Plant Mol Biol
DOI 10.1007/s11103-015-0329-7

The rice transcription factor *OsWRKY47* is a positive regulator of the response to water deficit stress

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Received: 16 March 2015 / Accepted: 30 April 2015

Abstract *OsWRKY47* is a divergent rice transcription factor belonging to the group II of the WRKY family. A transcriptomic analysis of the drought response of transgenic rice plants expressing P_{SARK}*::IPT*, validated by qPCR, indicated that *OsWRKY47* expression was induced under drought stress in P_{SARK}*::IPT* plants. A PCR-assisted site selection assay (SELEX) of recombinant OsWRKY47 protein showed that the preferred sequence bound in vitro is (G/T)TTGACT. Bioinformatics analyses identified a number of gene targets of *OsWRKY47*; among these two genes encode a Calmodulin binding protein and a Cys-rich secretory protein. Using *Oswrk47* knockout mutants and transgenic rice overexpressing *OsWRKY47* we show that the transcription of these putative targets were regulated by OsWRKY47. Phenotypic analysis carried out with transgenic rice plants showed that *Oswrky47* mutants displayed higher sensitivity to drought and reduced yield, while plants overexpressing *OsWRKY47* were more tolerant.

Keywords P_{SARK}*::IPT* · *OsWRKY47* · WRKY transcription factor · Delayed senescence · Drought tolerance

Introduction

WRKY transcription factors (TF) are defined by the presence of a 60 amino acid conserved region containing a WRKY and a zinc-finger-like motif. WRKY TFs have been classified in three groups according to the number of WRKY domains that they encode and to the zinc finger motif pattern. Members of group I exhibit two WRKY motifs and those of group II have only one motif (Eulgem et al. 2000); both groups I and II have a zinc finger type C_2–H_2 (C–X_{4-5}–C–X_{22-23}–H–X_1–H). A third group, group III, is formed by proteins with one WRKY motif and a zinc finger type C_2–HC(C–X_7–C–X_{23}–H–X_1–C). Sequence alignment tools and phylogenetic analyses have been used for the identification and classification of WRKY TFs in a few species such as *Arabidopsis thaliana* (Eulgem et al. 2000), *Oryza Sativa* (Wu et al. 2005; Xie et al. 2005; Ramamoorthy et al. 2008), *Hordeum vulgare* (Mangelsen et al. 2008) and *Heilanthus annuus* (Giacomelli et al. 2010), among others. In rice, the WRKY family comprises 102 proteins (Wu et al. 2005). All the known WRKY TFs tested so far, bind in vitro a sequence named W-box containing an invariant TGAC core, and variations outside this core (Eulgem et al. 2000).

WRKY TFs play pivotal roles in the complex signaling mediating plant responses to biotic and abiotic stresses (Ulker and Somssich 2004; Eulgem and Somssich 2007;

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Electronic supplementary material The online version of this article (doi:10.1007/s11103-015-0329-7) contains supplementary material, which is available to authorized users.

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Published online: 09 May 2015

Rushton et al. 2010; Chen et al. 2012; Tripathi et al. 2014; Schluttenhofer and Yuan 2015). Overexpression of soybean *GmWRKY13* in Arabidopsis conferred higher sensitivity to salinity and osmotic stress in the transgenic plants (Zhou et al. 2008). On the other hand, the expression of the barley *HvWRKY38* in *Paspalum notatum Flugge* increased the drought tolerance of the transgenic plants (Xiong et al. 2010). Similar results were obtained when the *Glycine Soja GsWRKY20* was expressed in *Arabidopsis* (Luo et al. 2013) and the maize *ZmWRKY58* was overexpressed in transgenic rice (Cai et al. 2014). In rice, the overexpression of *OsWRKY7* (Ramamoorthy et al. 2008), *OsWRKY05*, *OsWRKY43*, *OsWRKY1*, *OsWRKT2* (Berri et al. 2009) and *OsWRKY11* (Wu et al. 2009) was shown to improved drought tolerance.

A number of mechanisms associated with the ability of plants to tolerate water deficit stress have been described (Chen et al. 2012; Reguera et al. 2012; Suzuki et al. 2014). They involve regulatory molecules, TFs and hormones such as ABA, cytokinin (CK), ethylene and their crosstalk mechanisms (Peleg and Blumwald 2011). Among these, the CK-induced delayed senescence has been shown to be effective in increasing the tolerance of plants to water deficit (Rivero et al. 2007). Transgenic plants expressing the *IPT* gene, encoding a key enzyme in CK synthesis, under the control of *SARK* (a stress- and maturity-induced promoter) displayed enhanced photosynthesis and high yields under water stress (Rivero et al. 2007; Peleg et al. 2011b; Reguera et al. 2013). A Gene expression profile of flag leaves from wild-type plants and transgenic rice plants expressing P_{SARK}*::IPT*, highlighted the differential expression of *OsWRKY47* in the P_{SARK}*::IPT* plants under water stress (Peleg et al. 2011b). The correlation between the enhanced stress tolerance of the transgenic P_{SARK}*::IPT* plants and the enhanced *OsWRKY47* expression suggested a possible role of *OsWRKY47* in water stress tolerance.

OsWRKY47 is a TF divergent from its more related proteins in rice and other species. It presents 50 and 42 % similarity with *WRKY38* and *WRKY70* of *Setaria italica,* respectively; 42 % similarity with *BdWRKY70* of *Brachypodium distachyon* and 46 % similarity with the barley *HvWRKY4*. The *Arabidopsis* TFs most related to OsWRKY47 are AtWRKY46, AtWRKY54 and AtWRKY70; however, the sequence similarity with these proteins is relatively low. *AtWRKY70* and *AtWRKY54* have been associated with leaf senescence in Arabidopsis (Besseau et al. 2012). *Atwrky70* mutant plants displayed an early senescence phenotype, and a role as a negative regulator of senescence was suggested for this TF (Ülker et al. 2007). In spite of the high sequence and structure similarity between *AtWRKY70* and *AtWRKY54*, *Atwrky54* mutant plants did not show changes in senescence (Besseau et al. 2012). Nevertheless, double *Atwrky54-Atwrky70* knockout mutant plants showed an increased early senescence as compared to that displayed by *Atwrky70*, thus suggesting a possible cooperative interaction between *AtWRKY70* and *AtWRKY54* negatively regulating leaves senescence (Besseau et al. 2012).

