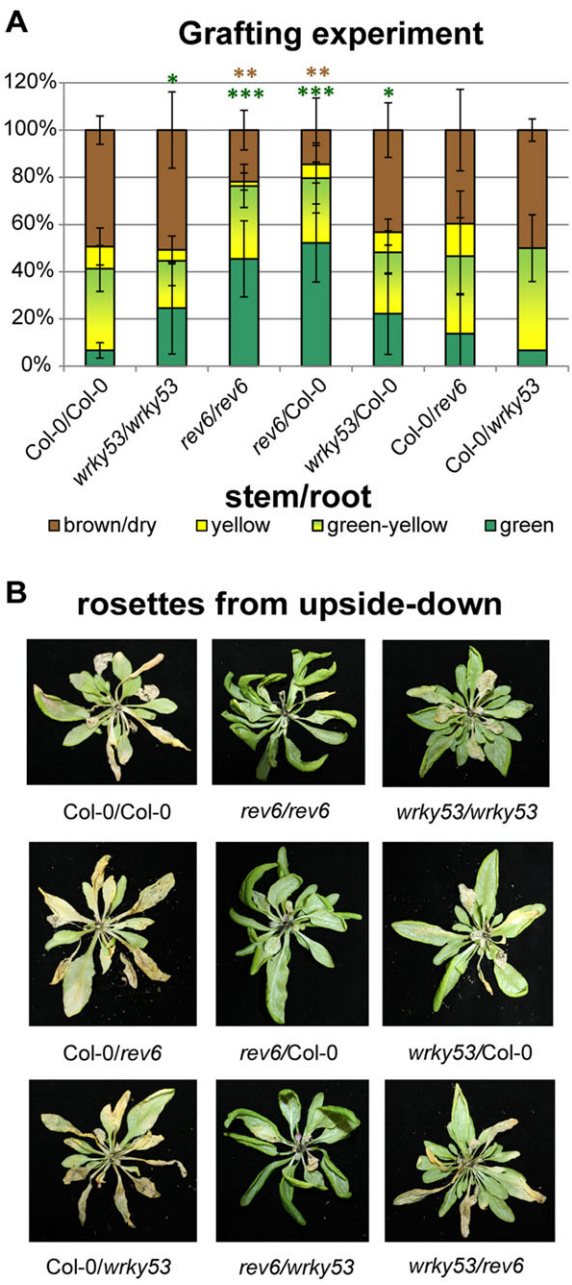

Fig. 8. Grafting experiments and senescence phenotype. (A) Nine combinations of grafted plants were generated between the wild-type and mutant plants (*rev6* and *wrky53*), including three self-grafted controls, e.g. wild type to wild type (Col-0/Col-0; scion/root). Error bars indicate s.d. (n=4-6 independent grafted plants with the exception of Col-0/wrky53, where we achieved only two successful grafts). The quantitative evaluation of leaf senescence of the non-grafted plants is shown in Fig. 5. Asterisks represent significant differences from the Col-0/Col-0 graft, as determined using Student's t-test (*P<0.05, **P<0.005, ***P<0.0005). (B) The leaf-senescence phenotypes of grafts. Photographs were taken 7 weeks after grafting.

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 now 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

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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-ZIPIII* 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-ZIPIIIs 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-ZIPIIIs 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-ZIPIIIs 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_2O_2 is more effective in senescence induction than peroxisomal or mitochondrial H_2O_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-ZIPIIIs 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

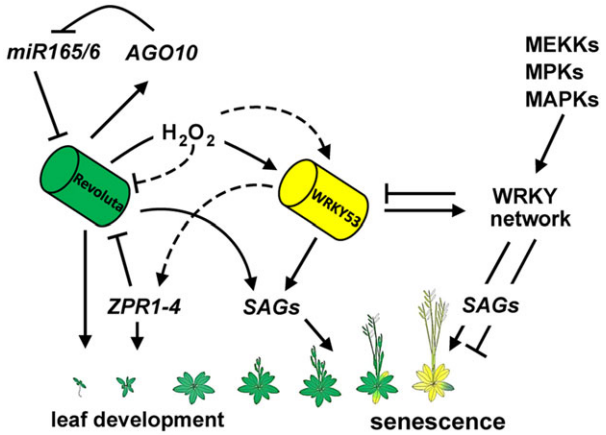

Fig. 9. Model HD-ZIPIII/senescence. A model summarizing our findings and showing the relationship between early leaf development processes and senescence. Both REV and WRKY53 intersect to regulate the late stages of leaf development.

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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 relies 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 mutants introgressed in Col-0 (Prigge 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 under long-day conditions (16 h of light) with only moderate light intensity (60-100 µmol s^{-1} m^{-2}) to slow down development for better analyses. Under these conditions, the plants developed bolts and flowers within 5-6 weeks. During growth and development of the leaves, the respective positions within the rosette were color coded with different colored threads, so that even at very late stages of development, individual leaves could be analyzed according to their age. Plants were harvested in a weekly rhythm and samples were always taken at the same time in the morning to avoid circadian effects. For the evaluation of leaf senescence phenotypes, leaves of at least six plants were categorized in four groups according to their leaf color: (1) 'green'; (2) leaves starting to get yellow from the tip as 'yellow-green'; (3) completely yellow leaves as 'yellow'; and (4) dry and/or brown leaves as 'brown/dry'. Exogenous hydrogen peroxide treatment was conducted by spraying 1%, 0.1% or 0.01% hydrogen peroxide solution including 0.1% Tween20. Grafting experiments were carried out according to Marsch-Martínez et al. (2013).

Intracellular hydrogen peroxide measurements

After stress treatment, leaf 7 (0.1% H_2O_2 treatment) and leaf 8 (heat stress, 2 h at 39°C) were harvested and incubated for exactly 45 min in DCFDA working-solution (2′,7′-dichlorodihydrofluorescein diacetate, 200 µg in 40 ml MS-Medium, pH 5.7-5.8). Leaves were then rinsed with water and 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 (Brand et al., 2010). Crude extracts were pre-incubated with different concentrations of DTT and H_2O_2 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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Supplementary material

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bZIPs and WRKYs: two large transcription factor families executing two different functional strategies

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bZIPs and WRKYs are two important plant transcription factor (TF) families regulating diverse developmental and stress-related processes. Since a partial overlap in these biological processes is obvious, it can be speculated that they fulfill non-redundant functions in a complex regulatory network. Here, we focus on the regulatory mechanisms that are so far described for bZIPs and WRKYs. bZIP factors need to heterodimerize for DNA-binding and regulation of transcription, and based on a bioinformatics approach, bZIPs can build up more than the double of protein interactions than WRKYs. In contrast, an enrichment of the WRKY DNA-binding motifs can be found in WRKY promoters, a phenomenon which is not observed for the bZIP family. Thus, the two TF families follow two different functional strategies in which WRKYs regulate each other's transcription in a transcriptional network whereas bZIP action relies on intensive heterodimerization.

Keywords: bZIPs, WRKYs, DNA-binding, heterodimerization, regulatory mechanisms, G/C box accumulation, W-box accumulation

INTRODUCTION

Due to their sessile nature, plants cannot move to avoid unfavorable conditions as animals do, thus they are forced to cope with their immediate environment, whatever this is. Since the potential environmental variability covers a continuum range from the optimal growth conditions to the toughest stress, a complementary number of possible physiological responses have evolved in order to respond in the most convenient manner to any possible scenario. This process involves transcription factor (TF) networks modulating the expression of a huge number of responding genes.

Unraveling how these networks operate is a major field in plant research, since the comprehensive understanding of the regulatory circuits will allow us to modify them in a beneficial way in the current context of growing food demand and global climate change. Many efforts are focused on deciphering the structure of specific networks by identifying up- and downstream components, however, the comparative analysis of the general features of the regulation of whole families of TFs is still challenging. Granted that TFs within the same family are evolutionary closely related, they are likely regulated by common mechanisms. The recognition of these strategies, shared by entire families of TFs, can provide useful clues to better characterize the function of members of these families.

In this review, we summarize the major regulatory mechanisms characterized so far for WRKYs and bZIPs, two of the largest TF families in plants. Although they have a comparable size, 75 bZIPs and 76 WRKYs can be found in the TAIR database, and they regulate critical physiological processes, such as plant defense, stress responses, or development including senescence; they appear to follow different regulatory strategies. Whereas WRKYs are strongly regulated at the transcriptional level by each other, bZIPs are regulated predominantly at the post-translational level via the formation of heterodimers. This distinction can be inferred from a bioinformatics approach whereby all the *Arabidopsis* bZIPs and WRKYs IDs gathered from the TAIR were used as an input for the *Arabidopsis* Interaction Viewer in the BAR webpage (http://bar.utoronto.ca/welcome.htm). The 76 WRKYs resulted in 170 interactions, while the 75 bZIPs yielded in 389, more than the double than WRKYs. In addition, the WRKY binding motifs (W-boxes) are found to be enriched in the WRKY gene promoters compared to the average occurrence over all *Arabidopsis* genes. In comparison, C- and G-boxes, the preferred bZIP binding motifs in plants, are not enriched in the bZIP promoters.

THE bZIP TFs AND THEIR REGULATION

This family of dimeric TFs is present in all eukaryotes, from *Saccharomyces cerevisiae* (17 bZIP genes) to human (56 bZIP genes).

Abbreviations: ABAR, magnesium-protoporphyrin IX chelatase H subunit (CHLH)/putative ABA receptor; ABF, ABA responsive element binding factor; ABI, ABA-insensitive; AREB, abscisic acid responsive elements-binding protein; BBX25, B-box domain protein 25; bZIP, basic region/leucine zipper; CCA1, circadian clock associated 1; CKII, casein kinase II; COP1, constitutive photomorphogenic 1; EEL, enhanced em level; EmBP-2, ABRE-binding factor Embp-2 (*Zea mays*); FLS2, flagellin-sensitive 2; GBF, G-box binding factor; HDA19, histone deacetylase 19; HIS1, histone H1; HY5, elongated hypocotyl 5; HYH, HY5 homolog; ICS1, isochorismate synthase 1; LSD1, lesions simulating disease resistance 1; MEKK, MAP kinase kinase kinase; MKK, MAP kinase kinase; MPK, MAP kinase; NPR1, nonexpressor of PR genes 1; OREB1, ABRE binding factor OREB1 (*Oryza sativa*); PR1, pathogenesis related 1; RBCS1a, ribulose bisphosphate carboxylase small chain 1a; SIB, sigma factor-interacting protein; SIPK, salicylic acid-induced protein kinase; SnRK, SNF1-related protein kinase; *SUVH2*, su(var)3-9 homolog 2; TGA, TGA factor family; TRAB1, ABRE binding factor TRAB1 (*Oryza sativa*); WRKY, WRKY transcription factor; YAP, yeast AP-1-like transcription factor; ZmBZ-1, bZIP transcription factor 1 (*Zea mays*).

bZIPs have been described in *Arabidopsis* (75), rice (89), sorghum (92), soybean (131), and recently in maize (125; Wei et al., 2012). In plants, they are involved in important processes such as pathogen defense (Alves et al., 2013), abiotic stress signaling (Fujita et al., 2005), hormone signaling (Choi et al., 2000), energy metabolism (Baena-González et al., 2007), as well as development, including flowering (Abe et al., 2005), senescence (Smykowski et al., 2010), and seedling maturation (Alonso et al., 2009).

The name of the family is derived from the basic region/leucine zipper (bZIP) domain present in all its members. This domain consists of an uninterrupted α-helix comprising a basic region (BR) which is necessary and sufficient to bind the DNA, followed by a C-terminal leucine zipper (LZ) motif responsible for the dimerization (Schumacher et al., 2000; Miller et al., 2003). The bZIP family was subdivided according to sequence similarities and functional features resulting in 10 groups named A to I, plus S in *Arabidopsis* (Jakoby et al., 2002; Nijhawan et al., 2007; Wei et al., 2012). While many bZIPs can form homodimers, bZIP members classified in different groups can be combined through heterodimerization to form specific bZIP pairs with distinct functionalities.

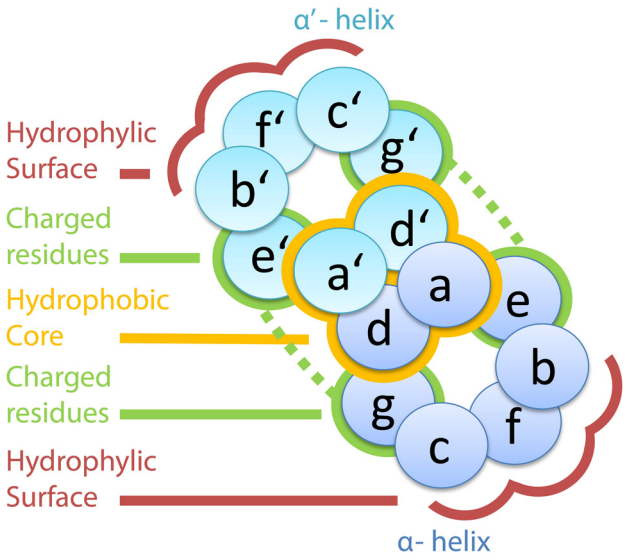

FIGURE 1 | Outline of a section through two a helices interacting via leucine zippers. The amino acids in positions ***a*** and ***d*** configure the hydrophobic core, which is indicated with the yellow halo. Charged residues in positions ***e*** and ***g*** generate electrostatic forces, represented by the dashed green lines. The hydrophilic surface is formed by the amino acids in positions ***b***, ***c***, and ***f***.

THE bZIP STRUCTURE DETERMINES THE DIMERIZATION SPECIFICITY

By means of dimerization, a limited number of monomers can generate a wide pool of different dimers with singular properties, thereby expanding the repertoire of regulatory responses (Amoutzias et al., 2006, 2008). However, protein interaction has to be selective in order to grant the appropriate response to each situation. In agreement to that, Newman and Keating (2003) showed that, in human and yeast, only 15% of all possible interactions actually take place between bZIP proteins. This specificity relies on the constitution of the LZ, which is composed of structural repetitions of the so called heptads. In each heptad, seven amino acids are arranged around two α-helix turns, in which two definite positions are occupied by leucines or other hydrophobic amino acids. These residues expose their side chains to the same side of the helix, thus resulting in an amphipathic structure. Based on this conformation, hydrophobic forces created between the non-polar sides of two LZs drive their dimerization (Vinson et al., 2002). However, the remaining composition of the heptad is decisive in determining if the interaction will actually take place.

