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Heterologous expression of *OsWRKY23* gene enhances pathogen defense and dark-induced leaf senescence in *Arabidopsis*

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Abstract WRKY proteins are a superfamily of plant transcriptional factors with potential regulatory roles pertaining to a variety of biotic and abiotic stress responses. In this study, we investigated the expression profiles of *OsWRKY23* under different developmental stages, pathogen infection, continuous-dark and hormone treatments. Under normal growth conditions, *OsWRKY23* expressed exclusively in roots and senescing leaves. Under biotic and abiotic stresses treatments, *OsWRKY23* was markedly induced by continuous-dark-induced leaf senescence and infection by rice pathogen *Pyricularia oryzae* Cav as well as salicylic acid (SA). Further analysis of *35S:OsWRK23 Arabidopsis* plants showed that over-expression of *OsWRKY23* resulted in enhanced expression of the pathogenesis-related (PR) genes and increased resistance to the bacterial pathogen *Pseudomanas syringae*. Furthermore, over-expression of *OsWRKY23* accelerated leaf senescence in darkness. The senescence-associated marker genes *SAG12* and *SEN1* were altered in darkness in *35S:WRKY23 Arabidopsis* plants. In conclusion, these results suggest that *OsWRKY23* is a novel modulator of pathogen responses as well as dark-induced leaf senescence.

Keywords *Arabidopsis thaliana* · Dark-induced senescence · *Oryza sativa* · Pathogen defense · Transgenic plant · WRKY transcription factor

Abbreviations

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ET	Ethylene
HR	Hypersensitive response
JA	Jasmonate
MAPK	Mitogen-activated protein kinase
MeJA	Methyl jasmonic acid
PAMPs	Pathogen-associated molecular patterns
PCD	Programmed cell death
PR	Pathogenesis related
SA	Salicylic acid
SAR	Systemic acquired resistance

Introduction

In plants, many developmental processes and stress responses contain complex regulatory mechanisms. These regulatory mechanisms are often controlled at the level of transcription factors. The WRKY superfamily of plant transcription factors is one such group of proteins with potential regulatory roles pertaining to a variety of biotic and abiotic stress responses. Defined by the conserved amino acid WRKYGQK sequence followed by a Cys2His2 or Cys2HisCys zinc-binding motif (Eulgem et al. 2000; Babu et al. 2006), WRKY proteins regulate expression of their target genes by binding specifically to the core or stringent W box elements (TTGAC or TTGACC/T) located in the promoter regions (Maleck et al. 2000; Dong et al. 2003).

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A number of studies have shown that WRKY family proteins can function as either transcriptional activators or repressors of plant signaling pathways involved in pathogen defense responses. The *Arabidopsis WRKY29* has been identified as an important downstream component of a mitogen-activated protein kinase (MAPK) pathway involved in innate immune system that confers resistance to both bacterial and fungal pathogens (Asai et al. 2002). Xu et al. (2006) indicate that *AtWRKY18*, *AtWRKY40*, and *AtWRKY60* function redundantly and cooperatively as negative regulators in SA-dependent pathways but play a positive role in jasmonic acid (JA)-mediated pathways. Recently another report shows that activation of MLA proteins by pathogens blocks *HvWRKY1/2* activity, allowing for rapid and stronger disease resistance responses (Shen et al. 2007). Shen et al. (2007) further demonstrate that *AtWRKY18*, *AtWRKY40* and *AtWRKY60* show the highest sequence relatedness to *HvWRKY1/2* and they are negative regulators of defense genes transcription in the plant innate immune system.

In addition to pathogen defense responses, WRKY transcriptional factors also play important roles in environment stresses and plant development processes. As the final stage of leaf development, senescence is highly regulated and genetically programmed. Senescence occurs in response to aging and under certain stresses and hormones, including drought, heat, darkness and the hormones abscisic acid (ABA) and ethylene (ET). Despite the importance of the senescence processes in plants, our knowledge on regulatory mechanisms of senescence is still very poor. It is known that some WRKY proteins are involved in regulating senescence. *AtWRKY6* was shown to be expressed during pathogen defense, wounding and senescence, and was shown to act as activator on *PR1* expression (Robatzek and Somssich 2001). *AtWRKY6* could regulate plant senescence by binding specifically to the W box in the promoter of the senescence-induced receptor kinase (also named as flg22 induced receptor like kinase 1) in *Arabidopsis* (Robatzek and Somssich 2002; Asai et al. 2002). Another important gene, *AtWRKY53*, plays complicated regulatory roles in the events of leaf senescence (Hinderhofer and Zentgraf 2001; Miao et al. 2004, 2007; Miao and Zentgraf 2007).