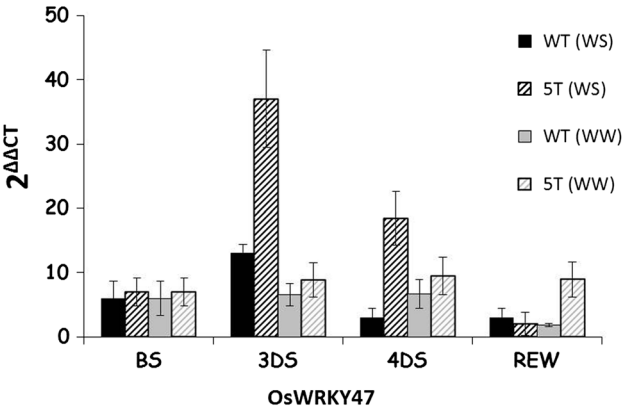

Fig. 1 Relative expression of *OsWRKY47* in WT and transgenic P_{SARK}*::IPT* plants. *BS* before stress (BS), 3DS and 4DS, days of water stress and ReW, 3 days after re-watering. *WW* well-watered, *WS* water-stress, *WT* wild type rice; 5T, P_{SARK}*::IPT* plants. Samples were harvested at the indicated periods. Values were calculated and normalized using the rice transcription elongation factor as internal control. Values are the mean ± SD (n = 6)

Here, we describe experiments aimed at elucidating *OsWRKY47* function(s) in the enhanced drought tolerance of transgenic P_{SARK}*::IPT* rice plants.

Results

OsWRKY47 expression is induced in water stressed P_{SARK}*::IPT* plants

Transcriptome analysis of wild-type (WT) and transgenic P_{SARK}*::IPT* rice plants (Peleg et al. 2011b) showed induced expression of *OsWRKY47* under WS in P_{SARK}*-IPT* plants. qPCR validation showed that the *OsWRKY47* transcript levels increased sixfold after 3 days of WS and decreased during the 4th day of stress in P_{SARK}*-IPT* plants (Fig. 1). These levels were significant higher than those shown by the WT plants under WS. The *OsWRKY47* levels of expression returned to WW levels after re-watering (Fig. 1).

OsWRKY47 preferentially binds the sequence GTTGACC in vitro

In order to identify signal pathway(s) components associated with *OsWRKY47* in P_{SARK}*-IPT* plants, we identified DNA sequences that could be specifically bound by *OsWRKY47*. A PCR-assisted binding site selection (SELEX) assay was

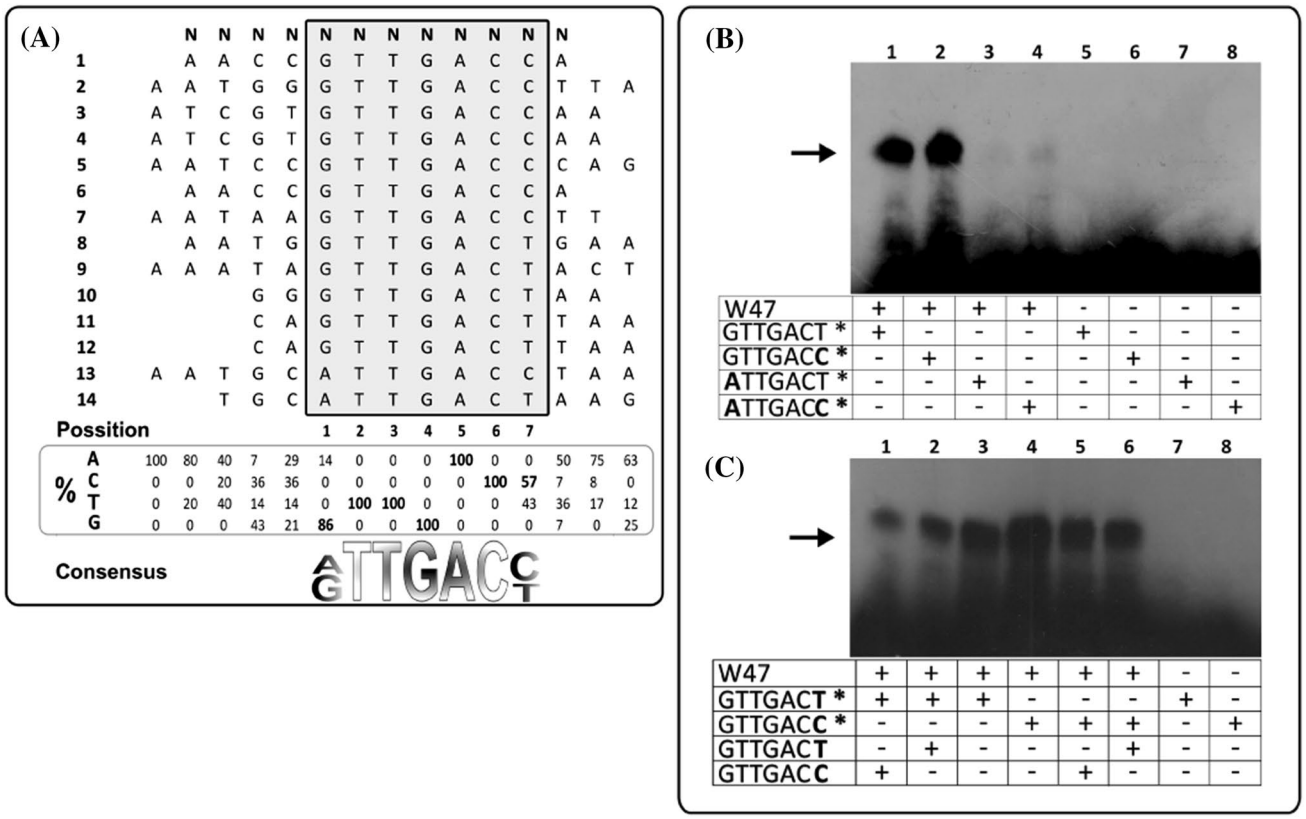

Fig. 2 *OsWRKY47* binds in vitro the sequence (G/A)TTGAC (T/C) containing a canonical W-box. **a** Compilation of the sequences of random clones obtained after cloning the selected oligonucleotide population. The sequences of 14 different clones, obtained from the last SELEX step performed with the recombinant *OsWRKY47*, were aligned to obtain the best consensus (G/A)TTGAC(T/C). Clone numbers (arbitrary) are indicated on the *left*. Below, a table indicating nucleotide frequencies at each position and the derived consensus sequence. **b** EMSA assay performed with OsWRKY47 with four variants of the consensus obtained after SELEX. *Lane 1* GTTGACT, *Lane 2* GTTGACC, *Lane 3* ATTGACT, *Lane 4* ATTGACC. **c** Competition EMSA assay performed with *OsWRKY47* with labeled oligonucleotides containing GTTGACT or GTTGACC alone or in the presence of the same unlabeled oligonucleotides. Each double stranded oligonucleotide (10,000 cpm) was incubated during 20 min as described in Methods with equal aliquots of W47 before loading. In the competition assays 100-fold unlabeled double-stranded oligonucleotides containing the indicated sequences were incubated with the protein during 10 min before the labelled oligonucleotide was added