Understanding the forces governing the bZIP dimerization has been a field of intensive research in recent years. To this end, the amino acid positions within a heptad are designated by a specific nomenclature with a letter ranging from ***a*** to ***g*** (Deppmann et al., 2006). According to that, positions ***d*** and ***a*** carry the hydrophobic residues and define the hydrophobic face; whereas positions ***b***, ***c***, and ***f*** are located on the opposite side, the hydrophilic one (**Figure 1**). Based on this codification and the already described interactions, rules governing the interaction have been formulated (Vinson et al., 2002) and even methods for dimer predictions have been created (Fong et al., 2004). Accordingly, the amino acids in positions ***a***, ***d***, ***e***, and ***g*** are the ones with a greater impact on determination of the specificity of the interaction (Deppmann et al., 2004). First, the primary hydrophobic forces are established between ***a*** and ***d*** positions of a heptad and their counterparts in the other LZ disposed in parallel (Vinson et al., 2002), in which the presence of leucines in the ***d*** positions is the most stabilizing factor for the dimerization (Moitra et al., 1997). Next, ***a–a′*** interactions contribute in determining the homodimerizing partners: asparagine residues in this position tend to interact rather with another asparagine, thus favoring the homodimer formation. Conversely, if this position is occupied by a lysine or serine, the heterodimer is favored as these two amino acids prefer residues other than themselves (Acharya et al., 2002). In addition, positions ***e*** and ***g*** are stabilizing the helix. These two positions act crosswise, so that ***e*** positions of one helix interact with ***g*** positions on the other one, and usually carry charged or polar amino acids. As a consequence, depending on the charge of these residues, attractive or repulsive forces are formed between the two LZs (Krylov et al., 1994). Overall, the amino acid composition of the LZ determines the energy of the interaction, making each dimer combination more or less likely to happen (Vinson et al., 2006).

Under the above mentioned rules, *Arabidopsis* bZIPs are predicted to form, almost exclusively, homodimers or quasihomodimers (dimers between two paralogs; Deppmann et al., 2006). Dimerization between bZIPs belonging to the G group (Shen et al., 2008), H group (Holm et al., 2002) or A group (Bensmihen et al., 2002) are in agreement with these predictions. In addition, bZIP are also able to heterodimerize specifically, as the following examples illustrate. The E group members bZIP34 and bZIP61 are unable to homodimerize due to the presence of a proline residue in their LZ, nevertheless they do form heterodimers with the bZIP51 (I group) or the bZIP43 (S group; Shen et al., 2007); G-box binding factor 4 (GBF4), belonging to the A group, interacts with members of the G group (Menkens and Cashmore, 1994); members of the

H group can heterodimerize with the G group bZIP GBF1 (Babu Rajendra Prasad et al., 2012); or even a whole heterodimerization network involving bZIPs from C and S groups has been described (Ehlert et al., 2006).

The preference of bZIPs to interact with more related partners reflects the selectivity of the dimerization. Because they perform similar, even overlapping, functions and can bind to the same *cis*-elements (Jakoby et al., 2002); they can interact laxly, for their ultimate function is not altered to a great extent. In contrast, heterodimerization between bZIPs which are more evolutionary distant is more restricted, as it brings together monomers with more disparate properties. Therefore, the specificity of the partner selection is of central importance because the composition of the dimer will define decisive functionalities such as transactivation potential or DNA-binding activity.

THE bZIP DIMER COMPOSITION DETERMINES THE DNA BINDING

The DNA recognition by bZIPs takes place with the two continuous α-helices wrapped around their LZ regions and pulled apart slightly on their N-terminus, forming a Y-shaped structure which embraces the DNA duplex (**Figure 2**). In this complex, each BR contacts the DNA along the major groove on opposite sides of the double helix, so that each monomer binds one-half of the DNA target sequence (Glover and Harrison, 1995). As a consequence of the dimeric arrangement of the bZIPs, the binding properties of each dimer are determined by its singular monomer composition.

The target sequences preferentially bound by bZIPs are palindromic or pseudo-palindromic hexamers with an ACGT core (Foster et al., 1994). The positions within a hexamer are designated with a number as established by Oliphant et al. (1989). Under this code, the bases are given a number radiating from the central positions, i.e., CG, which are both referred as 0. So, the 5′ half of the sequence are negative values, while the 3′ half are positive. Based on the nucleotide position +2, different kinds of ACGT-containing elements are classified as A-box, C-box, G-box, or T-box; among which C and/or G-boxes are preferentially bound by plant bZIPs (Izawa et al., 1993). Furthermore, the protein binding affinity is determined to a great extent by the nucleotides flanking the hexamer (Williams et al., 1992). The specificity of the DNA recognition arises, thus, as a result of variations in the *cis*-element sequence combined with the existence of unique BRs mixtures able to discriminate them.

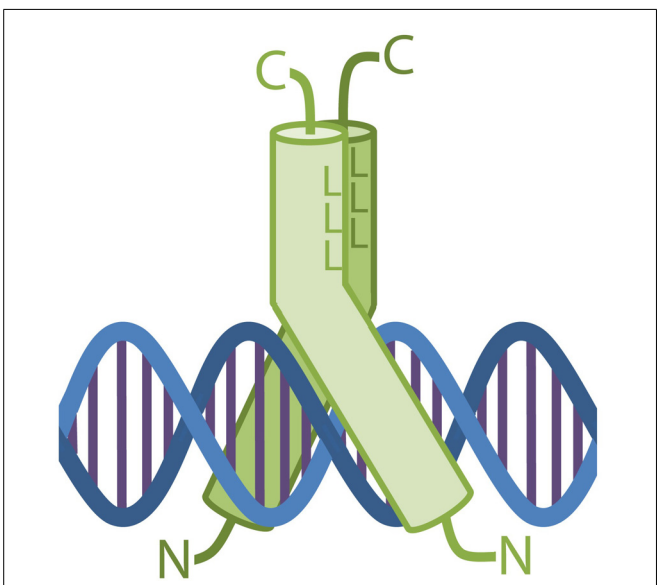

FIGURE 2 | Schematic drawing of a bZIP dimer bound to the DNA. The two proteins form a Y-shape structure which embraces a perpendicularly disposed DNA molecule. The major groove is contacted by both bZIPs via their DNA-binding domains. L represents the leucines forming the interface in the bZIP dimer.

Where exactly the specificity of the interaction relies on has been revealed from solved structures of bZIPs bound to DNA. The target sequence is contacted by only five residues in each BR all along 12 bp in the major groove and these contacts are extended by water molecules (Fujii et al., 2000). These key positions form part of an invariant sequence of nine amino acids (N-X7-R/K) which feature the BR. Granted that the BR is the most conserved region in bZIPs, the binding preferences of each monomer are determined by only subtle differences in its sequence. For instance, in mouse, bZIPs belonging to the CCAAT/enhancer-binding proteins (C/EBP) family, carry a valine residue in the position 5 of the signature sequence which discriminates against purines at position −3 of the DNA binding site (Miller et al., 2003). Therefore, unspecific interactions with similar DNA sequences are prevented by this single residue. In another case, in the AP-1-like TF (YAP) and CAMP response element binding protein-2 (CREB2) subfamilies of bZIPs in *Schizosaccharomyces pombe*, the presence of a hydrophobic residue in position 8 of the invariant sequence favors the contact with their AT-rich binding site targets (Fujii et al., 2000).

Besides the identity of amino acids directly contacting the DNA, the specificity of the DNA recognition is further modified by functional variability of the amino acids in the BR. This means that they can adopt different conformations depending on the accompanying residues, which creates a different set of contacts with the DNA bases (Miller et al., 2003). Beyond the BR, it is also known that the hinge region, the junction between the LZ and the BR, participates in determining the DNA-binding specificity (Niu et al., 1999). Likewise, the presence of ions between the dimer and the DNA (Schumacher et al., 2000), the redox status (Shaikhali et al., 2012), or even the DNA flexibility (Konig and Richmond, 1993) also affect the DNA-binding. On top of that, the BR is intrinsically unstructured in absence of DNA and the folding is only induced upon association with the double helix (Seldeen et al., 2008). Such lack of definite conformation allows the interaction with multiple *cis*-elements and facilitates post-translational modifications by better exposing the lateral chains, enhancing the regulatory possibilities (Dyson and Wright, 2005).

Granted that the specificity of the DNA recognition arises from the contribution of each BR individually, heterodimerization determines the manner in which the bZIP pairs recognize their target sequences. Through specific heterodimer formation, for example, the binding activity of bZIP53 to the albumin 2S2 promoter is significantly enhanced when combined with bZIP25 or bZIP10 (Alonso et al., 2009). Conversely, other bZIPs lose their

DNA-binding when associated to particular partners, as bZIP1, whose DNA-binding activity is prevented in combination with bZIP63 or bZIP10 (Kang et al., 2010).

THE DIMER COMPOSITION DETERMINES THE TRANSACTIVATION PROPERTIES

In addition to their role in the DNA recognition, each monomer contributes individually to the transactivation capacity (Miotto and Struhl, 2006). While some bZIPs have special domains acting as transactivators or as repressors, e.g., the proline rich domain in the G group (Shen et al., 2008), others require the presence of additional elements, such as coactivators (Rochon et al., 2006) or histone deacetylases (Kuo et al., 2000). Besides, the transactivation activity of the same bZIP can be further modified through the interaction with other proteins, e.g., the transactivation capacity of ELONGATED HYPOCOTYL 5 (HY5) is enhanced by the clock protein CCA1 (Andronis et al., 2008), but is inhibited when interacting with BBX25 (Gangappa et al., 2013). As a result of the peculiar transactivation properties of each bZIP, the composition of the dimer determines the outcome of target gene expression.

More important, if different dimers can affect the expression in a peculiar manner, they can compete for the same *cis*-element with other bZIP pairs, constituting an efficient mechanism to adjust the expression of a given gene. Such a system has been described controlling the expression of late-embryogenesis abundant genes in *Arabidopsis* by the A group bZIPs ABA-insensitive 5 (ABI5) and EEL. These two bZIPs compete for the same binding site, conferring antagonistic transactivation functions: ABI5 homodimers activate the gene expression, whereas EEL homodimer and ABI5–EEL heterodimer repress it (Bensmihen et al., 2002). Furthermore, gradation of the expression can be achieved through the formation of different heterodimers. So is the expression of RBCS1a modulated by HY5, HY5 homolog (HYH), and GBF1 in which GBF1 acts a repressor, HY5 and HYH act as inducers. However, the different heterodimers that can be formed show intermediate effects depending on the pair of monomers combined (Singh et al., 2012). In other cases, functional cooperation between monomers is established instead of competition. Indeed, heterodimerization appears to be a requirement for the induction of genes under control of bZIPs belonging to the C/S1 network. In other words, while these bZIPs are not able to activate the gene expression by themselves alone, certain heterodimers result in a strong activation of specific target genes (Weltmeier et al., 2006).

MONOMER AVAILABILITY IS A HOT SPOT IN bZIP REGULATION

Having established how relevant the identity of the monomers in each dimer is, the availability of monomers arises as a key point of regulation restricting the number of interactions that can take place. The expression of bZIP genes is, indeed, adjusted to control their abundance, showing tissue specificity (Fujita et al., 2005; Iwata et al., 2008; Weltmeier et al., 2008; Alonso et al., 2009), as well as developmentally regulated expression, including embryogenesis (Bensmihen et al., 2002; Weltmeier et al., 2008), flowering (Abe et al., 2005), or senescence (Breeze et al., 2011). Furthermore, changes in expression of bZIPs have been reported upon exposure to certain stresses. For example, Zn deficiency increases the transcription of bZIP23 and bZIP19 (Assunção et al., 2010), bZIP53 and bZIP10 are induced after an osmotic stress period (Weltmeier et al., 2006). The ABA-responsive element binding protein (AREB) subfamily of bZIPs is up-regulated by drought and salt in *Arabidopsis* (Uno et al., 2000) as well as in tomato (Hsieh et al., 2010; Orellana et al., 2010). Besides, a remarkable amount of studies relate changes in the bZIP expression to the energy status. These include the repression of bZIP1 and bZIP63 by sugars (Kang et al., 2010; Matiolli et al., 2011) and bZIP11 by darkness (Rook et al., 1998), or the induction of the expression of several bZIPs by the activation of the energy deficiency-related kinase SNF1-related protein kinase 1 (SnRK1; Baena-González et al., 2007). Beyond the transcriptional level, bZIPs are regulated by alternative splicing (Zou et al., 2007) and by controlling the translation initiation, e.g., the repression of translation by sucrose in bZIPs belonging to the subgroup S1 in *Arabidopsis* (bZIP1, bZIP2, bZIP11, bZIP44, bZIP53; Wiese et al., 2004; Weltmeier et al., 2008).

After the protein synthesis, specific control of the protein turnover has been found regulating the abundance of some bZIPs such as GBF1 (Mallappa et al., 2008), ABF1 and ABF3 (Chen et al., 2013), or TGAs (Pontier et al., 2002). In addition, the amount of functionally active monomers in the nucleus is regulated by subcellular partitioning. In order to be targeted to the nucleus, bZIPs carry a nuclear localization signal (NLS) which is located within the BR, overlapping with the invariant DNA binding sequence and consisting of two clusters of lysines/arginines (Miller, 2009). Nevertheless, few bZIPs have been found outside of the nucleus being retained by different means. For instance, bZIP10 is retained in the cytoplasm by the zinc-finger protein lesions simulating disease resistance 1 (LSD1). This protein interferes with the NLS-mediated nuclear import of bZIP10 (Kaminaka et al., 2006). In other cases, bZIPs are actively shuttled out of the nucleus due to the presence of a nuclear export signal (NES; Tsugama et al., 2012) and they stay in the nucleus only when the NES gets masked (Li et al., 2005). Finally, extra-nuclear retention can be achieved by attachment to membranes. The so called membrane associated bZIPs are anchored via an N-terminal trans-membrane domain and are transferred to the nucleus after proteolytic cleavage. In *Arabidopsis*, bZIP17 (Liu et al., 2008), bZIP28 (Liu et al., 2007a), and bZIP60 (Iwata et al., 2008) have been found to be membrane associated so far.

bZIP ACTIVITY IS BROADLY REGULATED BY PHOSPHORYLATION

The activity of the available bZIP monomers can be further regulated by phosphorylation. This kind of post-translational modification can modify all the above-mentioned mechanisms controlling the TF function. First, dimerization specificity can be altered through phosphorylation of the LZ (Lee et al., 2010). Next, the DNA-binding of the bZIPs to their target sequences can be prevented by the addition of a phosphate group into the BR, which contributes with a negative charge creating repulsive forces with the DNA molecule (Deppmann et al., 2003; Kircher et al., 2010). Besides, phosphorylation within other regions of the protein can trigger conformational changes required for the activation of the protein (Lee et al., 2010). In addition to the

direct effect on the bZIP activity, phosphorylation can adjust the monomer abundance by altering the protein turnover. For example, phosphorylation of ABF3 creates a binding site for a 14-3-3 protein which protects ABF3 from rapid turnover (Sirichandra et al., 2010). Likewise, phosphorylation of HY5 prevents its degradation by impeding the interaction of this bZIP with the E3-ubiquitin-protein ligase COP1 (Hardtke et al., 2000). Finally, phosphorylation can control the bZIP subcellular localization, targeting a bZIP either for nuclear import (Djamei et al., 2007) or for cytoplasmic retention (Ishida et al., 2008).