Although there is much information implicating *Arabidopsis* WRKY proteins in plant defense responses, evidence about the functions of rice WRKY proteins is limited. Rice is one of the most important food crops. More than half of the world population depends on rice as a stable food resource. Many biotic or abiotic stresses interact together to threaten the world's food supply. Rice exposed to such stresses shows marked changes in gene expression, including many transcriptional factors (Zhou et al. 2002). As a big transcriptional factors family, the rice WRKY proteins were first named and classified by Zhang et al. (2004). To respect the original work of Zhang et al. (2004), our previous work continued to use and perfect this nomenclature and classification, and found that some rice WRKY proteins were induced by abiotic stresses (Qiu et al. 2004; Qiu and Yu 2009). Additionally, the nomenclature is consistent with what reported recently (Xie et al. 2005; Ross et al. 2007). A few research reports have shown that rice WRKY proteins play roles in defense responses. Rice *WRKY45* plays a crucial role in benzothiadiazole-inducible blast resistance (Shimono et al. 2007). Rice *WRKY13*, as an activator of the SA-dependent pathway and a suppressor of JA-dependent pathway, mediates rice resistance by directly or indirectly regulating the expression of a subset of genes acting both upstream and downstream of SA and JA (Qiu et al. 2007). Recently, a comprehensive transcriptional profiling of rice WRKY proteins has been examined under various abiotic and phytohormone treatments (Ramamoorthy et al. 2008). But, the important specific roles of most rice WRKY proteins have not been determined so far.

Functional redundancy within multigene families often complicates genetic attempts to define the role of individual member (Bouche and Bouchez 2001). In certain cases, overexpression of the respective gene can provide much information for gene function (Robatzek and Somssich 2002). Here, we report on the functional characterization of a transcription factor OsWRKY23 (also named as OsWRKY15 by Wu et al. 2005 or OsWRKY31 by Zhang and Wang 2005, respectively) by over-expression of it in *Arabidopsis* since network of gene regulation in *Arabidopsis* is far clearer than in any other plant species. Many reports have revealed that heterologous expression of stress-induced rice gene could influence corresponding stress responses in *Arabidopsis*. For example, over-expression of *OsDREB1A* in *Arabidopsis* induced over-expression of target stress-inducible genes of *Arabidopsis DREB1A* resulting in plants with higher tolerance to drought, high-salt, and freezing stresses (Dubouzet et al. 2003). Over-expression of *OsMYB3R-2* increased tolerance to freezing, drought, and salt stress in transgenic *Arabidopsis* (Dai et al. 2007). Over-expression of *OsWRKY45* enhanced disease resistance and drought tolerance in *Arabidopsis* (Qiu and Yu 2009). Thus, heterologous expression gives an opportunity to understand the function of previously uncharacterized genes. The present work shows that OsWRKY23 makes transgenic plant more resistant to pathogen infection and more sensitive to dark-induced senescence.

Materials and methods

Plant materials and growth conditions

Rice (*Oryza sativa* ssp., *japonica* cv. Nipponbare) seedlings were grown in the greenhouse under conditions

having 28°C day/23°C night temperature, 14 h light/10 h dark cycle. For expression profile analysis of various organs and at different developmental stages: 3-week-old roots, 15 days young leaves, 45 days mature leaves, 90 days senescing leaves, 15 days flag leaves and unfertilized young panicles were harvested for RNA extraction.

Arabidopsis thaliana ecotype Columbia was used in all transgenic experiments. The transgenic *Arabidopsis* lines expressing the *OsWRKY23* gene were grown under controlled conditions (23°C day/19°C night) on the same photoperiod of rice seedlings. The empty vector pOCA30 (Du and Chen 2000) was used to generate transgenic control plants in a similar manner. For in vitro analysis, seeds were surface sterilized and plated on 0.5 × Murashige and Skoog agar medium. After being stored in darkness at 4°C for 2 days, the seeds were germinated for 7 days and seedlings were transferred to soil.

Construct preparation of OsWRKY23 cDNA

In our previous research, we isolated the OsWRKY23 cDNA by screening a cold-treated cDNA library derived from rice seedlings (Qiu et al. 2004), followed by the sequencing of the resulting cDNA clones. Sequence analysis of the OsWRKY23 cDNA was confirmed to be identity to the cDNA clones AY870607, AY341845, and AK108909, respectively, from the Shanghai Academy of Agricultural Science of China and the National Institute of Agrobiological Sciences of Japan. To generate the *35S::OsWRKY23* construct, the OsWRKY23 cDNA was cloned into pOCA30 in the sense orientation and behind the 35S promoter. Then the construct was introduced into *Agrobacterium tumefaciens* strain GV3101 and then transformed into *Arabidopsis thaliana* ecotype Columbia plants. Independent transgenic lines were selected for kanamycin resistance and confirmed by RNA gel blot analysis.