applied as an experimental strategy. The binding domain of *OsWRKY47* was expressed in *E. coli* cells as a fusion with *S. Japonicum* glutathione S-transferase and purified by affinity chromatography. Seven SELEX rounds were performed, and since no additional improvement was detected after the 7th round, the PCR products were cloned and 14 individual clones were randomly selected for sequencing. The sequences were aligned (Fig. 2a) and the alignments resulted in a 7 bp consensus (G/A)**TTGAC**(T/C) or its reverse complement (A/G)**GTCAA**(T/C), containing the canonical W box (T)**TGAC**(C/T). Notably, in addition to this conserved core, positions −4, −5, +9 and +10 (taking the first G/A as +1) showed a highly conserved A, extending the target to a 14 bp sequence **AA**NNN(G/A)**TTGAC**(T/C)N**AA** (Fig. 2a).

In order to solve the slight incertitude in the central core flanking nucleotides (Fig. 2a), synthetic double strand oligonucleotides bearing variants 1 (**G**TTGAC**C**), 2 (**G**TTGAC**T**), 3 (**A**TTGAC**T**) or 4 (**A**TTGAC**C**) of the consensus sequence were labeled and confronted to the recombinant OsWRKY47. EMSA assays performed with and without unlabeled competitors (Fig. 2b, c) indicated that G was clearly preferred than A in the +1 position (Fig. 2b) while C was slightly preferred as compared to T in position 7 (Fig. 2c). These results were consistent with the abundance of each nucleotide observed when the analysis of the SELEX was carried out (Fig. 2a). Altogether these results indicated that GTTGACC was the OsWRKY47 preferred sequence, at least in vitro.

Identification of *OsWRKY47* targets

Transcriptome analysis of the transgenic P_{SARK}::*IPT* rice plants, combined with the SELEX results, allowed the

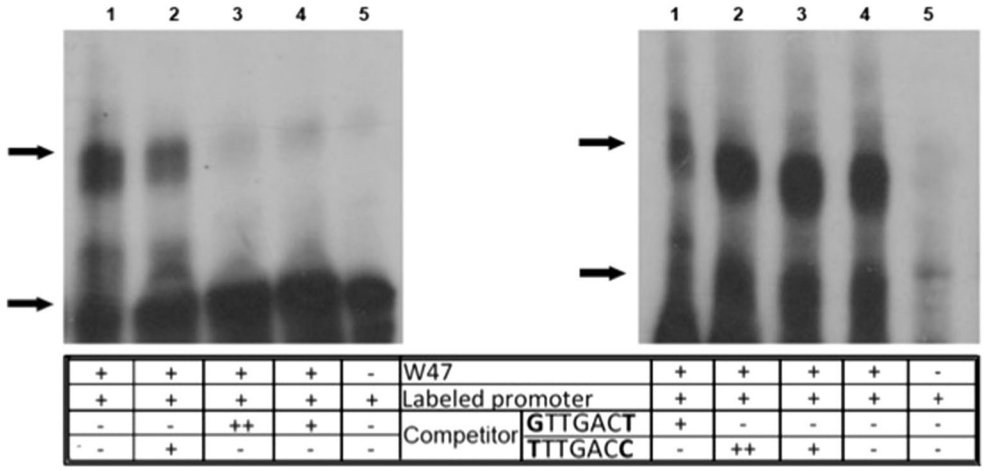

Fig. 3 The CRRSP-rich protein and the CaBP promoters are bound by OsWRKY47 in vitro. Segments of the CRRSP-rich protein (137 bp) and calmodulin-binding protein (142 bp) promoters, containing the W-boxes were labeled and used as probes in EMSA assays. *OsWRKY47* was confronted during 20 min with the DNA labeled segments (10,000 cpm each) as described in Methods. For competition assays 100-fold unlabeled DNA containing the sequences indicated below was incubated with the protein during 10 min before adding the labelled promoter

identification of few putative target genes. For the identification of putative targets of *OsWRKY*, the transcriptome of transgenic P_{SARK}::*IPT* was analyzed aiming at the identification of genes with expression patterns similar to *OsWRKY47*. We considered genes exhibiting up- or down-regulation under WS in P_{SARK}::*IPT vs.* WT or in P_{SARK}::*IPT* plants under WS vs. WW. This first selection resulted in 356 genes (Fig. S1). These 356 genes were investigated for the presence of *OsWRKY47*-bound sequences in their promoters, specifically for the presence of **G**TTGAC**C** or **G**TTGAC**T** domains. This second selection further reduced the number of candidate genes to 82 (Fig. S1; Table S1). An in silico analysis using Blas2GO (www.blast2go.com) and Mapman (www.mapman.gabipd.org) did not contribute to the identification of specific process/metabolism enriched with these 82 genes. In addition, we examined genes that were co-expressed with *OsWRKY47* in other tissues/organs using in silico analysis of publicly available rice microarray databases. This analysis reduced the putative targets to 26 genes (Table S2), of which eight targets were selected for validation by qRT-PCR (Fig. S2). These genes included: Cys Rich Repeat Secretory Protein 55 Precursor (*CRRSP*; LOC_Os03g16950); Calmodulin-Binding Protein (*CaMBP*; LOC_Os12g36110); Receptor-like Kinase (LOC_Os06g36270); Metal (Zn) Cation Transporter (LOC_Os03g29850); Cys Rich domain containing protein (LOC_Os03g01210); Rhodanese-Like (LOC_Os02g06290); Receptor-like protein kinase (LOC_Os05g25350) and Brassinosteroid insensitive 1-associated receptor kinase 1 (*BAK1*; LOC_Os08g07760). Two of these genes, *CaMBP* and *CRRSP* were selected for further analysis.

OsWRKY47 binds in vitro and in vivo the promoters of *CaBP* and *CRRSP*

Segments of the promoters of *CaMBP* and *CRRSP* containing the DNA sequences identified as bound by *OsWRKY47* were labeled and used in EMSA assays with the recombinant *WRKY47* protein. The protein was able to specifically bind to the segment GTTGACT in the *CaMBP* promoter and the sequence TTTGACT in the *CRRSP* promoter (Fig. 3). Increasing concentrations of the unlabeled DNA fragment, used as a competitor, significantly diminished the binding while the competition with other unlabeled sequences did not, supporting the specificity of the binding (Fig. 3).