Above all, the manner in which the activity of a bZIP is regulated is specific meaning that the same kinase enhances the activity of some bZIPs, but diminishes the action of others. Such a situation has been described for instance for EmBP-2 and ZmBZ-1 phosphorylated by CKII (Nieva et al., 2005). The specific effect of the phosphorylation for each bZIP allows the customized regulation of multiple genes by the action of few upstream kinases. This is an optimal feature for the control of responsive pathways and, indeed, bZIPs are frequently found to be involved in such networks like, e.g., the deciphered ABA-responsive pathway in rice, which involves the action of a SnRK, namely SnRK2, activating the transcription of the ABA responsive genes through the phosphorylation of the bZIP proteins OREB1 and TRAB1 (Kagaya et al., 2002; Kobayashi et al., 2005; Chae et al., 2007). Similarly, bZIPs belonging to the S and C groups coordinate the activation of the metabolic response to low energy stress in combination with SnRK1 (Baena-González et al., 2007; Hummel et al., 2009; Dietrich et al., 2011; Cho et al., 2012).

THE WRKY TFs AND THEIR REGULATION

The WRKY TF family is found in the plant kingdom and belongs also to the 10 largest families of TFs in higher plants. Like bZIPs, the WRKY family is divided into different subgroups, but in contrast to the ten bZIP groups, the WRKY family is only divided into three groups. WRKY factors are also found in the unicellular eukaryote *Giardia lamblia* and the slime mold *Dictyostelium discoideum* (Ulker and Somssich, 2004; Zhang and Wang, 2005), but there is no hint that WRKY TFs exist in animals. However, former analyses have shown that WRKY TFs belong to a WRKY-GCM1 (glial cell missing 1) superfamily which is a widespread eukaryote-specific group of TFs (Babu et al., 2006).

Almost two decades have already passed since their discovery (Ishiguro and Nakamura, 1994; Rushton et al., 1995, 1996) and by now a lot of different functions have been attributed to the WRKY TFs. They participate in the regulation of many plant processes including the responses to pathogen infestation (Pandey and Somssich, 2009; Birkenbihl et al., 2012; Hu et al., 2012; Chujo et al., 2013), abiotic stresses (Jiang and Deyholos, 2009; Rushton et al., 2010; Scarpeci et al., 2013; Wang et al., 2013), trichome development (Johnson et al., 2002), and senescence (Zentgraf et al., 2010; Zhou et al., 2011; Besseau et al., 2012). Northern blot analysis revealed that in *Arabidopsis* around 70% of the *WRKY* genes were differentially expressed in plants after infestation with an avirulent strain of the bacterial pathogen *Pseudomonas syringae* or treatment with salicylic acid (Dong et al., 2003) emphasizing their importance in pathogen response. A more recently described physiological activity of WRKY factors is their participation in the biosynthesis of alkaloids (Suttipanta et al., 2011; Yamada and Sato, 2013; Yang et al., 2013).

WRKY STRUCTURAL FEATURES

The WRKY factors are named after their characteristic DNA-binding domain (DBD) of approximately 60 amino acids. This domain contains a highly conserved WRKYGQK motif at the N-terminus and a zinc-finger structure at the C-terminus called the WRKY domain. There are two possibilities how the zinc-finger structure of this domain can be formed, either $Cx_{4-5}Cx_{22-23}HxH$ (C2H2) or $Cx_7Cx_{23}HxC$ (C2HC), in which the cysteine and histidine residues bind one zinc atom and generate a finger like structure. Both, the WRKYGQK motif and the zinc-finger structure are necessary for the DNA-binding activity of WRKY TFs. Mutations in the invariable WRKYGQK motif significantly reduced the DNA-binding activity and substitutions of the conserved C and H residues of the zinc-finger even abolished the DNA-binding (Maeo et al., 2001).

All WRKY proteins contain one or two of these DNA-binding WRKY domains and are categorized into three subgroups dependent on their number of WRKY domains and the zinc-finger structure. Group I WRKY proteins are marked by two WRKY domains with a C2H2 zinc-finger structure. Group II and III WRKY proteins consist of only one WRKY domain with a C2H2 and a C2HC zinc-finger structure, respectively. The group II WRKY proteins were originally further divided into IIa, IIb, IIc, IId, and IIe based on their primary amino acid sequence, but later, phylogenetic analyses have shown, that the subgroups IIa and IIb are combined to IIa + b, and IId and IIe to IId + e (Eulgem et al., 2000; Zhang and Wang, 2005; Rushton et al., 2010).

Recently, it was shown for *Solanum lycopersicum* that even sequence variants for the highly conserved WRKYGQK motif exist. WRKYGKK is the most common variant, but WRKYGMK, WSKYGQK, WQKYGQK, and WIKYGEN have also been described. Furthermore, it was found that also novel zinc-finger variants exist, namely $Cx_{29}HxH$ and $Cx_7Cx_{24}HxC$ (Huang et al., 2012). Moreover, Mangelsen et al. (2008) could also detect variants of the WRKYGQK motif (WRKYGKK, WQKYGQK, WRKYGEK, and WSKYGQM) in *Hordeum vulgare*.

The WRKY domain binds to a so called W-box (TTGACC/T) in the promoters of target genes. This sequence is the minimal core element necessary for binding of a WRKY protein to DNA (Rushton et al., 1996; Ciolkowski et al., 2008). W-boxes can be found in the promoters systemic acquired resistance related (SAR) genes, including *isochorismate synthase 1*, *non-expressor of PR genes 1*, and *pathogenesis related 1* (Fu and Dong, 2013); or ABA signaling-related genes such as *ABI4*, *ABI5*, and *ABA responsive element binding factor 4* (Rushton et al., 2012). Often there are several W-boxes in one promoter, and even motif clusters can be found. Remarkably, W-boxes are also found in the promoter of *WRKY* genes, suggesting a potentially strong transcriptional networking between WRKY proteins.

The elucidation of the solution structure of WRKY proteins in contact with the DNA will help to understand the mechanism of DNA-binding. In 2005, the solution structure of the C-terminal WRKY domain of *Arabidopsis* WRKY4 (a group I WRKY protein) was discovered by NMR (Yamasaki et al., 2005). Yamasaki

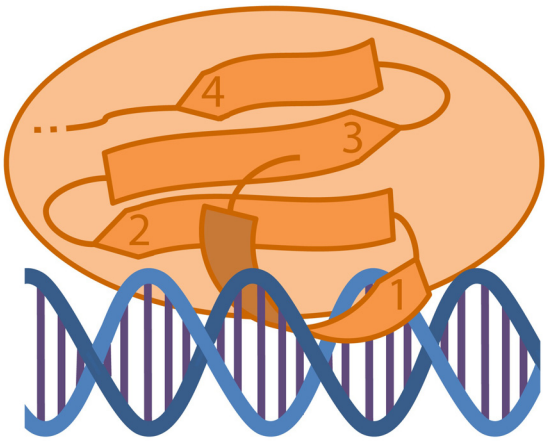

FIGURE 3 | Diagram of a WRKY C-terminal domain interacting with the DNA. The C-terminal WRKY domain consists of a four-stranded antiparallel β-sheet (1–4). The DNA recognition takes place along the major grove by the β1-strand containing the WRKYQK motif.

et al. (2012) could dissolve the structure of the same domain in complex with a W-box. The C-terminal WRKY domain consists of a four-stranded antiparallel β-sheet, in which the β1-strand, that comprises the WRKYGQK motif, contacts the major DNA groove (**Figure 3**). Residues of the WRKYGQK motif recognize the DNA mainly through apolar contacts with methyl groups of the T bases of the W-box. The DNA in this model is B-formed. Another model for the protein–DNA structure formation was proposed in 2007 by Duan et al. (2007). They investigated the crystal structure of the WRKY domain of Arabidopsis WRKY1 (also a group I WRKY protein), but the domain attribution used for WRKY1-C was longer. They found that this WRKY domain consists of a five-stranded antiparallel β-sheet with β2 and β3 forming the DNA-binding sites. The zinc-binding site was found between β4 and β5. By using a similar domain attribution like Yamasaki et al. (2012) the structure between WRKY4-C and WRKY1-C was comparable.

THE W-box, SURROUNDING SEQUENCES AND THE WRKY DOMAIN DETERMINE THE DNA-BINDING SPECIFICITY

WRKY TFs bind W-boxes in the promoters of target genes to regulate their expression. But almost all WRKY factors bind W-boxes raising the question, how specificity is achieved between certain promoters and different WRKY TFs.

Binding studies revealed that only the presence of W-boxes is not sufficient for a DNA–protein interaction. By using gel shift experiments, Miao et al. (2004) could show a specific DNA-binding activity of WRKY53 to promoter fragments of its target gene *senescence-induced receptor-like kinase* in which a complex was only formed with a fragment containing four W-boxes, whereas no DNA–protein interaction was found when the fragment consisting of only three W-boxes. However, reporter gene assays showed, that these three boxes are necessary for the induction of a reporter gene by WRKY6 (Robatzek and Somssich, 2002). This indicates that the presence of W-boxes is not sufficient for specific binding and that most likely the surrounding sequences and the overall structures are important. A more detailed study for five different WRKY TFs toward their DNA-binding selectivity depending on neighboring sequences was performed by Ciolkowski et al. (2008). They found differences in the binding site preferences of WRKY6, WRKY11, WRKY26, WRKY38, and WRKY43 by gel shift experiments. WRKY6 (group IIb) and WRKY11 (group IId) show high binding affinity to sequences with a G residue directly adjacent 5′ to the W-boxes, whereas WRKY26 (group I), WRKY38 (group III), and WRKY43 (group IIc) bind more efficiently with a C, A, or T in the direct 5′ neighborhood. Interestingly, the binding of these three WRKYs was enhanced by exchanging the first T base in 5′ direction. Ciolkowski et al. (2008) concluded again that for a specific transcriptional regulation the adjacent sequences to W-boxes are important. Besides, there are some reports of WRKY proteins binding to non-W-box sequences. In a reporter gene assay, WRKY6 can regulate the reporter gene expression under the control of the *WRKY42* promoter lacking perfect W-boxes (Robatzek and Somssich, 2002). WRKY53 can also directly interact with a W-box lacking fragment of the same promoter (Miao et al., 2004) and with clustered imperfect W-boxes only consisting of the TGAC core elements of a W-box (Potschin et al., 2013) indicating more diversity in sequence affinities of WRKY TFs. Moreover, binding to a PRE4 element (TACTGCGCTTAGT) and to a W-box containing element was shown for *Os*WRKY13 of rice (Cai et al., 2008).

A DNA–protein interaction enzyme-linked immunosorbent assay (DPI-ELISA) screen was developed by Brand et al. (2013) to elucidate WRKY DNA-binding specificities in a more general view. They used only the WRKY DBDs for the DPI-ELISAs with the aim to unravel the DNA-sequence specificity for each WRKY DBD. The DBDs of *At*WRKY50, *At*WRKY11, and *At*WRKY33 (C-terminal DBD and N-terminal DBD) were tested and, in fact, they found sequences that seem to be DBD-specific. Remarkably, they could show that both DBDs of group I WRKYs are functional and can bind to DNA, even though the binding of the N-terminal DBD was weaker than that of the C-terminal DBD. Although homology modeling revealed a potential binding ability for both domains, the N-terminal domain always showed weaker or even no binding (Eulgem et al., 1999; Maeo et al., 2001; Duan et al., 2007). However, the actual function of the N-terminal WRKY domain is still unclear.

As mentioned above, there are sequence variants for the highly conserved WRKYGQK motif of the WRKY domain. The *Arabidopsis* WRKY50 factor has the slightly different amino acid sequence WRKYGKK in the WRKY domain (Eulgem et al., 2000; Brand et al., 2013). Brand et al. (2013) chose the WRKY domain of this WRKY TF and the *Arabidopsis* WRKY11 DBD with a conserved WRKYGQK motif to investigate, if there is a difference in the DNA recognition caused by this single amino acid exchange (lysine and glutamine) in the DNA-binding site. The amino acid glutamine prefers to bind nucleobases due to its partial negative charge, whereas lysine prefers to bind the phosphate backbone due to its partial positive charge. In fact, these WRKY domains showed preferences for distinct DNA target sequences, depending on this amino acid exchange in the conserved WRKYGQK motif. They mutated the conserved motif of WRKY50 to WRKYGQK$^{(KQ)}$ and this of WRKY11 to WRKYGKK$^{(QK)}$ and tested these mutated

WRKY domain proteins in DPI-ELISAs. WRKY50^{mut} showed a similar DNA-binding affinity like WRKY11^{wt} and WRKY11^{mut} like WRKY50^{wt}, suggesting that these amino acids in the WRKY domain are important for specific DNA recognition.

W-boxes IN WRKY GENE PROMOTERS ENABLE TRANSCRIPTIONAL NETWORKING

An interesting point that has emerged in promoter analysis of WRKY TFs is the enrichment of W-boxes in their own promoters as indicated by Dong et al. (2003). They analyzed the 1.5 kb promoter sequence upstream of 72 WRKY genes in *Arabidopsis*, finding that 83% of the WRKY genes contain at least two perfect W-boxes (TTGACC/T) and 58% contain even four or more TTGAC core elements suggesting a regulatory network between the WRKY factors.

Further detailed studies of several WRKY promoters also confirmed the presence of multiple W-boxes. For example, two W-boxes were found in the promoter of *AtWRKY6*, four and five W-boxes in the promoters of the two homologous genes of *Coffea arabica*, five W-boxes in the promoter of *AtWRKY18*, and three perfect W-boxes plus an additional TGAC cluster in the promoter of *AtWRKY53* (Robatzek and Somssich, 2001; Petitot et al., 2013; Potschin et al., 2013). Some WRKYs even carry 11 or 12 (*AtWRKY66*, *AtWRKY17*) TTGAC core elements in an analyzed 1.5 kb promoter fragment (Dong et al., 2003). In order to compare the bZIP family with the WRKY family in this aspect, we analyzed bZIP promoters for C and G-boxes and WRKY promoters for W-boxes. We could easily verify this enrichment analyzing the 3-kb upstream fragments of 76 WRKY genes, which led to similar results: 72% of the WRKY genes contain two or more W-boxes. Furthermore, we found that 40% of the WRKYs have three or more W-boxes in their promoters, which is clearly above the average found for all annotations in the TAIR database (**Figure 4**). In contrast, no enrichment of C- or G-boxes could be detected in the bZIP promoters compared to the overall distribution of these *cis*-elements.