RNA gel blot analysis

Total RNA (20 μg) was separated on agarose–formaldehyde gels, stained with ethidium bromide and blotted to nylon membrane. α-^{32}P-dATP-labeled gene specific probes were used and hybridized at 68°C for 16 h. RNA samples from each experiment were analyzed in at least two independent blots, and each experiment was repeated at least twice. Probes for *PR1*, *PR2*, *PR5*, *SAG12* and *SEN1* were obtained from PCR amplification using specific primers: *PR1*, 5′-TCTTCCCTCGAAAGCTCAAG-3′ and 5′-ACACCTCACTTTGGCACATC-3′; *PR2*, 5′-TCCCTTGCTCGTGAATCTCT-3′ and 5′- TCGGTGATCCATTCTTCACA-3′; *PR5*, 5′-CAGTTGTGCGTACACCGTTT-3′ and 5′-CGGTGAATTCCGCTAGAGTC-3′; *SAG12*, 5′-GAGGATGTCCCGGTTAATGA-3′ and 5′-TCCACTTTCTCCCCATTTTG-3′; *SEN1*, 5′-AACATGTGGATCTTTCAAGTGCC-3′ and 5′-GTCGTTGCTTTCCTCCATCG-3′.

Abiotic stress treatment

For abiotic stress treatment, 4-week-old rice (*Oryza sativa* ssp., *japonica* cv. Nipponbare) seedlings were exposed to continuous-dark. Rice leaves in continuous-dark were harvested at 0, 0.25, 1, 3 and 6 days after treatment.

For hormone treatments, 3-week-old rice seedlings were subjected to various hormone solutions (including MeJA, ACC and SA with the same concentration at 100 μM). Samples were then collected in 0, 1, 4, 8 and 24 h intervals, respectively.

Pathogen infections

For the expression profiling of *OsWRKY23* during biotic stress, 3-week-old rice seedlings were inoculated with the pathogen *Pyricularia oryzae* Cav in the control condition with 100% humidity. Leaf tissues were harvested 0, 1, 2, 3, 5, 6 and 7 days after infection.

For transgenic plants, pathogen infection was performed by infiltration leaves (4-week-old) of 35S::*OsWRKY23* transgenic and vector control *Arabidopsis* plants with *Pseudomonas syringae* pv. *tomato* DC3000 (OD_{600} = 0.001). Inoculated leaves were harvested 72 h after infiltration and homogenized in 10 mM $MgCl_2$. Diluted leaf extracts were plated on King's B medium (100 μg/ml rifampicin and 25 μg/ml kanamycin) and incubated at 28°C for 2 days to titer the bacteria. Colony-forming units of 4–6 individual plants were determined from each time point. This pathogen infection experiment was repeated three times with similar results.

For inoculation with *Botrytis cinerea*, 4-week-old plants were infected by spraying or single-leaf drop inoculation as described by Zheng et al. (2006). The spray-inoculated plants were harvested 0, 1 and 2 days after infection.

Continuous-dark induced leaf senescence

More than 100 detached, green rosette leaves (leaves 5, 6 and 7) of 4-week-old transgenic and vector control *Arabidopsis* plants, respectively, were placed onto dishes containing 100 ml of distilled water under continuous-dark (23°C).

ACC-induced leaf senescence

Four-week-old transgenic and vector control *Arabidopsis* plants were sprayed by 100 μM ACC solutions. The plants were harvested at 0, 2 and 4 days.

Measurement of chlorophyll content

Chlorophyll was extracted with 80% acetone from detached leaves. Chlorophyll content was determined at 663 and 645 nm according to Lichtenthaler (1987).

Cell death measurement

Cell death was visualized in senescing detached leaves by lactophenol-trypan blue staining as described (Koch and Slusarenko 1990). Cell death rate was detected by Evans blue staining as described (Guo and Crawford 2005).

Results

Sequence analysis of OsWRKY23

In our previous study, we isolated a full-length cDNA clone for *OsWRKY23* (Os01g53260; Qiu et al. 2004). Sequence analysis showed that the full length of this cDNA clone was 765 bp, encoding a protein of 254-amino acids containing a single WRKY domain (Fig. 1). Within this single WRKY domain was a characteristic Cys_2His_2 zinc-finger-like motif at its C-terminus. WRKY proteins are classified into three groups in light of their structures (Eulgem et al. 2000). OsWRKY23 falls into group II. We clarified its sequence homology by using BLAST program with sequence of OsWRKY23 as query and found it shares 75.3, 59.6 and 51.4% identity at the amino acid level with *AtWRKY43* (At2g46130), *AtWRKY56* (At1g64000) and *AtWRKY24* (At5g41570), respectively. Mangelsen et al. (2008) further revealed that *AtWRKY43* and *AtWRKY56* were more closely related to *OsWRKY23* according to their similar expression profiles in seed stages. All these proteins have not been well characterized so far and analysis of *OsWRKY23* here would provide some novel meaningful information for their functions.