To confirm these interactions in vivo, *Nicotiana benthaniana* leaves were transiently co-transformed with P_{35S}::*WRKY47* and different constructs bearing the *CaMBP* or the *CRRSP* promoters directing the reporter *GUS* expression or the same promoters where the putative active W-boxes were mutated or deleted (Fig. 4). GUS activity was evaluated fluorometrically (Fig. 4a) and histochemically (Fig. 4b). GUS activity was detected when tobacco leaves were transformed with the constructs bearing either the *CaMBP* or the *CRRSP* promoter directing it even in the absence of OsWRKY47, suggesting that other WRKY TFs could be also involved in the activation of *CaMBP* and *CRRSP*. However, GUS activity was clearly increased (threefold to fourfold change) when P_{35S}::*WRKY47* was used to co-transform the leaves. The *GUS* expression strongly decreased when the core of the W-boxes (4 bp) was deleted or when the C in position +6 was mutated to T, indicating the specific binding of OsWRKY47 to this sequence activating *GUS* expression (Fig. 4). Positive and

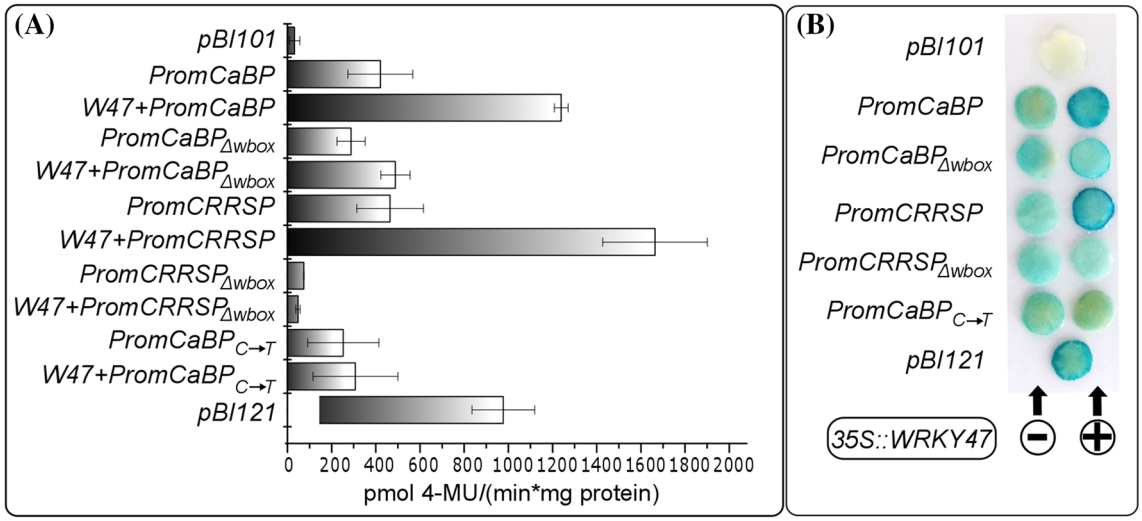

Fig. 4 *OsWRKY47* regulates the expression of CaBP and CRRSP in transient co-expression assays **a** GUS activity was evaluated by fluorometry after transient co-expression assays of tobacco-leaf disks. An empty vector *35S::null* (negative control) or *35S::OsWRKY47* (W47) were co-transformed with the complete promoters of the cys-rich protein (Prom Cys, 1448 bp upstream the +1) or of the calmodulin-binding-protein (Prom Cal, 667 bp upstream the +1) or mutated versions of the same promoters (Prom CaBP del, Prom CRRSP del, Prom CaBP C1464→T) all fused to the *GUS* reporter gene. **b** Illustrative photographs of the leaf-disks assayed by GUS histochemistry

negative transient transformation controls were performed using *35S::GUS* and *35S::null* constructs, respectively, yielded the expected results (Fig. 4).

CaMBP and *CRSSP* transcript levels were evaluated by qRT-PCR in a *WRKY47* knockdown mutant (*Oswrky47*, termed *Osw47-1*) and in plants overexpressing *WRKY47*. We obtained *Osw47-1*, a *WRKY* mutant showing a transposon-DNA insertion in the first intron of the *OsWRKY47* gene (Fig. 5a), resulting in a significant decrease in *OsWRKY47* transcripts (Fig. 5b, c). Both *CaMBP* and *CRSSP* genes were down-regulated in the *Osw47-1* mutant (Fig. 5d, e) and up-regulated in the *WRKY47*-overexpressing plants (Fig. 5g, h), as compared to the rice WT plants, supporting the notion of the regulation of *CaMBP* and *CRSSP* expression by *WRKY47*. Both *CaMBP* and *CRSSP* displayed higher transcript levels in OE-1 and OE-2 plants even before the drought treatment, indicating their responsiveness to *WRKY47* (Fig. 5g, h). Consistent with this notion, *CaMBP* and *CRSSP* expression was repressed in *Osw47-1* grown under well-watered conditions and remained low during the stress episode and after re-watering (Fig. 5d, e).

Effects of silencing and overexpressing *OsWRKY47* on drought tolerance

Two independent transgenic rice plants (OE-1 and OE-2) expressing the P_{Ubi}*::OsWRKY47* were tested for drought tolerance (Fig. 6). Under WW conditions, the transgenic rice plants did not showed significant morphological or developmental differences as compared with the WT plants (Fig. 6a). However, a yield reduction of ~20 % was observed at the end of the experiment (Fig. 6c). Under WS conditions WT plants displayed a significant yield reduction of ~70 %, whereas the transgenic plants displayed only a 15–20 % yield reduction (Fig. 6c).

Under WW conditions, no phenotypical differences were observed between WT and *Osw47-1* mutant plants (Fig. 7a). The WS episode induced a typical leaf rolling phenotype (Fig. 7b) with a small reduction in the total chlorophyll content of the flag leaves of the Osw47-1 plants (Fig. 7d), and a slight reduction (albeit not significant) in grain yield (Fig. 7e). After re-watering, the WT chlorophyll content remained constant after 6 d of re-watering (Fig. 7c), while it decreased further in *Osw47-1* flag leaves (Fig. 7d). Under WS conditions, grain yield of the transgenic *Osw47-1* plants showed a significant reduction (~60 %) as compared with only 20 % reduction in the WT plants (Fig. 7e). It should be noted that when the response to WS of the *Osw47-1* and WT plants was compared, watering was halted for only 3 days because the *Osw47-1* plants displayed early stress symptoms (i.e. leaf rolling). Hence, the apparent differences in yield penalty of the WT plants in Fig. 6c (5 days of WS) versus Fig. 7e (3 days of WS).