In agreement with the W-box enrichment in their own promoters, it has been demonstrated that the WRKY proteins act on the promoters of their own genes and on other WRKY genes in cotransfection assays resulting in activation or repression of a reporter gene (Robatzek and Somssich, 2002; Petitot et al., 2013; Potschin et al., 2013). In addition, a pull-down analysis of WRKY53 with genomic DNA resulted in a list of putative target genes of WRKY53 including eight different WRKY genes (Miao et al., 2004). Furthermore, the analysis of *wrky* mutant or overexpression plant lines revealed that the expression of other WRKY genes is altered in these lines. Loss of the *At*WRKY22 protein increased the expression of *AtWRKY70* after dark treatment, whereas overexpression of the *At*WRKY22 protein decreased the expression of *AtWRKY70* under normal conditions in comparison to wild type plants. When *AtWRKY70* is mutated, the expression of *AtWRKY22* is decreased compared to wild type plants after dark treatment (Zhou et al., 2011). Moreover, a double-knock out mutant of *Atwrky11 wrky17* showed increased transcript levels of *AtWRKY70* and *AtWRKY54* (Journot-Catalino et al., 2006). Microarray analyses of stressed *Atwrky33* mutant plants compared to the wild type revealed lower expression of *AtWRKY28*, which was also confirmed by qRT-PCR (Jiang and Deyholos, 2009). Alteration on the expression of certain WRKY genes was also shown for *Atwrky18* mutant compared to wild type plants in microarray analyses (Wang et al., 2006). Based on 2000 *Arabidopsis* microarray experiments, it was found that more than 70% (45 out of 61) of the WRKY genes are co-regulated with other WRKYs (Berri

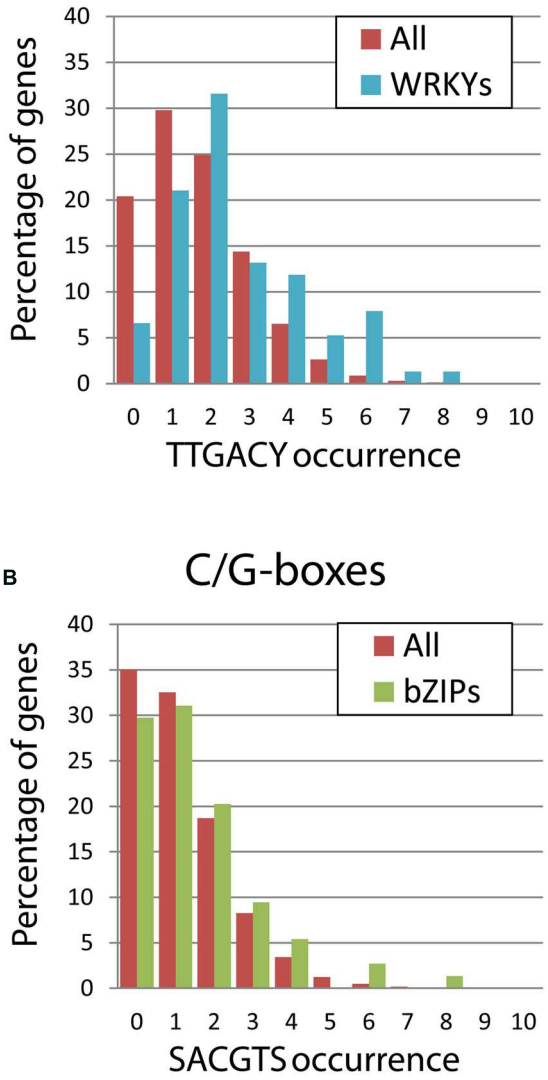

FIGURE 4 | Frequency of *cis*-element distribution within the 3-kb upstream of coding sequences. The occurrence of the *cis*-elements was calculated only for the positive strand using the Patmatch tool in the TAIR website. Red bars refer to the whole set of 33,602 annotations of the TAIR10 Genome Release, while green and blue bars indicate the subsets including only bZIPs or only WRKYs genes, respectively. **(A)** The occurrence of W-boxes was determined using a TTGACY motif, where Y indicates pyrimidine. The enrichment of W-boxes in the WRKY promoters is remarkable, while only few WRKY promoters carry no W-boxes. **(B)** The occurrence of the G- and C-boxes, the preferred *cis*-elements bound by plant bZIPs, was determined using a SACGTS motif, where S indicate strong bases (C and G). In contrast, no increase of bZIP-binding sites in their promoters can be observed.

et al., 2009). In addition, ChIP resolution scanning of the parsley *PcWRKY1* promoter with an antiserum that detects most of the WRKY factors showed that the W-boxes of this promoter are constitutively occupied by WRKY factors (Turck et al., 2004). Therefore, it seems that the WRKY factors act in a network with mutually regulation of their own expression.

WRKY PROTEINS CAN INTERACT WITH MULTIPLE PARTNERS

In addition to transcriptional networking, WRKY proteins can also form dimers and are also capable to form heterodimers. Furthermore, many other proteins have been characterized to form protein complexes with WRKY proteins thereby regulating their function. An excellent overview on protein interaction partners of WRKY proteins was recently published by Chi et al. (2013). Here, we focus on the heterodimer formation between WRKY factors and their impact on transcription.

The growing number of discovered interaction partners reveals that there is also a networking between the WRKY factors on the protein level. Moreover, there is some evidence that these WRKY heterodimers act in a different way on transcriptional regulation than homodimers or monomers. Recently, a participation of *At*WRKY18 in the senescence process was discovered (Potschin et al., 2013). *At*WRKY18 can physically interact with *At*WRKY53, an important regulator of early senescence, leading to different transcriptional activation in a reporter gene assay of the heterodimer in comparison to the single proteins. A well-investigated network exists between the three *Arabidopsis* WRKY factors WRKY18, WRKY40, and WRKY60. It was shown by Xu et al. (2006) that these three WRKYs interact with each other in a yeast two-hybrid assay and form homo- and heterodimers. In gel shift assays, WRKY18 and WRKY40 heterodimers bind much stronger to different W-box carrying sequences than the respective homodimers. In contrast, if WRKY40 is mixed with WRKY60 proteins the binding affinity declines. Since WRKY60 alone shows almost no binding activity for the used DNA sequences the effect has to be due to heterodimer formation. An example for the regulation activity of WRKY18/WRKY40 heterodimers is given by Chen et al. (2010). *WRKY60* is expressed after ABA treatment and this induction is almost lost in the *wrky18* and *wrky40* mutants, suggesting that *WRKY60* is regulated by WRKY18/WRKY40 in the ABA signaling pathway. In addition, they could show activation of the *WRKY60* promoter by WRKY18/WRKY40 heterodimers in a reporter gene assay, whereas the homodimers had no effect (Chen et al., 2010). These three WRKY proteins participate in the ABA signaling pathway through direct regulation of ABI4 and ABI5. Interestingly, not only different binding effects to these two genes were observed for the heterodimers, by using fragments of the ABI4 and ABI5 promoters in gel shift assays, binding activity of a combination of all three WRKYs together was sometimes completely abolished binding, although all possible heterodimers could bind to the same fragment. This indicates that an interaction between all three WRKY proteins takes place and that this higher order complex has again a distinct functionality (Liu et al., 2012).

An example of different binding activity for heterodimers between WRKYs and non-WRKY proteins is given by Lai et al. (2011). *At*WRKY33 can interact with SIGMA FACTOR-INTERACTING PROTEINS 1 and 2, two VQ motif-containing proteins that stimulate the DNA-binding activity of WRKY33. It was shown in a yeast two-hybrid assay using deletion mutants of WRKY33 that this interaction is mediated by the C-terminal WRKY domain of WRKY33. WRKY33 belongs to group I with the characteristic of two WRKY domains in which in general the C-terminal WRKY domain carries out the DNA-binding. However, the C-terminal WRKY domain is also responsible for mediating protein–protein interactions (Lai et al., 2011), so that these two functions overlap in this domain. Besides, in a yeast two-hybrid screen with *Arabidopsis* VQ and WRKY proteins, the C-terminal WRKY domain of group I WRKY proteins and the sole WRKY domain of group IIc WRKY proteins seem to be important for protein–protein interactions (Cheng et al., 2012). Group IIa WRKY proteins contain canonical LZ sequences and many other group II and III WRKYs have at least multiple leucine, isoleucine or valine residues at their N-termini, forming a similar structure of a LZ for protein–protein interactions (Chi et al., 2013).

W-boxes in the promoters of target genes are often clustered. Since one WRKY DBD is thought to bind one W-box, such W-box clusters in the DNA can mediate a complex formation of higher order protein complexes between different WRKY proteins. Depending on the orientation and the number of nucleotides between the W-boxes, the WRKY DNA-binding protein complex is composed of WRKY proteins with specific conformations. However, higher order complex formation does not only refer to clustered W-boxes, but also to separated W-boxes through DNA loop formations (Chi et al., 2013), enhancing again the variety of WRKY TF activity. In contrast to the bZIP factors that need to dimerize for DNA-binding, the mode of DNA-binding seems to be more diverse for the WRKY factors. They appear to bind as monomers, dimers or even as trimers (Xu et al., 2006; Ciolkowski et al., 2008; Liu et al., 2012). But although single WRKY proteins were usually used in gel shift experiments, it is still possible that these WRKYs form homodimers. The isolation of the solution structure of a WRKY protein in complex with DNA revealed that monomer binding occurs, although they did not use the whole protein for structure analysis.

WRKY ACTIVITY IS ALSO MODULATED BY PHOSPHORYLATION BUT THROUGH DIFFERENT KINASES

As already described for bZIPs, WRKY TFs activity can also be modulated by phosphorylation. In the case of WRKYs, phosphorylation can be mediated through the mitogen-activated protein kinase (MAPK) pathway (Asai et al., 2002). Normally, a MAP kinase kinase kinase (MEKK) phosphorylates and activates a MAP kinase kinase (MKK) that in turn phosphorylates a MAPK responsible for phosphorylation and regulation of different effector proteins. An entire MAPK signaling cascade was characterized for the response of plant cells to the bacterial component flagellin which is sensed by the flagellin receptor FLS2 (flagellin-sensitive 2), a leucine-rich-repeat (LRR) receptor kinase. The MEKK MEKK1 is activated by the FLS2 kinase, MEKK1 activates MKK4/MKK5, two MKKs that activate MPK3/MPK6, two MAPKs that activate the effector proteins WRKY22/WRKY29 resulting in an immune response. Activation of this MAPK cascade

confers resistance to both bacterial and fungal pathogens (Asai et al., 2002). Phosphorylation-dependent activation in immune responses was also shown for *Nicotiana benthamiana* WRKY8. *Nb*WRKY8 increases its DNA-binding activity after incubation with salicylic acid-induced protein kinase (SIPK), a MAPK that is also able to phosphorylate *Nb*WRKY8. Additionally, phosphorylation of *Nb*WRKY8 resulted in an enhanced transactivation activity in a reporter gene assay (Ishihama et al., 2011). For its homolog *At*WRKY33, a regulation through phosphorylation was also shown. Transcriptomic analysis of *wrky33* and wild type plants upon *Botrytis cinerea* infection discovered a strong transcriptional reprograming mediated by *At*WRKY33 in plant pathogen responses (Birkenbihl et al., 2012). *At*WRKY33 is a substrate of MPK3/MPK6, two MAPKs important for the induction of camalexin biosynthesis (Ren et al., 2008), the major phytoalexin in *Arabidopsis*, and is therefore responsible for growth inhibition of certain pathogens (Glawischnig, 2007). Mutation of five potential phosphorylation sites in WRKY33 in the *wrky33* mutant background blocks the ability of WRKY33 to restore the induction of camalexin production (Mao et al., 2011). *At*WRKY53, a positive regulator of senescence is phosphorylated by MEKK1 although this kinase is upstream in the MAPK signal cascade and appears to take a short cut. The phosphorylation enhances DNA-binding activity of *At*WRKY53 *in vitro* and transcription of a reporter gene *in vivo* (Miao et al., 2007). Phosphorylation is often mediated by clustered Pro-directed Ser residues (SP-cluster) in the N-terminal region of several group I WRKY proteins. In addition, some group I WRKYs harbor a so called D-domain [$(K/R)_{1-2}$-x_{2-6}-(L/I)-x-(L/I)] important for the interaction with MAPKs (Ishihama et al., 2011). However, interaction with MAPKs is not restricted to group I WRKY proteins. Popescu et al. (2009) found in protein microarrays a lot of WRKYs from different groups as interaction partners of diverse MAPKs with most of the WRKYs carrying SP-cluster.

WRKY EXPRESSION IS ALSO UNDER EPIGENETIC CONTROL

In eukaryotic cells, nuclear DNA wraps around histone proteins forming nucleosomes that are finally packaged into chromatin. Whereas euchromatin is the loosely packaged form accessible for the transcription machinery, heterochromatin is tightly packaged and transcriptionally inactive. These two states are not static but can be converted into each other providing an essential mechanism of regulating gene expression. Conversions are predominantly achieved through modifications of the histones by acetylation, methylation, and phosphorylation. Acetylation of a histone results in a more loosely form of the nucleosome and an easier access of the transcription machinery for gene expression. This kind of modification is mediated by histone acetyltransferases, which add acetyl groups to activate gene expression, and histone deacetylases, which remove acetyl groups to inactivate gene expression. Kim et al. (2008) could show that *At*WRKY38 and *At*WRKY62, two negative regulators of plant defense, interact in the nucleus with histone deacetylase 19 (HDA19), a positive regulator of plant defense. Both WRKYs show transactivation activity in a reporter gene assay which is abolished by HDA19 suggesting that *At*WRKY38 and *At*WRKY62 induce the expression of genes negatively regulating plant defense and this is inhibited by HDA19. Epigenetic control was also observed for *AtWRKY53* during senescence. For this WRKY gene, specific histone methylations are necessary for correct gene expression and progression of senescence (Ay et al., 2009). Methylation of histones can either activate or repress transcription depending on the methylated site mediated by histone methyltransferases and histone demethylases. Plants overexpressing *SUVH2*, a histone methyltransferase, have a different status of histone methylation, whereby the expression of *AtWRKY53* is repressed (Ay et al., 2009). But also histone acetylation seems to be important for *AtWRKY53* expression since the promoters of *AtWRKY53* and *AtWRKY6* are enriched with acetyl groups (Luna et al., 2012). Recently, yeast two-hybrid and bimolecular fluorescence complementation assays showed that banana *Ma*WRKY1 could interact with *Ma*HIS1, a linker histone H1 protein (Wang et al., 2012).

WRKY FUNCTION CAN BE TRIGGERED BY SUBCELLULAR LOCALIZATION

Most WRKY TFs are located in the nucleus for direct transcriptional regulation (Robatzek and Somssich, 2001; Zhang et al., 2004; Zheng et al., 2006; Liu et al., 2007b). However, an interesting example for WRKY TFs that regulate gene expression by changing their subcellular localization is given by Shang et al. (2010). Usually, WRKY40 inhibits expression of ABA-responsive genes in the nucleus. Triggered by high concentrations of ABA, *At*WRKY40 interacts strongly with magnesium-protoporphyrin IX chelatase H subunit [CHLH]/putative ABA receptor (ABAR) inhibiting further regulatory function of *At*WRKY40 in the nucleus. ABAR is localized predominantly in the outer chloroplast membrane, with its N- and C-terminus exposed to the cytosol. ABAR binds ABA and appears to be an ABA receptor (Shen et al., 2006). *At*WRKY40 interaction with the C-terminus of ABAR in the cytosol releases inhibition of ABA response genes in the nucleus and ABA response can occur. Furthermore, the expression of *AtWRKY40* is repressed after ABA treatment (Shang et al., 2010).