MENLQLQGDDHDDEALPHFPYFAVPSPPPLAVAP 34

AASATTSDGHQHGPLEVLEQPPCSNNLHPDGLVD 68

GPQLAATTAVPMMLPAMTSLDWQSLLQTCLQVPP 102

PVLEQQQPAAAAQADQYSGENDHGDLQAAESSGA 136

GNKEKQVMAKGGAGRPSGTKKKASRPRFAFQTRS 170

WRKY DNA-binding motif

DNDILDDGYR**WRKYGQK**AVKNSKHPRSYYR**C**THH 204

T**C**NVKKQVQRLAKDTSIVVTTYEGV**H**N**H**PCEKLM 238

EALTPILKQLQFLSQF 254

Fig. 1 A deduced amino acid sequence of OsWRKY23. The highly conserved WRKYGQK sequence and the C_2H_2 zinc-finger residues (WRKYGQK-Xn-C-X_{4-5}-C-Xn-HXH) are underlined

Expression patterns of OsWRKY23

In order to better understand the biological function of *OsWRKY23* gene, we first analyzed *OsWRKY23*'s expression pattern in various organs and at different developmental stages. RNA gel blot analysis showed that *OsWRKY23* gene was only expressed in roots and in senescing leaves, but not in all other organs tested (Fig. 2a, b). This expression pattern revealed a novel and strong association of *OsWRKY23* with the process of senescence.

To analyze the involvement of *OsWRKY23* in plant basal defense, we analyzed its expression in response to the pathogenic fungus *Pyricularia oryzae* Cav. It is interesting that expression of *OsWRKY23* can be induced markedly in leaves under treatments, although it did not express consistently in this organ. *OsWRKY23* transcripts were elevated substantially within 1 day after pathogen inoculation, and then declined gradually to low level at 6 days; whereas at 7 days, the expression level of *OsWRKY23* increased to the level of 2 days (Fig. 2c). Expression profiling using different plant tissues revealed a strong association of *OsWRKY23* with senescence. Leaf senescence, a form of programmed cell death (PCD), could be induced by continuous-dark conditions (Yoshida 2003; Lin and Wu 2004). When under continuous-dark, the transcripts of *OsWRKY23* started to accumulate at 3 days post-treatment, reaching a maximal level at 6 days (Fig. 2d). We also analyzed the induction of *OsWRKY23* using RNA blots by defense-inducing molecules SA, 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ET biosynthesis, and methyl jasmonic acid (MeJA). The *OsWRKY23* gene was induced rapidly and strongly by SA (Fig. 2e). The transcript level of *OsWRKY23* increased rapidly but slightly within 1–4 h after treatment with ACC, and then returned to basal levels within 8 h (Fig. 2e). JA treatments did not induce *OsWRKY23* expression (Fig. 2e). These results suggest that *OsWRKY23* is involved in plant defense response pathways possibly including pathogen response and leaf senescence. The enhanced induction of *OsWRKY23* by SA suggests that SA signaling pathways may have an important role in *OsWRKY23* involved stress responses.

Over-expression of *OsWRKY23* gene in *Arabidopsis* enhanced plant disease resistance

To further analyze the biological functions of the *OsWRKY23* gene, we generated more than 30 independent