Discussion

WRKY TFs were first identified by their ability to bind the cis-element W-box, (T)(T)TGAC(C/T), a core with

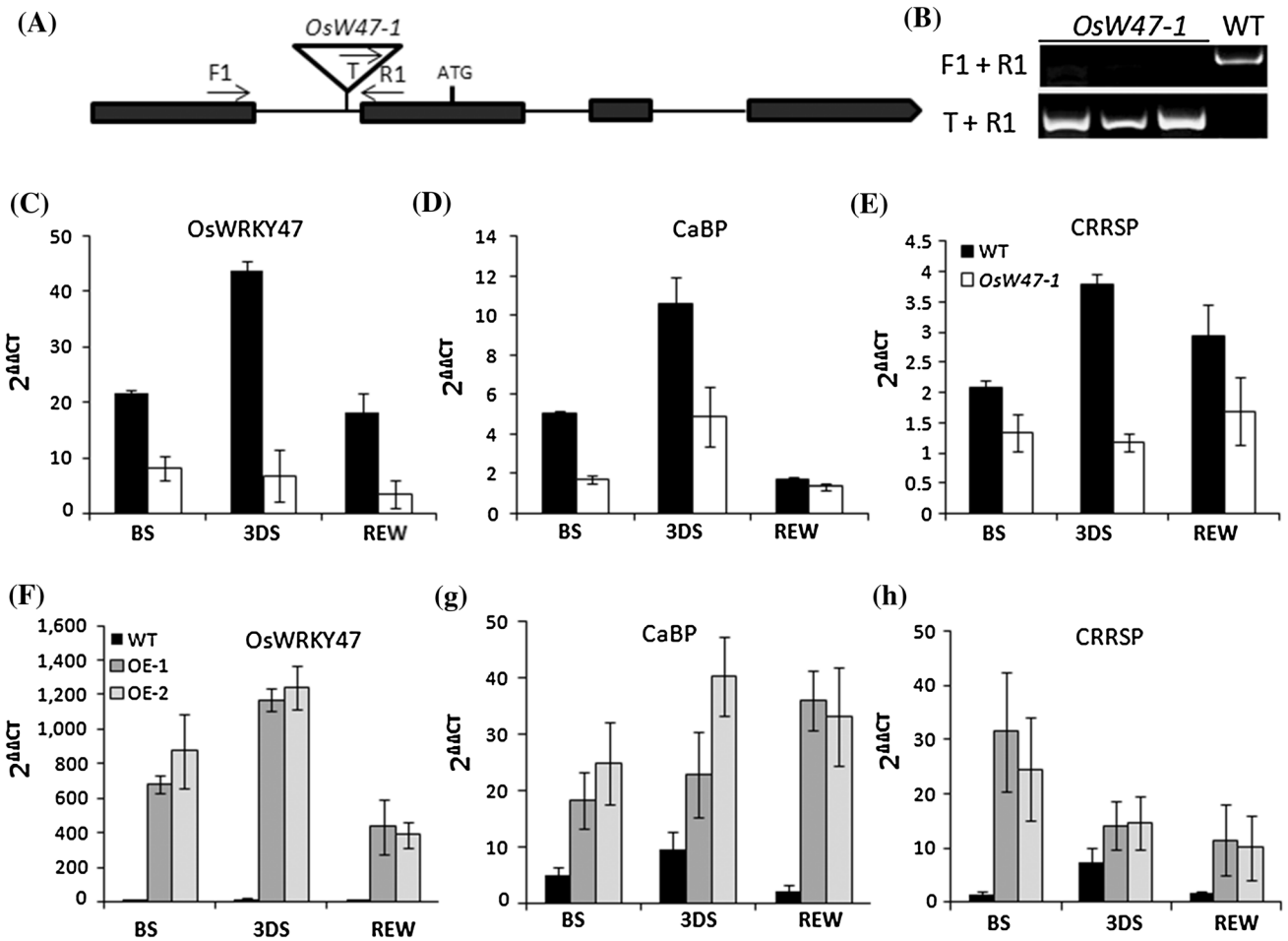

Fig. 5 CaBP and CRRSP are repressed in *OsWRKY47* mutants and induced in overexpressors OsWRKY47. **a** *OsWRKY47* gene structure and position of the transposon insertion. Exons are represented by *boxes* and introns by *lines*. Insertion positions of the transposon insertion in mutant *Osw47-1* and start codon (ATG) are shown. **b** PCR detection of transposon insertion in the mutant *Osw47-1* using the primers F1, R1, and T shown in (**a**). **c** *OsWRKY47* transcript levels in WT and in mutant *Osw47-1* plants. **d** Calmodulin binding protein (*CaMBP*) transcript levels in WT and in mutant *Osw47-1* plants. **e** Cysteine-rich repeat secretory protein (*CRRSP*) transcript levels in WT and in mutant *Osw47-1* plants. **f** *OsWRKY47* transcript levels in WT and in transgenic plants overexpressing *OsWRKY47* (OE-1 and OE-2) plants. **g** *CaMBP* transcript levels in WT and in transgenic plants overexpressing *OsWRKY47* (OE-1 and OE-2) plants; **h** *CRRSP* transcript levels in WT and in transgenic plants overexpressing *OsWRKY47* (OE-1 and OE-2) plants. The plants were subjected to 5-day water stress and then re-watering. Samples were harvested before treatment (BS), 3 days post stress (3DS) and after re-watering (REW). Values were calculated and normalized by using the transcription elongation factor as internal control. Values are the mean ± SD (n = 6)

varying flanking sequences (Rushton et al. 2010). Only, a few exceptions in the binding sequence specificity have been reported (Sun et al. 2003; Verk et al. 2008). Flanking sequences have been shown to be specific determinants for binding. Here, we demonstrated that *OsWRKY47* specifically binds **G**TTGAC**T/C**, at least in vitro, while it is also able to bind, albeit with lesser affinity, **A**TTGAC**T/C**. Although SELEX assays could not absolutely distinguish between these sequences, EMSA assays clearly indicated that an A in the 5′ flanking position was almost forbidden while competition experiments showed that T or C were similar in the 3′ flanking position, although C seemed to be preferred. Binding preferences of WRKY TFs have not been deeply explored so far. However, some interesting examples are available in the literature; *AtWRKY18* which is involved in ABA signaling and salt tolerance (Chen et al. 2010; Shang et al. 2010) binds the sequence (C/A)TTGAC(T/G). *AtWRKY6*, a negative regulator of the response to low Pi levels and a positive regulator to low brassinosteroid levels (Chen et al. 2009; Kasajima et al. 2010), binds specifically the sequence **G**TTGACC, a sequence similar to that bound by *OsWRKY47* (Ciolkowski et al. 2008). The same cis-element bound by *OsWRKY47* is also bound by *AtWRKY11* (Ciolkowski et al. 2008) which