CONCLUSION

The hitherto characterized regulatory mechanisms controlling the function of TFs belonging to the bZIP and the WRKY families have been summarized in order to offer a comparative view. Not surprisingly, the major known mechanisms controlling protein activity have been found regulating members of both families. However, the prevalence of certain regulatory mechanisms reveals preferences in the manner how the activity of the proteins in each family is controlled, what we designate as a general regulatory strategy (**Figure 5**). In the case of the bZIPs, networking on the protein level by heterodimerization appears to be the preferred tool to adjust and fine-tune bZIP function. Regarding the WRKYs, controlling transcription of each other stands out as networking strategy for this family in synergism with the epigenetic control of their promoters.

It is tempting to speculate about the implications of using these different strategies. It can be argued that the bZIP strategy of heterodimerization with a strong component of post-translational regulation would enable very fast crosstalk between different input signals but at the same time imply keeping a pool of "ready to use" monomers. Such an energetically expensive strategy must grant counteracting advantages in order to be maintained during the evolution. Besides the fact that the bZIP strategy allows for

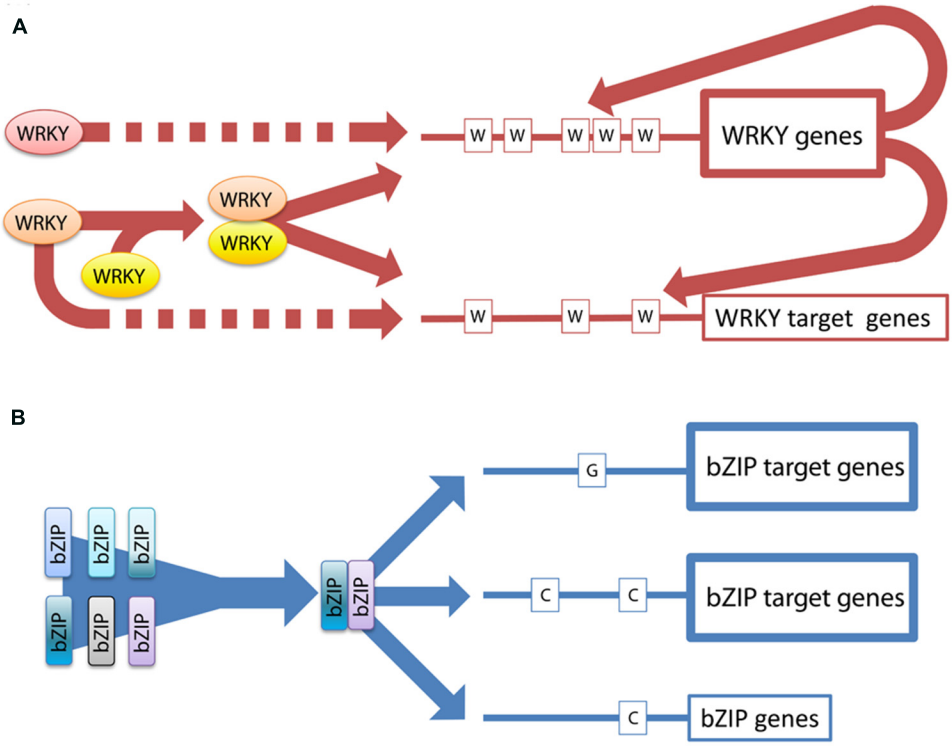

FIGURE 5 | Model featuring the main differences between the regulatory strategies of the two families. (A) The expression of WRKY TFs is strongly regulated at the transcriptional level. The promoters of the WRKY genes are enriched in W-boxes, which are bound by WRKY proteins indicating transcriptional networking and also feed-back regulations. The dotted lines indicate that WRKY possibly interact with the DNA also in a monomeric stage. In addition, WRKYs can form dimers and thereby increase the variability in regulating specific target genes. **(B)** In the bZIP family heterodimerization is extensively used to increase variability in target gene regulation. The activity of the bZIPs is regulated via specific dimerization which determines the target specificity. There are no indications for transcriptional networking.

rapid responses, factor combination confers enhanced integration capacity and flexibility: a limited pool of monomers allows multitudes of responses. The looseness of the dimerization guarantees a certain degree of graduation and fine-tuning of several responses at the same time. In another sense, the WRKY strategy seems to actively strive for autocontrol by a decisive regulation of the own expression. Although this strategy results in slower responses, for it requires *de novo* synthesis of proteins, it ensures the proper timing and the steadiness of the response. These kinds of responses are expected to be rather long-term ones, so that they become buffered once they have been triggered. In agreement with these conjectures, bZIPs seem to have a more prominent role regarding stress adaptation, which require dynamic, adaptive responses; whereas WRKYs are frequently related to longer lasting situations, like pathogen defense or the senescence progression.

To sum up, we suggest that bZIPs and WRKYs follow different regulatory strategies and we hypothesize that these reveal different control methods, either the "adjustable kind" or the "slow-but-sure" one. Although there are some facts which are undisputed, as the enrichment of WRKY binding sites in their own promoters, further data will be required to support our hypothesis. To this end, the identification and characterization of further response pathways involving WRKYs and bZIPs as well as system biology approaches combined with bioinformatics and modeling will help to unravel the network strategies of the two families in more depth. However, new *in vivo* approaches will be necessary to follow also the dynamic of these processes. In addition, deciphering molecular evolution of the two TF families in more detail might also provide inside into the strategies that these gene families pursue.

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Chapter 16

Study of Hydrogen Peroxide as a Senescence-Inducing Signal

Stefan Bieker, Maren Potschin, and Ulrike Zentgraf

Abstract

In many plant species, leaf senescence correlates with an increase in intracellular levels of reactive oxygen species (ROS) as well as differential regulation of anti-oxidative systems. Due to their reactive nature, reactive oxygen species (ROS) were considered to have only detrimental effects for long time. However, ROS turned out to be more than just toxic by-products of aerobic metabolism but rather major components in different signaling pathways. Considering its relatively long half-life, comparably low reactivity, and its ability to cross membranes, especially hydrogen peroxide, has gained attention as a signaling molecule. In this article, a set of tools to study hydrogen peroxide contents and the activity of its scavenging enzymes in correlation with leaf senescence parameters is presented.

Key words Hydrogen peroxide, Leaf senescence, *Arabidopsis*, Oilseed rape, Plants, Catalase, Ascorbate peroxidase, Superoxide dismutase, Lipid peroxidation, SAGs, SDGs, Guaiacol, Chlorophyll contents, Anti-oxidative systems

1 Introduction

Senescence is an age- and development-dependent process which can take place on tissue, organ as well as on whole organism level. In the following, we will focus on leaf senescence, which is controlled by two underlying key processes: (1) sequential leaf senescence which reallocates nutrients from old to newly developing leaves. This is achieved by a metabolic shift from anabolic to catabolic processes. Sequential leaf senescence is mainly under the control of the growing apex and is arrested when no more new leaves develop and the plant starts to flower and later sets fruits and seeds. (2) During flower induction and anthesis, the second process takes over, and monocarpic leaf senescence governs the remobilization of nutrients from the leaves to the now developing flowers and fruits. This process is crucial for fruit and seed development as it has a major impact on yield quantity and quality. In many cases, reproductive development has control over leaf senescence, since

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removal of reproductive organs can lead to regreening of already senescent leaves. This so called correlative control can be observed particularly in soybean and pea, while *Arabidopsis* does not show such a behavior [1].

In general, leaf senescence can be divided into three phases. During the *initiation phase,* the interplay between hormones and environmental and developmental cues determines the time point to trigger senescence. The correct timing is crucial, as too early induction would reduce the plant's capability to assimilate CO_2, while too late onset of leaf senescence would narrow the time frame for remobilization of nutrients to the developing fruits. During the *reorganization phase,* a shift from anabolic to catabolic processes occurs, and massive transcriptomic changes take place. Almost 6500 differentially regulated genes have been identified via reverse-genetic approaches and large-scale transcriptome profiling [2]. The progression of reorganization is easily visible to the naked eye as besides other macromolecules chlorophyll is degraded, thus converting the leaf's color from green to yellow. This phase entails detoxification of degradation intermediates and by-products and the remobilization of salvaged nutrients, and it is also accompanied by a loss of anti-oxidative capacity. The *terminal phase* completes the process of leaf senescence. During this phase, the remaining cellular components which have formerly been necessary to maintain control of the whole process are degraded. The nuclear DNA is fragmented; membranes are deteriorated; the remaining compartments like, e.g., mitochondria and nuclei, are disintegrated; and thus cell integrity and viability are irreversibly lost [3].

Reactive oxygen species (ROS), especially hydrogen peroxide, play a pivotal role throughout all three mentioned phases. During initiation and reorganization phase, hydrogen peroxide has been shown to be necessary for a successful induction and progression of the senescence program. Living cells balance production and scavenging of ROS to keep ROS levels in all cellular compartments under tight control. For *Arabidopsis* and oilseed rape, an increase in intracellular hydrogen peroxide concentrations correlates with the onset of senescence. A temporal loss of anti-oxidative capacity during senescence initiation can be observed and appears to be mainly achieved by a loss of CATALASE2 (CAT2) and ASCORBATE PEROXIDASE 1 (APX1) activities. In the case of *Arabidopsis*, this activity loss is accomplished by the transcriptional repression of the *CAT2* gene by the transcription factor G-box binding factor 1 (GBF1) [4]. As a consequence, the increased levels of hydrogen peroxide lead most likely to an inhibition of APX1 activity on the protein level. APX appears to be rendered sensitive against its own substrate exactly at this time point [5]**.** This loss of anti-oxidative capacity culminates in an intracellular accumulation

of hydrogen peroxide which coincides with leaf senescence induction. Several senescence-associated transcription factors have been shown to be highly responsive to hydrogen peroxide (*see*, e.g., [6–8]), and, additionally, scavenging of hydrogen peroxide has been shown to have severe senescence-delaying effects [9]. During the terminal phase of the senescence program, an even more substantial second increase of hydrogen peroxide contents occurs in some plants. These ROS are thought to be mainly originating from macromolecule degradation processes like, e.g., lipid degradation via β-oxidation and membrane deterioration or the disruption of the electron transport chains. In addition, this higher production of ROS is reinforced by a decreasing anti-oxidative capacity in senescent tissues.

A fairly new method for the estimation of relative hydrogen peroxide contents is the fluorescent sensor HyPer [10]. To create this sensor, the regulatory domain from the bacterial hydrogen peroxide-sensing transcription factor OxyR was implemented in a circularly permutated YFP (cpYFP). This regulatory domain undergoes conformational changes upon oxidation via H_2O_2 or reduction via the glutaredoxin (GRX) and thioredoxin (TRX) systems, thus also inducing a conformational change in the cpYFP. When reduced, the HyPer protein has its excitation maximum at 420 nm, and when oxidized at 500 nm, emission is in both cases at 516 nm. Sequential excitation at both wavelengths followed by emission ratio calculation allows the determination of relative hydrogen peroxide contents.

The system has been shown to be able to detect short-term ROS bursts in plants as well as in animal systems [11–13]. Unfortunately, this sensor cannot be used to assess senescence-specific long-term changes in hydrogen peroxide contents since oxidation of the HyPer protein scavenges hydrogen peroxide molecules which are essential to trigger senescence. Although the kinetics of reduction have been shown to be very slow [10, 14], it seems to be sufficient for effective scavenging in the case of a slow increase in hydrogen peroxide contents as given during senescence, and thus inducing a delay in senescence induction [9]. Nevertheless, when used under inducible promoter systems and as localized variants, this tool might shed further light on the senescence-specific H_2O_2-signaling cascade in the future.

Considering the impact of ROS during senescence induction and progression, the study of these molecules has become more and more important over the past. However, due to ROS inherent reactivity and instability, specific measurement of ROS is still considered to be problematic. Therefore, a set of tools to measure and estimate ROS contents in correlation with other senescence parameters is presented here to describe this complex process in detail.

2 Materials

2.1 H_2DCFDA

1. Stock solution: 0.4 mg carboxy-H_2DCFDA solved in 400 µL DMSO, dilute 1:1 with distilled water.
2. Working solution: 400 µL of stock solution are added to 39.6 mL MS medium.
3. MS medium: 4.3 g MS medium without vitamins, 30 g sucrose, solve in 600 mL distilled water, adjust pH to 5.7–5.8 with KOH, and add water to 1 L.
4. 40 mM Tris-HCl, pH 7.0.
5. Hydrogen peroxide (30%).
6. 0.5 M NaOH.
7. Fluorescence reader: excitation filter ~480 nm, emission filter ~520 nm.

2.2 Guaiacol-Based H_2O_2 Measurement Buffer (Modified After Tiedemann [15] and Maehly and Chance [16])

Reaction buffer: 50 mM potassium phosphate buffer pH 7.0, 0.05% guaiacol (v/v), add shortly before use, 2.5 µmL^{-1} horseradish peroxidase.

2.3 Catalase Zymograms (After Chandlee and Scandalios [17])

1. Protein extraction buffer: 100 mM Tris, 20% glycerol (w/v), pH 8.0; add 30 mM DTT final concentration shortly before use.
2. Stacking gel: 3.5% acrylamide, 0.5 M Tris, pH 6.8.
3. Separating gel: 7.5% acrylamide, 1.5 M Tris, pH 8.8.
4. PAGE running buffer: 25 mM Tris, 250 mM glycine, pH 8.3.
5. Staining solution 1: 0.01% hydrogen peroxide solution, set up just before use.
6. Staining solution 2: 1% $FeCl_3$ and 1% $K_3[Fe(CN)_6]$ (w/v), stir at least 1 h before use.

2.4 APX Zymograms (After Mittler and Zilinskas [18])

All buffers are best prepared shortly before use.

1. Stacking gel: 5% acrylamide, 0.5 M Tris-HCl, pH 6.8, 10% glycerol (v/v).
2. Separating gel: 10% acrylamide, 1.5 M Tris-HCl, pH 8.8, 10% glycerol (v/v).
3. Electrophoresis buffer: 25 mM Tris, 250 mM glycine, 2 mM ascorbic acid, pH 8.3.
4. Protein extraction buffer: 50 mM potassium phosphate, pH 7.8, 2% Triton-X 100 (v/v), 5 mM ascorbic acid, 35 mM β-mercapto-ethanol, 2% polyvinylpyrrolidone (w/v).

5. Staining solution I: 50 mM potassium phosphate, pH 7.0, 2 mM ascorbic acid.
6. Staining solution II: 50 mM potassium phosphate, pH 7.0, 20 mM ascorbic acid, 0.5 μM hydrogen peroxide.
7. Developer solution: 50 mM potassium phosphate, pH 7.8, 14 mM TEMED, 2.45 mM nitroblue tetrazolium (NBT).