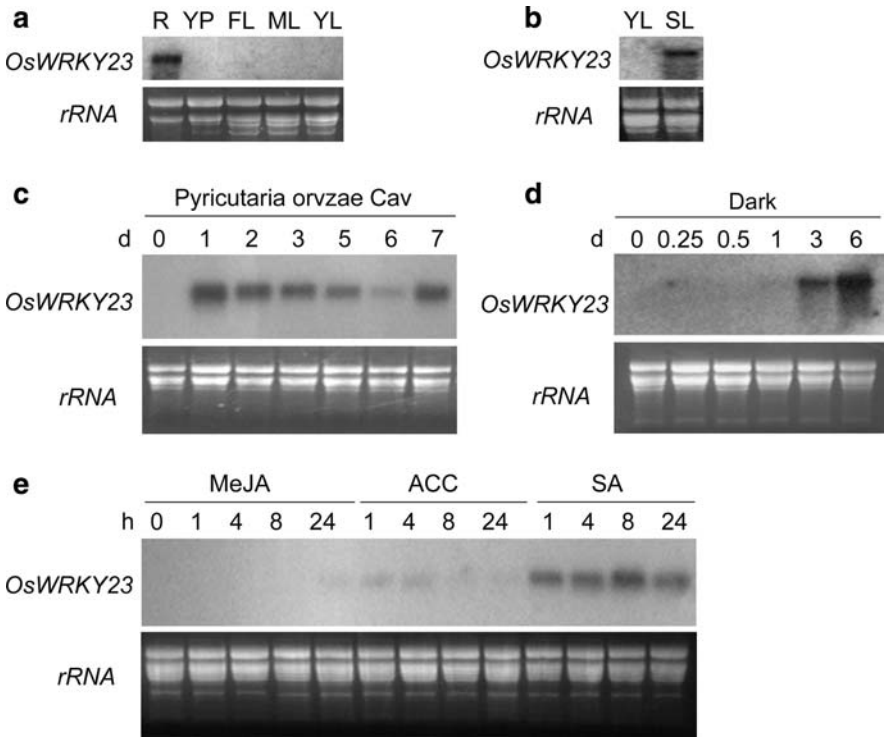

Fig. 2 Expression profiles of *OsWRKY23* gene. **a** *OsWRKY23* gene expression in various plant organs. R: 3-week-old roots; YP: unfertilized young panicles; FL: 15 days flag leaves; ML: 45 days mature leaves; YL: 15 days young leaves. **b** Expression of *OsWRKY23* in young leaves and senescing leaves. YL: 15 days young leaves; SL: 90 days senescing leaves. **c** Time course of *OsWRKY23* expression in response to pathogen inoculation. **d** Time course of *OsWRKY23* expression in response to continuous-dark stress. **e** Time course of *OsWRKY23* expression in response to plant hormone. To avoid the conserved WRKY DNA-binding domain and ensure the hybridization probe is specific enough, we used a 320 bp long fragment of 5′ specific region of *OsWRKY23* cDNA digested by Kpn1 as gene specific probe

transgenic *Arabidopsis* lines, each harboring the *OsWRKY23* cDNA under the control of the cauliflower mosaic virus 35S promoter. Among the 10 lines analyzed by RNA gel blot hybridization, two transgenic lines of 35S-W23-L5 and 35S-W23-L8 exhibited the greatest expression level of *OsWRKY23* gene. The transgenic line 35S-W23-L7 also expressed *OsWRKY23* at a high level, but lower than that of either 35S-W23-L5 or 35S-W23-L8 (Fig. 3a). Thus, 35S-W23-L7 (moderate expression) and 35S-W23-L8 (strong expression) were selected for further analysis regarding defense responses to biotic and abiotic stresses. Besides the level of ectopic *OsWRKY23* expression, no other obvious different morphological or growth phenotypes were observed between the vector control line and the *35S:OsWRKY23* transgenic lines (Fig. 3b).

To determine the effect of *OsWRKY23* over-expression on disease resistance in *Arabidopsis*, We examined the response of vector control plants and *35S:OsWRKY23* transgenic plants to the virulent bacterium *Pseudomonas syringae* pv. *tomato* DC3000. Four-week-old plants were inoculated with the bacteria and the growth of the pathogen was monitored. The inoculated leaves of the transgenic plants displayed less chlorosis than vector control plants at

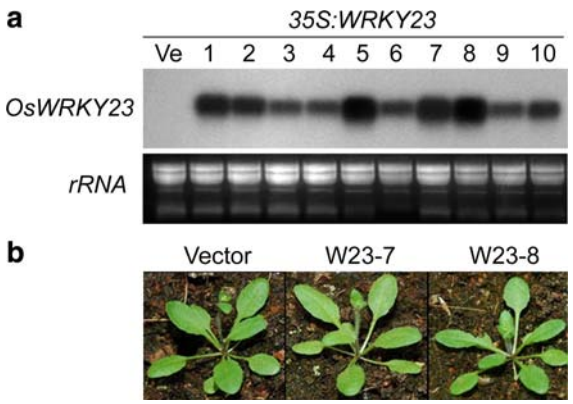

Fig. 3 *OsWRKY23* over-expression *lines*. **a** Over-expression of *OsWRKY23* gene in transgenic *Arabidopsis* plants. Total RNA was isolated from both untreated 4-week-old vector control plants and the T2 progeny of transgenic *lines* containing the *35S:WRKY23* construct and probed with an *OsWRKY23* specific fragment. Ve: vector **b** Growth of transgenic *lines* and vector control plant

3 and 7 days after inoculation (Fig. 4a). Consistently, 3 days after infiltration with PstDC3000, the *35S:WRKY23* over-expression lines supported levels of bacterial growth that was significantly lower than the vector control plants.