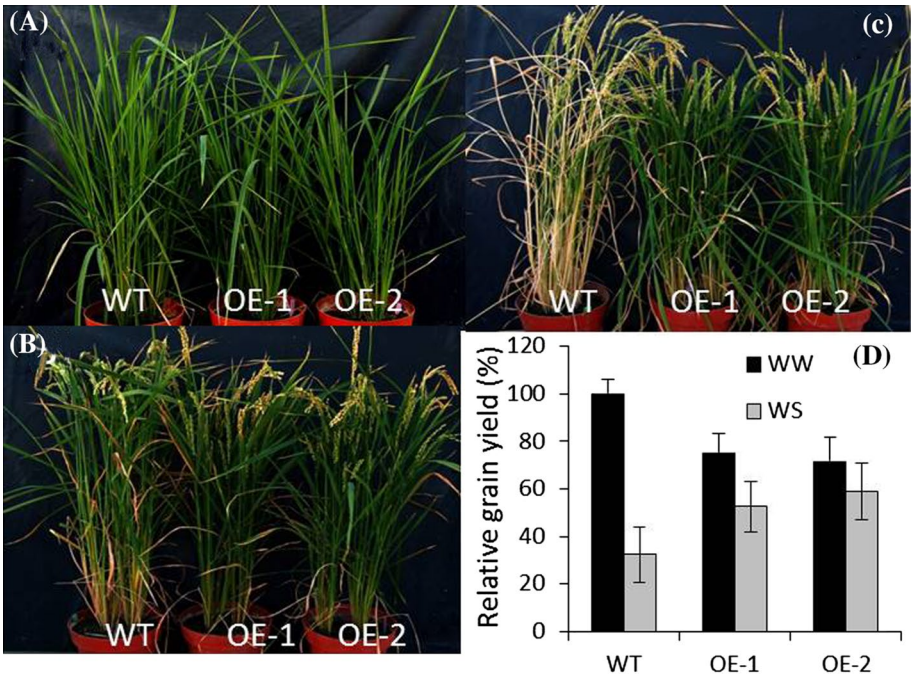

Fig. 6 *OsWRKY47* overexpressors display higher yield than controls under drought stress. **a**–**c** The phenotype of WT and OsWRKY47 overexpressors (OE-1 and OE-2) subjected to 5-day water stress (*left*) and then re-watered (*right*). Photographs were taken before treatment (**a**), 3 days post stress (**b**) and after re-watering (**c**). **d** Relative grain yield of wild type (WT) and mutant *Osw47-1* plants subjected to water stress (WS) or well watered (WW). The average grain yield of WT plants under water well condition was set as 100 %. The values (mean ± SD) were calculated relatively to the average grain yield of WT plants. Twenty plants were tested for each line. *Asterisks* indicate significance levels as compared to WT ($P \leq 0.001$)

is a positive regulator of the drought response (Wu et al. 2009). Although other WRKY TFs have been associated

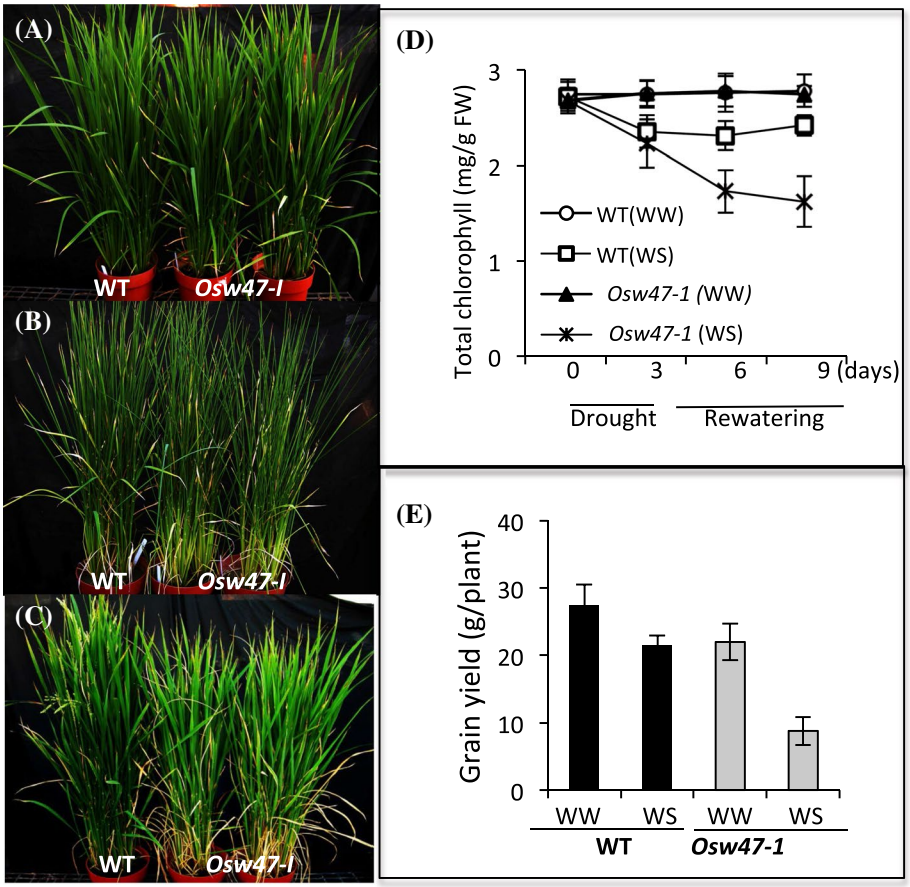

Fig. 7 Mutant *Osw47-1* plants exhibit lower yield than controls after drought stress. **a**–**c** The phenotype of WT and mutant *Osw47-1* plants subjected to 3-day water stress (*left*) and then re-watered (*right*). Photographs were taken before treatment (**a**), 3 days post stress (**b**) and after re-watering (**c**). **d** Total chlorophyll content of flag leaves. The values are the Mean ± SD (n = 20). *Asterisks* denote significance levels as compared to WT ($P \leq 0.05$)

with enhanced plant tolerance to drought, no binding assays for these WRKY TFs have been reported.