2.5 SOD Zymograms (After Baum and Scandalios [19])

1. Protein extraction buffer: 100 mM Tris, 20% glycerol (w/v), pH 8.0; add 30 mM DTT final concentration before use.
2. Stacking gel: 5% acrylamide, 0.5 M Tris-HCl, pH 6.8.
3. Separating gel: 13% acrylamide, 1.5 M Tris-HCl, pH 8.8.
4. Electrophoresis buffer: 250 mM glycine, 25 mM Tris, pH 8.3.
5. Staining buffer: 50 mM potassium phosphate, pH 7.8, 1 mM EDTA, 0.2% *N*,*N*,*N*,*N*-Tetramethylethylenediamine (TEMED), 2.6 μM riboflavin, 1.2 mM NBT.

2.6 Catalase Assay Buffers (After Cakmak and Marschner [20])

1. Phosphate buffer: 25 mM potassium phosphate, pH 7.0, add 2 mM EDTA after titration.
2. 10 mM hydrogen peroxide solution.

2.7 APX Assay Buffers (After Nakano and Asada [21])

1. Extraction buffer: 100 mM potassium phosphate, pH 7.0, 5 mM ascorbic acid, 1 mM EDTA.
2. Reaction buffer: 25 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1 mM hydrogen peroxide, 0.25 mM ascorbic acid.

2.8 Lipid Peroxidation Buffers (After Hodges, DeLong [22] and Janero [23])

1. Extraction buffer: 0.1% trichloroacetic acid.
2. Reaction buffer 1: 20% trichloroacetic acid, 0.01% butylated hydroxytoluene.
3. Reaction buffer 2: 20% trichloroacetic acid, 0.5% thiobarbituric acid.

3 Methods

3.1 Plant Preparation and General Considerations for Senescence Phenotyping

Basically, two approaches can be followed: (i) all plants are sown at once and harvested periodically or (ii) the plants are sown in the desired intervals and harvested at once. In addition, stratification of imbibed seeds for 1–2 days at 4 °C should always be carried out to ensure synchronous plant development. What has to be considered concerning the planting regime are the planned experiments. Especially the here described hydrogen peroxide measurement with a fluorescent dye is very prone to high variations when carried out with different dye solutions; furthermore long-term storage of one batch of dissolved dye is not recommended.

For all of the methods described in the following, it is necessary to sample defined leaf positions. Although grouping of leaves into, e.g., old (position 1–4), middle aged (position 5–8), and young (position 9–12 and above) is possible, this will result in higher variance of the results. Therefore, the leaves should be color-coded according to their sequence of emergence during early developmental stages so that, even in later stages, positions within the rosette can clearly be assigned to each individual leaf. Figure 1 shows the typical arrangement and shape of the leaves. It goes without saying that the attached markings should in no way hinder growth and nutrient supply of or light incidence onto the leaf to ensure proper development.

When studying hydrogen peroxide as a senescence-inducing signal, growth conditions have to be considerably controlled. Too high light irradiation induces excess photon energy, giving rise to plastid ROS production. Suitable light conditions strongly depend on the used plant species and ecotype; in our case working with intensities below 110–120 μE m^{-2} s^{-1} has proven to be viable for *Arabidopsis* ecotype Col-0. Almost all biotic stresses (e.g., gnats feeding on the plants, fungal infestation, etc.) will elicit ROS signaling cascades as well as premature senescence induction. Abiotic stresses (e.g., drought, heat, or cold) also will induce signaling cascades including ROS production; thus continuous monitoring and logging of growth conditions are highly recommended.

Furthermore developmental indicators as, e.g., the time point of bolting and flowering as well as the development of first pods should be monitored. By that, plant lines with a general alteration in development can easily be distinguished from plant lines with altered senescence (*see* **Note 1**).

3.2 Chlorophyll Contents

As mentioned above, the loss of chlorophyll is the first indicator for the progression of senescence visible to the naked eye. Speaking in general terms, two different approaches are available to determine chlorophyll contents. Non-invasive measurements are relying on light transmittance through the leaf. The two most common devices for this are the atLEAF+ (FT Green LLC, Wilmington, DE, USA) and the SPAD-502 chlorophyll meter (Soil Plant Analysis Development, Minolta Camera Co., Ltd., Japan). Both devices operate in a similar manner, varying only slightly in the utilized wavelengths. The SPAD-502 measures light transmittance at 650 and 940 nm, while the atLEAF+ uses 660 and 940 nm. For a measurement, the leaf is clamped into the device; hence it is not necessary to remove it. Considering reliability and reproducibility, both devices have been shown to deliver comparable results [24]. Additionally, FT Green implemented the conversion from atLEAF+ values to SPAD values already in their software.

For reproducible monitoring of chlorophyll contents with one of these devices, a few simple points have to be kept in mind: (1)

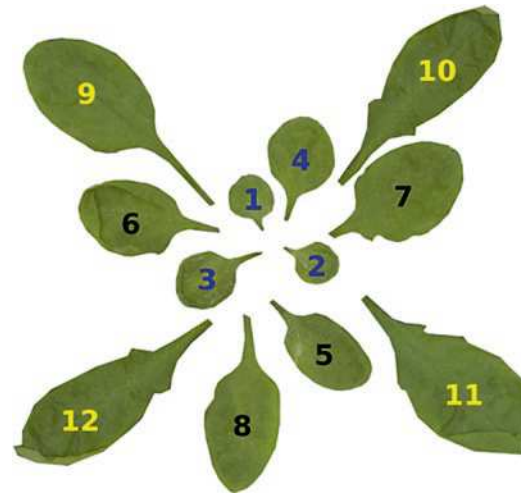

Fig. 1 *Arabidopsis* rosette. Numbers indicate sequence of leaf emergence. Suggested grouping indicated as follows: blue numbers = leaf 1–4/group old, black numbers = leaf 5–8/group middle aged, yellow numbers = leaf 9, and above/group young. Spiral growth direction is clockwise

Orientation of the measured leaves should always be the same. (2) To avoid positional effects, several measurements distributed over the whole leaf should be made. (3) The device's measuring area should always be completely covered by the leaf.

Chlorophyll extraction and the following photometric measurement has the advantage to deliver results formatted as μg chlorophyll per mg fresh weight as well as it gives the opportunity to determine Chl A to Chl B ratios. Furthermore, positional effects as they may occur with noninvasive measurements are avoided by homogenization of whole leaves. However, a major drawback of this approach is the necessity to remove the measured leaf, eliminating it for further analysis, and, in comparison with the noninvasive approach, it is more time-consuming.

Another quick and easy method to estimate senescence progression via chlorophyll contents is to assign each leaf on a plant to four categories according to its color (e.g., green, green-yellow, yellow, and brown) and calculate the proportion of each category in relation to the total number of leaves. The upside of this method is the minimal requirements in time and equipment and that the difference in total number of rosette leaves of individual plants is taken into account. The downside is that the categorization relies on the individual judgment of the experimenter.

The following results were gained using the atLEAF+ chlorophyll meter. Fifteen biological replicates were measured with at least three measurements per leaf. Despite the high number of replicates, standard deviation increases as soon as chlorophyll contents start to decrease. Another typical behavior can be observed in the

later stages. While chlorophyll degradation has not started in leaf 11 during week 6, 1 week later, its chlorophyll levels are almost as low as in leafs 5 and 7. This markedly faster and sudden decrease in chlorophyll contents usually marks the point, when sequential senescence is shifted to whole plant senescence.

A delayed senescence phenotype is accompanied with a lag in chlorophyll degradation. As an example, Fig. 2 also shows an *Arabidopsis* line expressing HyPer. These plants show a severe senescence delay, while the development of the plants remains untouched.

3.3 Hydrogen Peroxide Measurement

3.3.1 H_2DCFDA

The used dye is in a non-polar state enabling it to cross cellular membranes. An intracellular esterase deacetylates the dye, rendering the molecule polar and thus trapped inside the cell. Additionally, oxidation can only take place on the deacetylated dye; thus only intracellular oxidants will be measured, as extracellular oxidized dye is not able to enter the cell and will be rinsed off before homogenization. However, carboxy-H_2DCFDA is a general ROS indicator; although H_2O_2-specific dyes like di-amino-benzidine do exist, these dyes are often highly toxic. This is why using a non-toxic dye in combination with other analytic tools is preferred.

As mentioned above, sampling the same leaf position is crucial for reliable data. Also the measurement needs to be normalized to either leaf area or leaf weight. A combination of both (e.g., leaf discs with the same diameter which are weighed) usually gives the best results.

Weigh sample and/or measure sample area. Incubate sample for 45 minutes in working solution. Rinse sample twice in distilled water and dab off remaining fluid. Freeze sample in a new tube on liquid nitrogen. Homogenize sample in 500 µL 40 mM Tris pH 7. Centrifuge for 15 min at >12,000 × *g* and 4 °C. Measure empty plate and Tris to cover variation in plate and Tris buffer. Measure sample in plate reader (excitation, 480 nm; emission, 520 nm).

When working with several batches of dye solutions or to make independent experiments better comparable, calibration of the dye is needed. Two options are given; both include chemical deacetylation followed by oxidation of the dye via hydrogen peroxide. Here one has to keep an eye on bubble formation due to the generation of oxygen during the oxidation reaction, as these will strongly influence the measurement in a plate reader. Dilutions indicated here are adapted to our fluorescence plate reader (Tristar LB 941, Berthold Technologies); if too low values or values exceeding dynamic range of your reader occur, change dilutions after the dye oxidation.

Maximal oxidation: Mix 0.755 mL 0.5 M NaOH solution with 1 mL working solution (molar ratio 40:1) → dye deacetylation. Incubate 30 min at room temperature. Add 500 µL 30% hydrogen peroxide solution to 156 µL deacetylated dye. Shake at

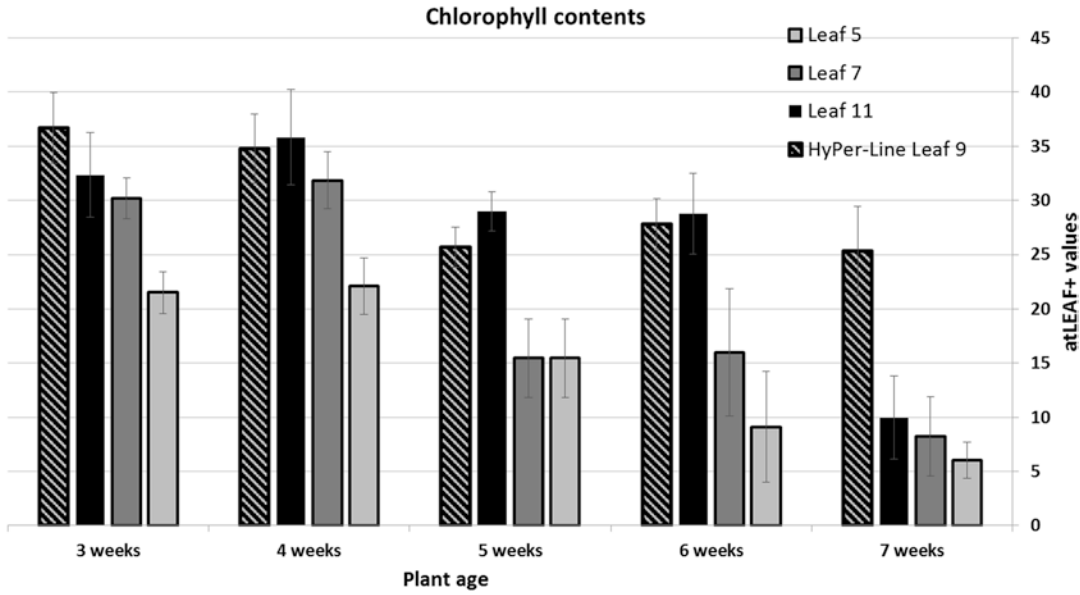

Fig. 2 atLEAF+ measurements over plant development. Leaf positions No. 5, 7, and 11 of *Arabidopsis* Col-0 plants and position 9 of *Arabidopsis* plants expressing HyPer were sampled in weekly rhythm as indicated. Values are mean of 15 or 10 biological replicates of wild-type and HyPer plants, respectively. Error bars indicate standard deviation. atLEAF+ values start decreasing at week 5 in wild-type plants, while chlorophyll contents of HyPer plants remain constant

room temperature for 1 h in the dark. Meanwhile measure deacetylated dye and all other used buffers (MS medium, 40 mM Tris working solution, etc.) to be able to correct variations in the used buffers. Measure fully oxidized, deacetylated dye. Values can be used to correct for variation in dye batches.

Calibration curve: Perform dye deacetylation as described above. Prepare tubes with different hydrogen peroxide concentrations in a volume of 24 µL (e.g., 5, 50, 75 and 100 µM final concentration hydrogen peroxide after the addition of 156 µL deacetylated dye to each). Add 156 µL deacetylated dye to each tube. Incubate at room temperature for 10 min in the dark. Add 700 µL 40 mM Tris pH 7 to each tube. Measure each hydrogen peroxide-dye mix and all other used solutions. Offset and slope can be used to correct for variation in dye batches.