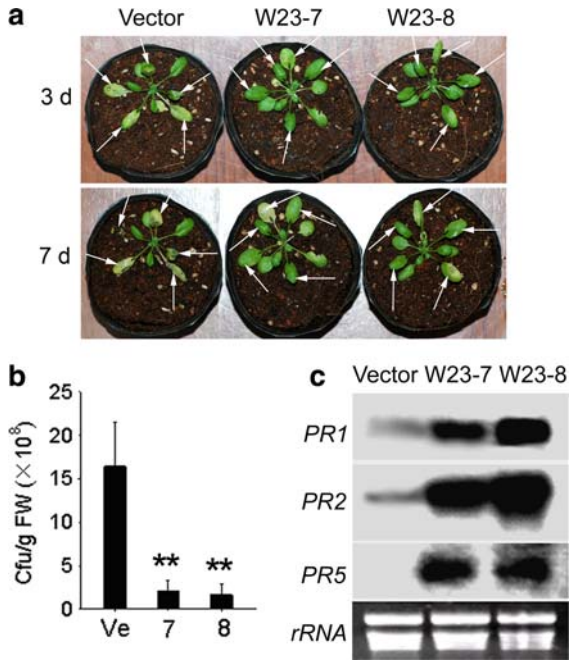

Fig. 4 Enhanced disease resistance in transgenic plants. **a** The images of *OsWRKY23* transgenic *lines* and *vector* control plants at 3 and 7 days after infection with PstDC3000. The infiltration leaves are marked with *white arrow*. **b** Bacterial titer in wild type and transgenic *Arabidopsis* plants at 3 days post inoculation with PstDC3000. Histograms are the average of triplicate bacterial disease assays and the *bars* indicate SD. Differences between vector control plants and transgenic *lines* were tested with one-way ANOVA followed by LSD post hoc test. ** differences for the transgenic plants compared with vector control plants are highly significant ($P < 0.01$, $n = 3$). Ve: vector; 7: W23-7; 8: W23-8. **c** Expression of *PR* genes in transgenic plants. Total RNA was isolated from both 4-week-old untreated vector control plants and the T2 progeny of two transgenic *lines* and probed with *Arabidopsis PR1*, *PR2* and *PR5* gene, respectively

A marked decrease (8–10 folds) in the bacterial growth was observed in the two transgenic lines relative to that of the vector control plants (Fig. 4b). These results show that heterologous expression of *OsWRKY23* leads to enhanced disease resistance in *Arabidopsis*. To further analyze the defense response in the *35S:WRKY23* over-expression lines, we compared their defense gene expression with that of vector control plants. SA-mediated defense plays a critical role in plant defense against the bacterial pathogen *P. syringae*. SA-mediated defense mechanisms are associated with the expression of *PR* genes, including *PR1*, *PR2*, and *PR5* (Li et al. 2004). Among these genes, *PR1* is often used as a reliable molecular marker for systemic acquired resistance (SAR). In healthy vector control plants, the basal expression levels of *PRs* were very low; whereas in healthy *35S:WRKY23* over-expression lines, the transcript levels of *PR1*, *PR2*, and *PR5* genes were significantly higher than those in the vector control plants (Fig. 4c). These results demonstrate that expression of these *PR* genes is positively regulated by *OsWRKY23* and the enhanced resistance of *35S:WRKY23* plants to the bacterial pathogen is associated with increased expression of SA-regulated *PRs*. We also evaluated the *35S:WRKY23* plants and vector control plants for responses to a necrotrophic fungal pathogen *Botrytis cinerea*. Disease symptoms and accumulation of important *Botrytis cinerea* resistance-related gene *PDF1.2* in *35S:WRKY23* over-expression lines did not differ from vector control plants (Supplementary Fig. 1). Thus, with no altered responses to *Botrytis cinerea*, over-expression of *OsWRKY23* enhanced the defense resistance to the bacterial pathogen *P. syringae* in *Arabidopsis*. Consistent with what Glazebrook (2005) have reported, these results suggest that different defense responses are required to combat different types of microbial pathogens.

OsWRKY23 accelerates dark-induced leaf senescence in transgenic plants

OsWRKY23 gene was expressed in senescing leaves and was induced by continuous-dark treatment, suggesting a possible role in leaf senescence (Fig. 2b, d). Senescence was induced by dark treatment of detached leaves, a procedure that is commonly used to artificially induce leaf senescence (Oh et al. 1996; Weaver et al. 1998). Under this condition, leaves of *35:WRKY23* transgenic plants senesced more rapidly than do vector control plants. Individual leaves from vector control plants became pale green after 4 days of dark treatment, whereas leaves from the transgenic lines showed severe yellowing and cell death (Fig. 5a, b). Measurement of chlorophyll content showed that chlorophyll was lost much more quickly in the leaves of transgenic lines than in vector control plants. The chlorophyll content reduced by 64 and 76% in 35S-W23-L7 and 35S-W23-L8 lines, respectively, whereas the vector

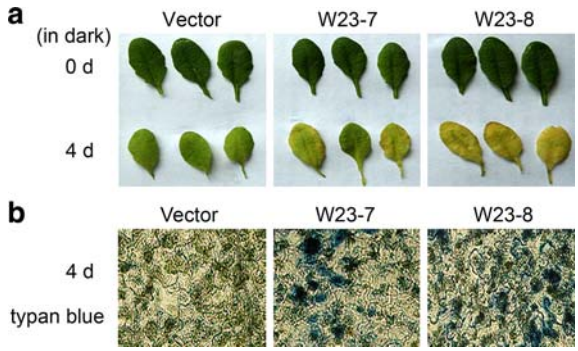

Fig. 5 Leaf senescence in control and transgenic plants under continuous-*dark* treatment. **a** Images of untreated leaves or leaves treated in continuous-*dark* for 4 days were taken. **b** Images of leaves stained with lactophenol-*trypan blue* at 4 days under dark treatment. The micrographs were taken with a light microscope at 200× magnification

control line lost only 51% after 5 days of continuous-dark treatment (Fig. 6a). Evans blue staining also showed that cell death rates were higher in the *OsWRKY23* transgenic lines: 47 and 57% for the 35S-W23-L7 and 35S-W23-L8 lines, respectively, whereas 30% for the vector control line after 5 days of treatment (Fig. 6b).