Among the genes containing a W-box that were co-expressed with *OsWRKY47* (Table S1), eight were chosen for confirmation by qPCR (Table S2 and Fig. S2), BAK1 (LOC_Os08g07760), the receptor-like protein kinase (LOC_Os06g36270), with an Arabidopsis homolog (*At3g46290*) (Guo et al. 2009), and the Cys-rich domain containing protein (LOC_Os05g25350) are associated with BR-mediated signaling (Li et al. 2007). The co-expression of genes involved in BR-regulation and signaling and *OsWRKY47* is noteworthy. A comparison between the P_{SARK}*::IPT* and WT plants revealed the enhanced expression of BR regulation- and BR signaling-associated genes (Peleg et al. 2011a, b). These results indicated an interaction between CK and BR (Peleg et al. 2011a, b). The CK-dependent induction of *OsWRKY47* and its association with BR-regulated genes support this notion.

The binding of OsWRKY47 to W-box containing genes was demonstrated in a gene encoding a CaM-binding protein (*CaMBP*, Os12g36110) and a Cysteine-Rich Repeat Secretory Protein 55 Precursor (*CRRSP*, Os03g16950). CaM can bind to a highly conserved Ca^{2+}-dependent CaM-binding domain (CaMBD) in the WRKY TFs belonging to the Group IId (Park et al. 2005). Although *OsWRKY47* does not contain typical CaM binding domains, its interaction with a CaMBP would suggest a possible role in the propagation of signals triggered by changes in cellular Ca^{2+} homeostasis. Three Arabidopsis genes (*At5g57580*, *At4g31000 and At5g26920*) displayed high similarity to *Os12g36110*. *At5g57580* belongs to the Group II of CaM-binding proteins playing a role during the heat shock-mediated elevation of cytosolic Ca^{2+} (Reddy et al. 2002). *At4g31000* was shown to be BR-regulated. *At5g26920* encodes a CaMBP (CBP60g) that conferred drought tolerance when overexpressed (Wan et al. 2012). Moreover, similarly to *OsWRKY47*, the transgenic plants overexpressing CBP60g displayed a higher tolerance to bacterial infection. *CRRSP* expression was induced by water stress in P_{SARK}*::IPT* rice plants (Peleg et al. 2011b). The expression of *At5g48540*, an Arabidopsis *CRRSP* homolog, was induced in the mutants *abi4* and *abi5* (Nakabayashi et al. 2005) and in plants subjected to hypoxia treatments (Klok et al. 2002). Similar phenotypes, i.e. increased biomass (Kerchev et al. 2011), were seen in both *abi4* and water stressed P_{SARK}*::IPT* rice plants.

For crop-plants the reproductive stage is the most drought sensitive developmental stage (Blum 2009; Peleg et al. 2011a). In the current study we applied a short water-stress treatment at the pre-anthesis stage (booting stage, panicle elongation), by withholding water until the stress symptoms appeared in the transgenic plants (leaf rolling, leaf senescence and reduction of photosynthetic activity), follow re-watering (Peleg et al. 2011b). The constitutive expression of *OsWRKY47* did not affect any morphological changes of flowering delay in the transgenic plants. Nevertheless the two transgenic lines expressing *OsWRKY47* displayed a yield reduction of about 20 % when the plants were well-watered. Negative effects are not uncommon when constitutive promoters (and not inducible promoters) drive the expression of key regulatory genes such as transcription factors, and pleiotropic effects on growth and development under control conditions have been reported (Reguera et al. 2012, and references therein). The expression of *OsWRKY47* resulted in a smaller stress-induced yield loss in the transgenic plants in comparison to the wild-type plants that lost 70 % of the grain yield. The role of *OsWRKY47* in water-deficit stress tolerance was further supported by the marked reduction in chlorophyll content in *Osw47-1* knockdown mutants and the reduction in grain yield after watering was held for 3 days during pre-anthesis.

In conclusion, the enhanced expression of *OsWRKY47* correlated well with the expression of proteins associated with CK- and BR-mediated signaling. Our data supports the involvement of *OsWRKY47* in DNA binding of these genes during the water-deficit stress episode and suggest role(s) of *OsWRKY47* activating genes associated with the inhibition of stress-induced senescence. Among the genes putatively regulated by OsWRKY47, a few encode proteins with regulatory roles including protein kinases, cation- and phosphate- transporters and TFs (Table S2). It has been shown that receptor-like-protein kinases (RLK) are targeted by WRKY TFs (Du and Chen 2000). AtWRKY6 is a TF mediating the induction of the senescence- and pathogen defense-associated PR1 and SIRK promoter activities. The latter encodes a receptor-like protein kinase whose expression is specifically induced during leaf senescence. *Atwrky6* knockout mutants showed a drastic growth reduction, and *AtWRKY6* overexpression led to increased *SIRK* expression. Notably, the *SIRK* promoter comprises a W-box indicating a direct activation by WRKY6 in vivo (Robatzek and Somssich 2002). WRKY53 has been related to senescence and several target genes, among these, other WRKY TFs, senescence-associated genes and others similar to the putatve OsWRKY47 targets, indicating similar functions of these Arabidopsis and rice TFs. (Miao et al. 2004).

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number: *OsWRKY47*: GenBank AK110900.1 and in MSU Rice Genome Annotation Project Database and Resource under accession numbers: OsCRRSPrich promoter LOC_Os03g16950 and Os*CaM* promoter LOC_Os12g36110.

Materials and Methods

Plant material and growth conditions

Seeds of the transposon insertion line RdSpm6084I_3.1 (termed as *Osw47-1*) were obtained from the Rice Sequence Indexed Transposon Insertion Library (http://www.plb.ucdavis.edu/Labs/sundar/projects/riceGenomics.html). The insertions are assumed to be stable, because the transposase was segregated away in the selection for insertion lines. Seeds of wild-type (WT) rice (*O. sativa japonica cv*. Kitaake), transgenic plants expressing P_{SARK}*::IPT* (Peleg et al. 2011a), transgenic plants overexpressing *OsWRKY47* (OE-1 and OE-2), and transposon insertion mutant *Osw47-1* were sown on moist germination papers for 4 d at room temperature in the dark. Seedlings were transplanted into 2 l pots filled with a mix of 80 % sands and 20 % peat. Greenhouse conditions were controlled at 12 h/12 h day/night under an illumination of 1200 µmol photons m^{-2} s^{-1} at 30 °C/20 °C. Water-stress (WS) treatments were carried out at the pre-anthesis stage (end of booting stage toward panicle emerging) by withholding water for 5 days. Plants were re-watered when visual stress symptoms (i.e. leaf rolling) appeared in the transgenic plants.