The senescence-specific increase in hydrogen peroxide levels coincides with the decrease in chlorophyll contents. Figure 3 shows the hydrogen peroxide contents of leaf 5 of the same plants as depicted in Fig. 2. In wild-type plants, hydrogen peroxide contents start to increase at the same time point when atLEAF+ values for leaf 5 starts to decrease. In consistence, the HyPer lines show a much less pronounced increase in hydrogen peroxide contents, and the decrease in chlorophyll contents is markedly delayed and also less pronounced. The abovementioned second increase in

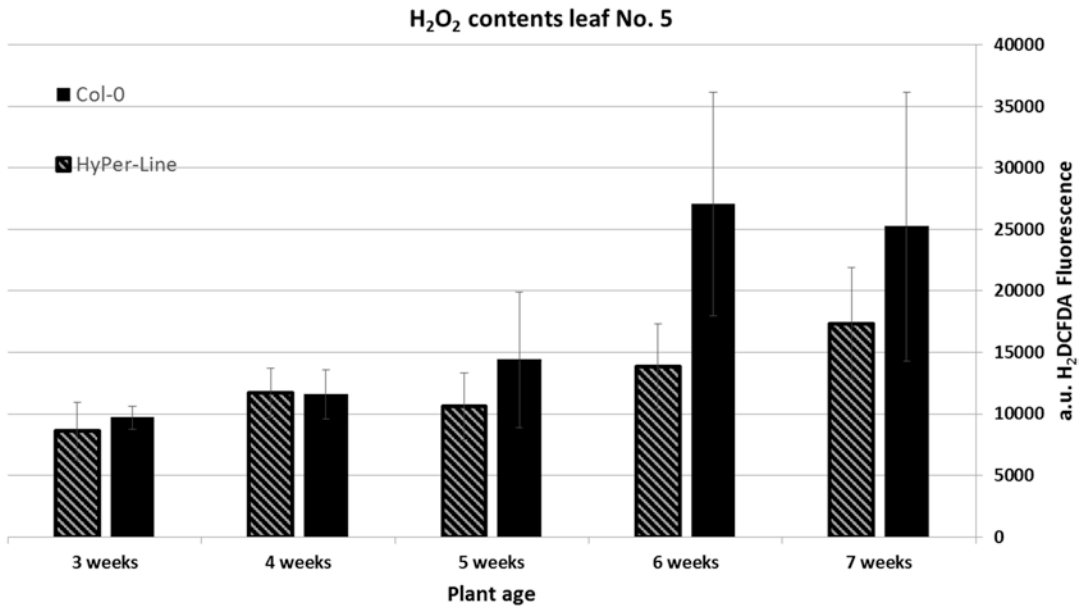

Fig. 3 Hydrogen peroxide contents over plant development. Leaf position No. 5 of *Arabidopsis* Col-0 and HyPer-expressing plants were sampled in a weekly rhythm as indicated. Values are mean of 15 or 10 biological replicates of wild-type and HyPer plants, respectively. Error bars indicate standard deviation. H_2O_2 contents start increasing in wild-type plants in week 5, while concentrations in HyPer start increasing in week 6. Additionally, increase in wild-type plants is much more pronounced than in HyPer plants

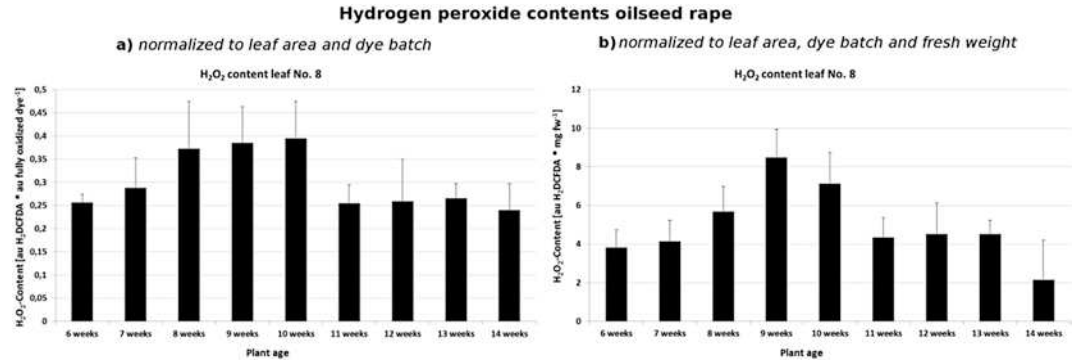

Fig. 4 Hydrogen peroxide contents during development of *Brassica napus* (cv. Mozart). Data represents mean values of at least three biological replicates. Error bars indicate standard deviation. For measurements, leaf discs with constant diameter were used. (**a**) Hydrogen peroxide contents normalized to leaf area. (**b**) Same data as in (**a**) but additionally normalized to mg fresh weight (fw) [9]

H_2O_2 contents is not observed here due to too short sampling time frame.

Transferring this knowledge onto other plant species, Fig. 4 shows a comparable pattern observed in *Brassica napus* (cv. Mozart). Also here, peak values of hydrogen peroxide contents coincide with the beginning of chlorophyll degradation (data not

shown). While in *Arabidopsis* hydrogen peroxide contents increase again in the latest stages of development, this could not be observed for oilseed rape.

3.3.2 Guaiacol-Based H_2O_2 Measurement (Modified After [15] and Maehly and Chance [16])

Punch 8–10 leaf discs with same diameter and determine fresh weight. Add 2 mL of reaction buffer to the leaf material. Add peroxidase. Incubate sample for 2 h in the dark. Take off supernatant. Determine OD_{470}. Hydrogen peroxide concentration can be calculated with the following formula:

$$C(H_2O_2)(\mu M) = \left(\frac{4OD_{470\,nm}}{26.6}\right) \times 1000$$

This assay gives the opportunity to estimate absolute concentrations. A major downside of this method is the necessity to injure the measured leaves. While using H_2DCFDA allows whole leaves to be incubated in the reaction buffer, thus restricting the area of inflicted injury only to the small diameter of the petiole, this method necessitates punching of leaf discs and, furthermore, long incubation times of the injured material. In that way, the injured area is extended as well as the time frame for possible stress responses, hence increasing the possibility of artifacts. Nevertheless, this method has been successfully used in our lab to determine senescence-specific changes in intracellular hydrogen peroxide contents (*see* Fig. 5).

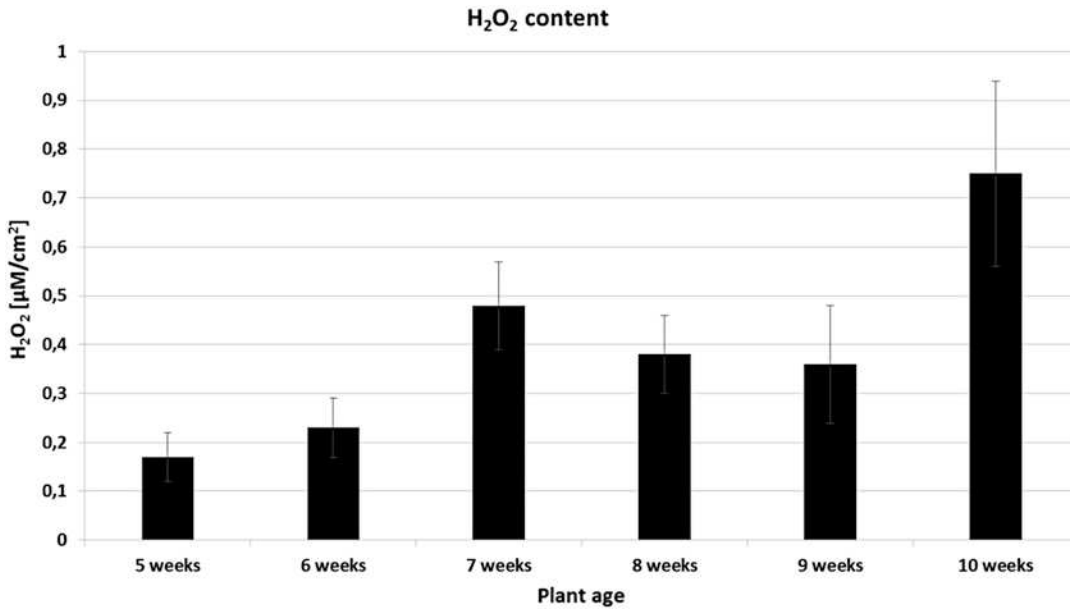

Fig. 5 Hydrogen peroxide measurements during plant development using guaiacol. Hydrogen peroxide content was measured in leaf discs and is indicated in µM/cm^2. Error bars indicate standard deviation of three replicates [5]

3.4 Anti-Oxidative Systems

An additional indicator for changing ROS contents is the differential regulation of the anti-oxidative enzymatic systems. A downregulation of these systems in most cases results in an increase of reactive oxygen species. An upregulation is a reliable indicator for already elevated ROS production most likely due to stress conditions. To monitor the activity of these enzymes, several possibilities are given; native PAGE and following in-gel activity staining gives the opportunity to visualize changes on isoform level, but quantification is not easily feasible in a reproducible manner. In contrast to in-gel activity assays, photometric assays can quantify enzyme activities, while an isoform-specific measurement is not possible.

3.4.1 Qualitative In-Gel Activity Assays: Catalases (After Chandlee and Scandalios [17])

Freeze ~100 mg leaf material in liquid nitrogen (samples can be kept at −80 °C for several months). Homogenize on ice in 400 µL extraction buffer. Centrifuge 30 min at >12,000 × *g* at 4 °C. Take off supernatant, and determine protein concentration. Load 10 µg protein on gel, and let gel run. Remove stacking gel, and incubate separating gel for exactly 2 min in staining solution 1. Rinse gel 2–3 times with distilled water. Incubate gel up to 10 min in staining solution 2; incubation should be stopped 1–2 min after bands become visible. Rinse gel in distilled water. Gels can be preserved in 7% solution of acetic acid.

As illustrated in Fig. 6, senescence-specific downregulation of CAT2 activity occurs, coinciding with the increase in hydrogen peroxide content (*see* Fig. 5). Furthermore, in addition to the CAT2 and CAT3 homotetramers, heterotetramers consisting of monomers of the different catalase isoforms can be detected. It is not known whether these heterotetramers also occur in vivo or whether they are formed during extraction. This method has already been successfully used for other plant species than *Arabidopsis* (*see*, e.g., [25]); as comparison a developmental series of *B. napus* (cv. Mozart) is shown in Fig. 7. Here, a severe downregulation of catalase activity is again coinciding with the increase of intracellular hydrogen peroxide contents (*see* above, Fig. 4). But other than in *Arabidopsis*, reconstitution of anti-oxidative capacity is less pronounced.

In the case of *A. thaliana*, identification of the different catalase isoforms can also be achieved via knockout (KO) lines (*see* Fig. 8). However, when studying other plant species where no or

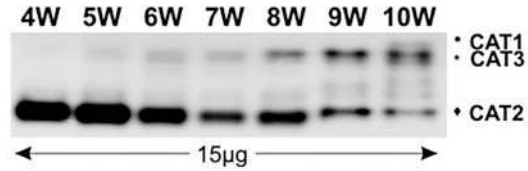

Fig. 6 Catalase activity zymogram overdevelopment of *A. thaliana*. CAT activity staining after native PAGE as described from *A. thaliana* Col-0 [5]

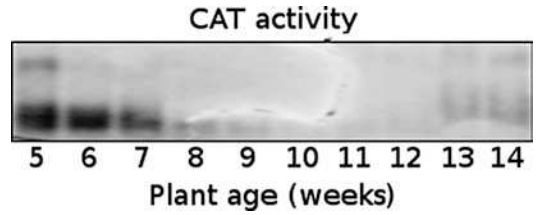

Fig. 7 Catalase activity zymogram overdevelopment of *B. napus*. CAT activity staining after native PAGE as described from *B. napus* (modified after [9])

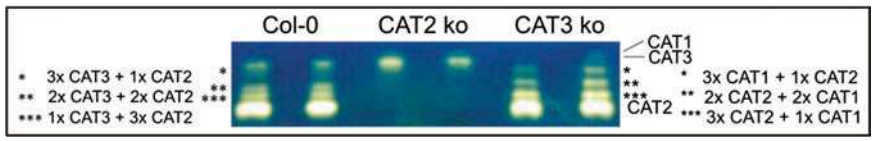

Fig. 8 Catalase zymogram of *Arabidopsis* knockout plants. Catalase extracts prepared from wild-type and catalase knockout plants. Activity staining after native PAGE was conducted as described. 10 μg of the raw protein extract were loaded. Isoforms and heterotetramer composition as denoted [29]

only a limited number of knockout lines are available, identification of CAT isoforms is most easily accomplished via zymograms combined with 3-Amino-1,2,4-triazole (3-AT) treatment. 3-AT is a catalase inhibitor acting more efficiently on CAT2 and CAT1 homologues than on CAT3 homologues [26]. Thus, when implemented in a catalase in-gel activity assay, first CAT2 activity will vanish, while CAT3 activity will decrease more slowly. Figure 9 shows a 3-AT-treated catalase zymogram from oilseed rape. Here, catalase extract was analogously prepared as described above, and 15 μg were loaded on each lane. The gel was then cut into four pieces. After incubation in a 10 mM 3-AT bath for the denoted periods, activity staining was carried out as mentioned above. Each band could be assigned according to 3-AT's known effectiveness on different catalase homologues (*see* Fig. 9).

3.4.2 Qualitative In-Gel Activity Assays: Ascorbate-Peroxidases (After Mittler and Zilinskas [18])

Homogenize 100–200 mg leaf material in liquid nitrogen. Add 300 μL protein extraction buffer and resuspend homogenate. Centrifuge for 30 min with >12,000 × *g* at 4 °C. Start gel preelectrophoresis (thus the ascorbic acid from the running buffer can run into the gel) for 20–30 min. Take off supernatant and determine protein concentration. Load 25 μg per sample on gel and let gel run. Incubate gel thrice for 10 min each in staining solution I. Incubate gel for 20 min in staining solution II. Put gel for 1 min in 50 mM potassium phosphate buffer pH 7.0. Develop gel for 5–10 min in developer solution. Rinse gel with distilled water. Gels can be preserved in 7% solution of acetic acid.

During senescence induction, APX1 activity declines in parallel to CAT2 activity loss. Despite the redundancy of the APX system in *Arabidopsis* (eight isozymes), APX zymograms of leaf material

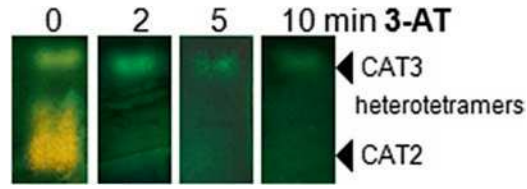

Fig. 9 3-AT-treated catalase activity zymogram of *B. napus.* Identification of isoforms in *B. napus* extracts. 3-AT incubation time prior activity staining is indicated above the lanes. Activity bands could be assigned to denoted catalases [9]

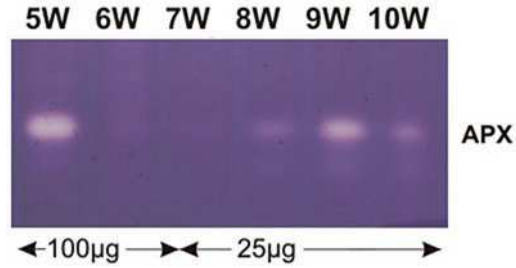

Fig. 10 Ascorbate peroxidase activity zymogram over development of *A. thaliana*. APX activity staining after native PAGE as described. Plant age is indicated in weeks. Amount of loaded raw protein extract is indicated below the lanes [5]

usually result in one prominent band representing the cytosolic APX1 (*see* Fig. 10).

For other plant species like, e.g., oilseed rape or tobacco, several activity bands occur, and a less clear senescence-associated regulation is observed. As example, zymograms prepared from oilseed rape (*B. napus* cv. Mozart) extracts are presented (*see* Fig. 11). Isoform identification without available KO plants is not easily feasible. Especially, as senescence-associated negative regulation of APX activity seems to take place on posttranslational level, thus rendering isoform-specific transcript quantification pointless.

3.4.3 Qualitative In-Gel Activity Assays: Superoxide Dismutases (After Baum and Scandalios [19])

Freeze ~100 mg leaf material in liquid nitrogen (samples can be kept at −80 °C for several months). Homogenize on ice in 400 µL extraction buffer. Centrifuge 30 min at >12,000 × *g* at 4 °C. Take off supernatant; determine protein concentration. Load 30–40 µg per lane. Incubate gel for 30 min in staining buffer. Rinse gel twice with water. Illuminate gel until staining is sufficient. Gels can be preserved in 7% solution of acetic acid.