Many genes up-regulated during the senescence process have been cloned as senescence associated genes, such as *SAG12* and *SEN1*. *SAG12* is a relatively common gene to emerge from differential screenings which encodes a cysteine protease (Lohman et al. 1994; Weaver et al. 1998). The *SEN1* gene encoding a senescence-associated protein of *Arabidopsis* has been used as a marker to characterize the senescence-associated response (Oh et al. 1996; Hanaoka et al. 2002). We compared the expression levels of *SAG12* and *SEN1* between transgenic and control plants. RNA gel blot analysis showed that *SAG12* was weakly induced in transgenic plants at 2 days after dark detachment whereas control plant had little transcript level (Fig. 7a). *SEN1* was induced more strongly in transgenic plants than in vector control plants at 2 and 4 days after dark detachment (Fig. 7b). This result provides molecular evidence for sensitivity of *OsWRKY23 Arabidopsis* transgenic plants to continuous-dark induced leaf senescence. These results suggest that heterologous expression of *OsWRKY23* enhances sensitivity of *Arabidopsis* transgenic plants to continuous-dark induced leaf senescence.

OsWRKY23 was induced slightly by ACC, the immediate precursor of ET biosynthesis. ET is an important hormone in regulation of diverse processes including pathogen attack, abiotic stresses and leaf senescence. So we also examined the responses of *35S:WRKY23* lines and vector control plants to ACC treatment. There was no altered phenotype and the transcripts of above two senescence associated genes in transgenic lines did not differ from vector control plants (Supplementary Fig. 2). Thus, whereas *35S:WRKY23* plants showed accelerated dark-induced senescence, it responded normally to ACC treatment.

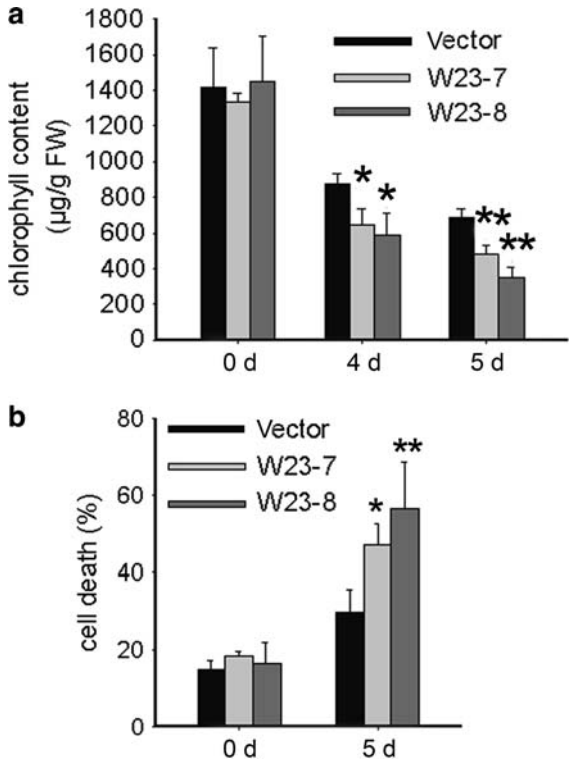

Fig. 6 Comparison of chlorophyll content and cell death rate between vector control and OsWRKY23 transgenic plants. **a** Chlorophyll content in control and transgenic plants in *dark* treatment. Histograms are the average of triplicate assays and the *bars* indicate SD. **b** Cell death rate was measured spectrophotometrically as Evans *blue* staining in detached leaves from control and transgenic plants as described in "Materials and methods". Histograms are the average of triplicate assays and the *bars* indicate SD. ** differences for the transgenic plants compared with vector control plants are highly significant ($P < 0.01$, $n = 3$). * differences for the transgenic plants compared with vector control plants are significant ($P < 0.05$, $n = 3$)

Discussion

Transcriptional regulation of plant defensive genes plays a central role in the activation of plant inducible defense responses. In the past years, several families of sequence-specific DNA binding transcription factors of plant defense genes have been identified, including WRKY factors which have been implicated in the regulation of pathogen defense, abiotic stress response and development process (Yu et al. 2001; Miao et al. 2004; Devaiah et al. 2007). Here we report functional characterization of *OsWRKY23* and suggest that *OsWRKY23* is likely to be involved in modulating resistance to bacterial pathogen infection and leaf senescence. The involvement of *OsWRKY23* in the modification of leaf senescence in addition to regulating pathogen response highlights the intricate network between these biological processes.