Superoxide dismutases convert superoxide anions to hydrogen peroxide. Thus enzymatic activity is a direct indicator for hydrogen peroxide production. Isoform identification can be achieved by incubation in 5 mM hydrogen peroxide or 2 mM KCN. Manganese SOD is insensitive to either treatment, CuZn-SOD is sensitive to CN^-, and the Fe-SOD is inhibited by H_2O_2 [27]. During *Arabidopsis* plant development, the activity of a plastid-localized

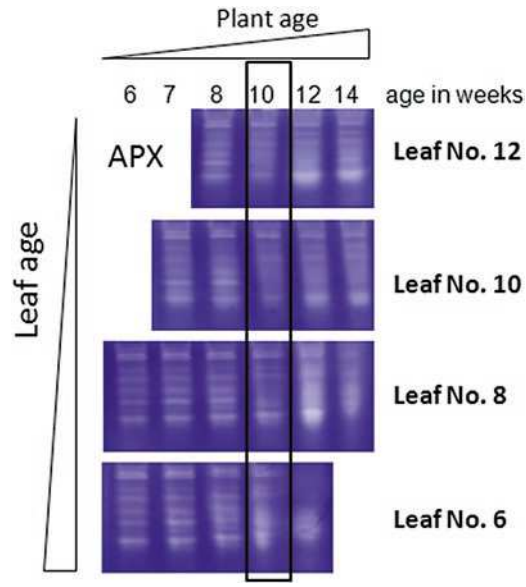

Fig. 11 Ascorbate peroxidase activity zymogram overdevelopment of *B. napus*. APX activity staining after native PAGE as described. Plant age is indicated in weeks [9]

isoform (Fe-SOD) is upregulated, while Mn-SOD isoform activity remains unchanged (for data *see* [28]).

3.4.4 Quantitative Assays: Catalase Assay (After Cakmak and Marschner [20])

Homogenize 2–4 leaves (ca. 50–150 mg; weigh before!) in 500–1000 µL phosphate buffer on ice. Centrifuge for 30 min with >12,000 × *g* at 4 °C. Take off supernatant and determine volume and protein concentration. Mix 50 µL protein extract with 850 µL phosphate buffer in a quartz cuvette. Add 100 µL 10 mM hydrogen peroxide solution (best when cuvette placed in photometer already). Measure OD_{240} for up to 5 min in the desired interval. Molar extinction coefficient ε of hydrogen peroxide is 43.6 mM cm^{-1} at 240 nm. Units per mg can be calculated via Lambert-Beer'sche formula (*see* below).

3.4.5 Quantitative Assays: Ascorbate-Peroxidases (After Nakano and Asada [21])

Homogenize 100 mg leaf material on liquid nitrogen. Resuspend in 200 µL protein extraction buffer. Centrifuge for 30 min at >12,000 × *g* and 4 °C. Take off supernatant and determine protein concentration. Mix 900 µL reaction buffer with 100 µL protein extract (best when cuvette placed in photometer already). Measure OD_{290} for up to 5 min in desired interval. Molar extinction coefficient ε for ascorbic acid is 2.8 mM cm^{-1} at 290 nm.

$$A = \frac{\Delta E / \min \times V}{\in \times d} (\times \text{Dilution})$$

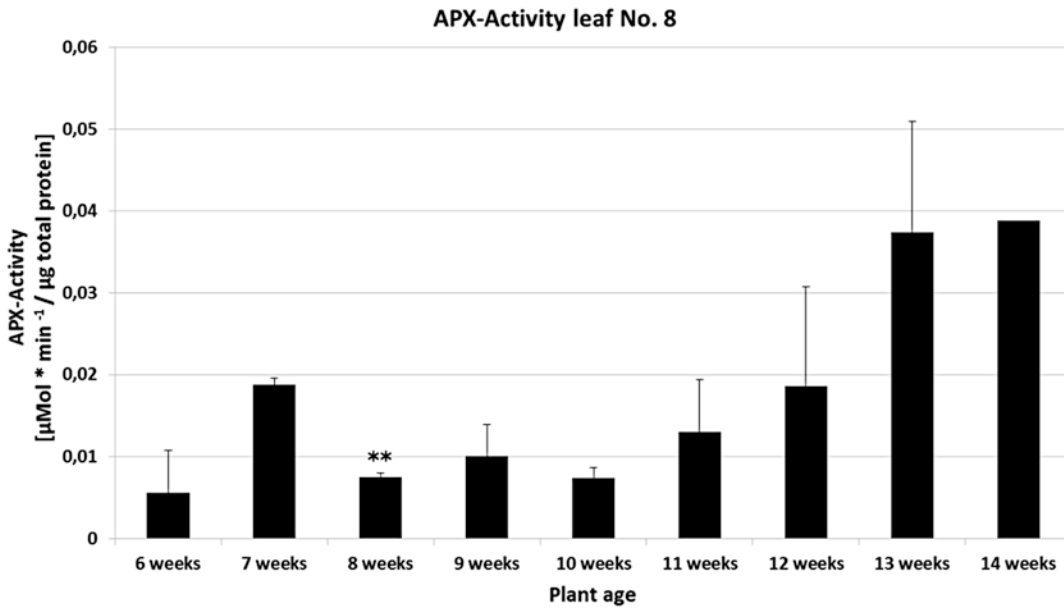

Fig. 12 Quantitative ascorbate peroxidase activity measurements during development of *B. napus*. Samples were taken from leaf No. 8. Data represents mean of at least three biological replicates. Error bars indicate standard deviation. Comparison of means and the determination of statistically differences rest on *T*-tests with $^{**}P < 0.01$. Tests were made pair wise (7w–8w, 8w–9w, 9w–10w, and 10w–11w) [9]

A = activity (U = µmol min^{-1}), V = total volume, d = Layer thickness, $\Delta E/\text{min}$ = change of absorbance per minute, ε = extinction coefficient.

In contrast to activity zymograms, with this assay downregulation in weeks 8 and 9 is very prominent and even upregulation in later stages is unmistakably visible (*see* Fig. 12).

3.5 Lipid Peroxidation (After Hodges, DeLong [22] and Janero [23])

Lipid peroxidation can be used as an indicator for oxidative stress in plants as well as membrane deterioration and senescence progression. The here utilized method is an improved thiobarbituric acid-reactive-substances assay (TBARS) developed by Prange et al. in 1999. Malondialdehyde is formed upon auto-oxidation and enzymatic degradation of polyunsaturated fatty acids. MDA in turn reacts with two molecules of TBA, yielding a pinkish-red substance with an absorbance maximum at 532 nm [22, 23]. To correct for non-TBA complexes also absorbing at 532 nm in the sample, besides the TBA reaction, another reaction without TBA is used. Additionally, besides lipid peroxides, sugars can complex with TBA. To take this into account, a further measurement at 440 nm is implemented. The molar absorbance of sucrose at 532 nm and 440 nm is 8.4 and 147, respectively, resulting in a ratio of 0.0571.

Weigh plant material (25–150 mg are recommendable). Homogenize plant material on liquid nitrogen. Add 500 µL 0.1% TCA solution. Centrifuge for 5–10 min at 10,000 × *g*. Add 200 µL supernatant to (1) 800 µL 20% TCA/0.01% butylated hydroxytoluene and (2) 800 µL 20% TCA/0.5% TBA. Vortex and incubate for 30 min at 95 °C (*attention*, pronounced formation of gases, open tubes every few minutes to prevent bursting or use screw-cap cups). Cool samples to room temperature on ice. Centrifuge again if needed. Measure absorption at 440, 532, and 600 nm. Calculate MDA concentration via following formulas:

1. $A = [(Abs_{532\ nm\ +\ TBA} - Abs_{600\ nm\ +\ TBA}) - (Abs_{532\ -TBA} - Abs_{600\ -TBA})]$.
2. $B = [(Abs_{440\ nm\ +\ TBA} - Abs_{600\ nm\ +\ TBA})\ 0.0571]$.
3. MDA equivalents (nmol * mL^{-1}) = (A-B/157000) 10^6

For example, in a comparison of the *Arabidopsis wrky53* KO line with wild-type plants, a decelerated senescence progression as well as a delayed induction of SAG expression (data not shown) can be observed. Chlorophyll contents initially decrease at the same rate from 31 to 38 DAS but during the later stages, it dramatically decrease in WT plants which is not the case for *wrky53* plants, thus hinting to a slower senescence progression rate. In consistence, lipid peroxidation starts increasing at the same time point in both plant lines, but again the slope is higher in WT plants (*see* Fig. 13a, b).

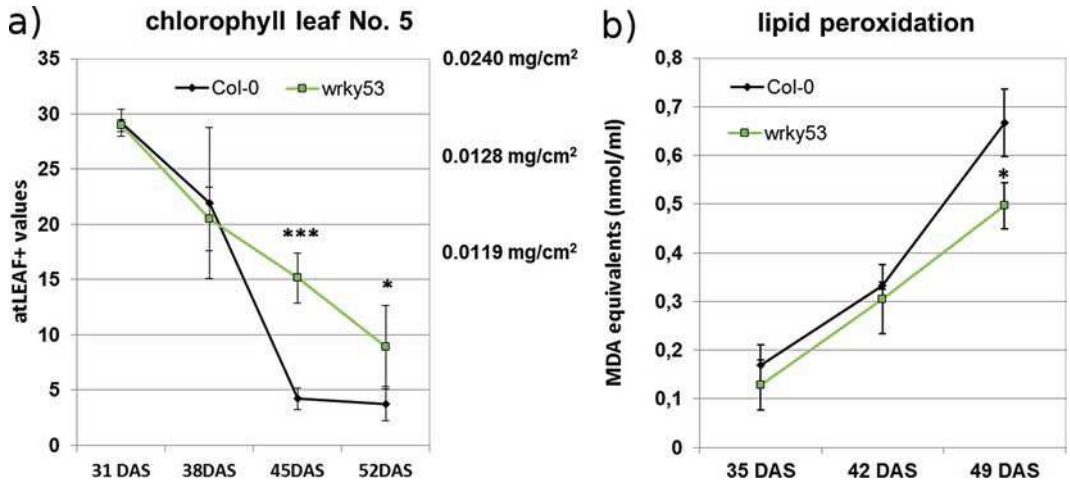

Fig. 13 Measurements of lipid peroxidation and chlorophyll contents during development of *A. thaliana*. (**a**) Chlorophyll contents of *Arabidopsis* Col-0 and *wrky53* plants. Left axis indicates atLEAF+ values, right axis the calculated chlorophyll contents in mg/cm^2. (**b**) Lipid peroxidation in Col-0 and *wrky53* plants. Plant age is indicated in days after seeding (DAS). Values represent mean of at least three biological replicates; error bars indicate standard deviation. Comparison of means and the determination of statistically differences rest on *T*-tests with $*P < 0.05$ and $***P < 0.001$

4 Notes

The methods described above are fairly robust and easy in terms of conduction. However, as all other methods, also these have some pitfalls ready at their fingertips. In the following, we want to point out the most common mistakes that can occur and give some advice how to circumvent these.

4.1 Catalase Zymograms

1. Band separation is insufficient/smearing: Used buffers might be at wrong pH/wrong glycine concentration → check pH of each buffer used; prepare new gel and running buffers. DTT in extraction buffer was not added/too old → add DTT shortly before usage. Gel might be overloaded → reduce amount of protein.
2. If background is dark green but no bands are visible: Proteins might be damaged → extraction should take place on ice at all times; extracts cannot be stored, always use freshly prepared extracts; vortexing of extracts is not recommendable. DTT in extraction buffer was not added/too old. Hydrogen peroxide concentration in the staining solution was too high, so that not all molecules were degraded by the amount of catalase loaded on the gel.
3. If background of gel is too bright to distinguish bands: Hydrogen peroxide concentration in the staining solution was too low → hydrogen peroxide solutions have to be prepared shortly before usage; prepare new staining solution 1; stock solution might be too old. Rinsing after incubation was conducted too extensively → reduce rinsing after incubation in solution 1.
4. If gel darkens after staining so that initially visible bands vanish: Incubation time in staining solution 2 might have been too long → reduce incubation time. Rinsing after staining was not conducted sufficiently → rinse gel after staining thoroughly.

4.2 Hydrogen Peroxide Measurement (H_2DCFDA)

1. Fluorescence readings are on background level: The dye batch may be expired → check functionality via calibration. Reader settings are wrong → increase lamp energy and/or counting time; use white plate to amplify signal. Sample incubation in working solution was insufficient → sample needs to be fully immersed during incubation.
2. Results have very high variation: Plants may have been exposed to different conditions → check light intensities in different positions in growth chamber; plants were standing too close to each other, dark-induced senescence may have occurred; check for pests; see log files for temperature, etc. Samples vary in weight and area/leaf position → ensure reproducible sampling.

The used dye batches may differ → correct for different batches via calibration. Working solution oxidized during sampling → keep working solution dark and cool until incubation with sample.

3. Calibration does not work properly: Deacetylation was insufficient → set up new sodium-hydroxide solution. Bubbles disturb measurement → ensure there was no bubble formation during measurement by tapping your plate until all bubbles are out. Oxidation was insufficient → use new hydrogen peroxide solution. Measured values exceed reader range → use different dilutions of oxidized dye to determine proper range for your reader.

4.3 Lipid Peroxidation

1. Measured values exceed dynamic range of photometer (usually results in 9.9999 values): Blank sample not set up properly → prepare new blank. Reader not set to zero for all wavelengths → re-blank. Bubble formation in cuvette disturbs measurement → tap cuvette several times before measuring to degas solution. Precipitate might have formed during incubation at 95 °C → centrifuge again before measuring.
2. Variation between replicates is very high: The used scale might be not accurate enough → switch scale or weigh out higher amounts of material. Homogenization of material might be insufficient → sample needs to be thoroughly homogenized for reproducible measurements. Bubbles or precipitate might have disturbed measurement → *see* above.
3. Measured values level at one maximal value: Too much plant material for one reaction was used → reduce used plant material or scale up reaction volume. Values might exceed dynamic range of reader → *see* above.

4.4 Gene Expression Analysis

1. When studying hydrogen peroxide as senescence-inducing signal, besides chlorophyll degradation, correlation to senescence-associated gene expression is also crucial. Therefore, analysis of SAG and SDG expression should be carried out with the same material used for phenotypic analysis. Many of these genes have proven their value, e.g., *SAG13* (At2G29350) encodes a short-chain alcohol dehydrogenase and serves as a reliable marker for early senescence stages. As further marker genes for early senescence, stages *CAT3* (At1G20620) and *WRKY53* (At4G23810) can be used. In contrast, expression of the cysteine protease *SAG12* (At5G45890) is upregulated in later stages of leaf senescence. There are many more genes with specific senescence-associated expression patterns which can be used for detailed classification of specific alteration of the senescence process like, e.g., delayed senescence induction and decelerated senescence progression. Combined with analysis of SDG expression, valu-

able datasets can be obtained, making interpretation of results gained with the methods mentioned above more reliable. Valuable SDGs are, for example, *CAB* (At1g29930), *CAT2* (At4G35090), and *RuBisCO* (At1G67090).

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