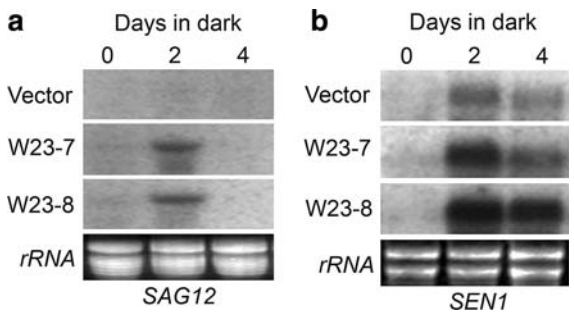

Fig. 7 RNA gel blot analysis of the transcripts of senescence related genes in vector plants and transgenic plants. **a** Senescence marker gene *SAG12* was weakly induced at 2 days in transgenic *lines* 35S-W23-L7 and 35S-W23-L8. **b** Strong up-regulation of *SEN1*

The innate immune system is the first line of inducible defense against infectious disease. In innate immunity, direct or indirect recognition of pathogen-associated molecular patterns (PAMPs) often leads to rapid activation of a hypersensitive response (HR) that confers a specific and effective resistance against all races of the pathogen. The pathogen-induced HR is often associated with activation of defense mechanisms in both local and distal parts of the plants, leading to SAR. The plant PR proteins serve as markers for monitoring local and systemic induced resistance. In *Arabidopsis*, the *PR1*, *PR2*, and *PR5* proteins are likely to play key roles in establishing SAR against a pathogen attack, controlled mainly by a SA-dependent transduction pathway (Dong 1998; Li et al. 2004). As an essential signal in pathogen defense, SA can activate expression of *PR* genes and enhance resistance to a broad spectrum of pathogens in distant and uninfected plant parts. In these responses to pathogen attack, WRKY proteins play important regulatory roles (Maleck et al. 2000; Chen and Chen 2002; Li et al. 2004; Catalino et al. 2006). Here, we have attempted to analyze the biological impacts of constitutive expression of a pathogen induced gene *OsWRKY23* in *Arabidopsis*. The fact that *OsWRKY23* was strongly induced by SA and was not significantly influenced by MeJA and ACC indicates it possibly involved in SA-dependent defense pathway. Consistent with our hypothesis, over-expression of *OsWRKY23* in *Arabidopsis* activated *PR* genes expression and enhanced resistance to *Pseudomonas syringae*. We also observed that, after *Botrytis* infection, the *35S:WRKY23* plants showed similar disease symptoms and similar expression of *PDF1.2*, a molecular marker of the JA- and ET-mediated defense response signaling pathways (Zheng et al. 2006). This observation suggests that *OsWRKY23* is not involved in response to *Botrytis* mediated mainly by JA/ET signaling. Thus, *OsWRKY23* might play a positive role in SA-mediated signaling pathways, and over-expression of the gene could have a positive impact on SA-mediated defense mechanisms and thus enhance plant resistance to *P. syringae*.

Although high levels of production of OsWRKY23 can be beneficial in plant pathogen responses, it also carries some costs. In this work, we have found that transgenic plants expressing high levels of this protein exhibited more sensitive to continuous-dark induced senescence. It has been suggested that the regulatory network governing leaf senescence has substantial crosstalk with plant defense signaling pathways (Buchanan-wollaston et al. 2005). Presently, we know very little about the regulatory factors of senescence-associated gene expression and repression. Expression profiling has revealed that WRKY factors constitute the second largest group of transcription factors of the senescence transcriptome (Guo et al. 2004). Thus, WRKY transcription factors may play a potential role in transducing the signals in both pathogen and senescence responses. Here, we used 'artificial senescence' with leaves detached from the plants and placed in darkness. This treatment is a very strong inducer of senescence and can induce leaf yellowing and chlorophyll loss paralleled the senescence associated genes induction. Previously, a dark-induced expression of *SAG12* and *SEN1* has been reported to occur after this dark detachment senescence, although *SAG12* only responded relatively weakly in older leaves (Weaver et al. 1998). In this study, we showed that transcripts of *OsWRKY23* were much higher in senescing leaves, suggesting it involved in regulation of leaf senescence. Expression of *OsWRKY23* also increased in darkness, which was consistent with *OsWRKY23* transgenic plants' early senescence phenotypes in darkness. The concomitant change in transcriptional activities of the senescence associated marker genes *SAG12* and *SEN1* in the *OsWRKY23* transgenic plants lends further support for a role of *OsWRKY23* in partly regulating steps within the process of leaf senescence. The normal responses of *35S:WRKY23* plants to ACC treatment suggest that leaf senescence is a complex process and *OsWRKY23* is only one gene partly involved in this important process. Establishment of the complex plant regulatory mechanisms remains a challenging endeavor.

In summary, our findings show that OsWRKY23, an induced regulatory factor, may be involved in pathogen and dark-induced senescence responses. This study could provide beneficial suggestions for further work about OsWRKY23 in two complex interlinked networks.

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