Nicotiana benthamiana seeds were grown on soil in a culture room at 28 °C under long-day photoperiods (16 h of illumination) with a mixture of cool-white and GroLux fluorescent lamps) at 300 µmol photons m^{-2} s^{-1}.

Constructs

ProCRRSP::GUS and ProCaMBP::GUS

The *CaMBP* and *CRRSP* promoter regions, identified in the OSIRIS database, were amplified by PCR using rice genomic DNA extracted from 45-d-old leaves as template and the oligonucleotides CysF/CysR and CalF/CalR, respectively. The amplified fragments of 1450 and 668 nucleotides, upstream the transcription initiation site, were cloned into the pGEM®-T Easy vector (Promega). Once verified, the promoter fragments were released by *Xho*I and *Xba*I restriction and cloned into *Sal*I and *Xba*I sites of the pKGWFS7 vector. The resulting constructs direct the expression of the reporter *GUS* by the specific promoters *CRRSP* and *CaMBP*.

$ProCRRSP_{\Delta wbox}$*::GUS*

The entire W-box of the CRRSP promoter was deleted following the technique described by Ho et al. (1989). Two flanking DNA segments surrounding the element to be deleted were amplified by PCR in separate reactions using the wild type CRRSP promoter as template and the oligonucleotides CysF and CysdelR and CysdelF and CysR respectively. CysdelR and CysdelF exhibited a 18 bp overlapped region. The resulting PCR products were mixed in a Taq polymerase buffer with 0.5 mM of each dNTP, 2.5 mM $MgCl_2$ and 5 units of Taq DNA polymerase. Hybridization and extension of the segments were carried out during 10 cycles of 30 s at 94 °C, 90 s at 62 °C and 2 min at 72 °C. A normal PCR amplification was performed using CysF and CysR as primers (Table S3). Once verified, the promoter fragments were digested with *XhoI* and *XbaI* restriction and cloned into *Sal*I and *Xba*I sites of the pKGWFS7 vector. The resulting constructs direct the expression of the reporter GUS by the specific promoter $CRRSP_{\Delta wbox}$.

$ProCaBP_{\Delta wbox}$*::GUS*

The mutated CaMBP promoter was obtained by the procedure described for $ProCRRSP_{\Delta wbox}$*::GUS* using the WT CaMBP promoter as template and the oligonucleotides CalF and CaldelR and CaldelF and CalR (Table S3). Once checked, the promoter fragments were digested with *XhoI* and *XbaI* restriction and cloned into *Sal*I and *Xba*I sites of the pKGWFS7 vector. The resulting construct direct the expression of the reporter GUS by the specific promoter $CaMBP_{\Delta wbox}$.

$ProCaBP_{C\rightarrow T}$*::GUS*

Substitution of C in position-1460 (inside the W-box) was obtained by the procedure described for $ProCRRSP_{\Delta wbox}$*::GUS* using the WT CaMBP promoter as template and the oligonucleotides CalF and CalsustR and CalsustF and CalR (Table S3). Once verified, the promoter fragments were digested with *XhoI* and *XbaI* restriction and cloned into *Sal*I and *Xba*I sites of the pKGWFS7 vector. The resulting construct direct the expression of the reporter GUS by the specific promoter $ProCaMBP_{C\rightarrow T}$.

ProCRRSPshort

A short version of the CRRSP promoter (147 bp) containing the Wbox was amplified by PCR with the CysFshort and CysFshort primers using the WT CRRSP promoter as template and cloned into the pGEM®-T Easy vector (Promega).

ProCaMBPshort

A short version of the CaMBP promoter (132 bp) containing the Wbox was amplified by PCR with the CalFshort and CalFshort primers using the WT CaMBP promoter and as template cloned into the pGEM®-T Easy vector (Promega).

pOsW47

The cDNA of *OsWRKY47* was cloned in pENTR/D-TOPO and the WRKY domain (W47, amino acids 23–219) was obtained by restricting with *Xho*I and *Sal*I, and inserted in frame into pGEX-4T-3 (Smith and Johnson 1988). To express the polypeptide, *E. coli* cells [strain BL21 (DE3)] bearing the corresponding plasmid were grown and induced with 1 mM IPTG as described previously (Palena et al. 1998). The recombinant protein was purified through GST-Sepharose (Research AG) as previously described (Smith and Johnson 1988), with a few modifications (Palena et al. 1998).

35S::OsW47

The complete *OsWRKY47* cDNA sequence was amplified by PCR with the W47R and W47F primers using the Oswrky47cd clone and as template cloned into the pBI122 vector *(*Capella et al. 2014) in the *BamH*I and *Kpn*I restriction sites (Table S3). This construct was used to transform tobacco leaves.

Ub::OsWRKY47

For overexpression in rice of *OsWRKY47* gene (LOC_Os07 g48260), we constructed the binary vector *pH7m24GW-Ub::OsWRKY47* through Gateway Cloning (Life Technologies). We cloned the Maize ubiquitin promoter from pCAMBIA-pMUb-CtHSR to entry vector pDONRP4P1r. OsWRKY47 were cloned by RT-PCR into entry vector pDONR207. These entry vectors were recombined with pH7m24GW by LR reaction. The constructed vector *pH7m24GW-Ub:OsWRKY47* was transformed in agrobacterium EHA105. The constructs *35S::OsW47*, *ProCRRSP::GUS*, *ProCaMBP::GUS*, $ProCaMBP_{\Delta wbox}$*::GUS*, $ProCRRSP_{\Delta wbox}$*::GUS* and $ProCaMBP_{C\rightarrow T}$*::GUS* were initially introduced in the BL21 (DE3) *E. coli* strain and then transferred to *Agrobacterium tumefaciens* (strain LBA4404) by electroporation using a gene Gene PulserTM (Bio-Rad).

Sequence analysis

The correct insertion and sequence of all the obtained clones was verified by sequencing (Macrogen Korea).

PCR-assisted binding site selection

To select DNA molecules specifically bound by the purified recombinant *GST::OsWRKY47*, the random oligonucleotide selection technique (SELEX; Oliphant et al. 1989) was applied, using procedures described by Blackwell and Weintraub (1990). A ^{32}P-labeled (30,000 cpm) 51-mer double stranded oligonucleotide containing a 12-bp random central core [5′-GATGAAGCTTCCTGGACAAT(12N) GCAGTCACTGAAGAATTCT-3′] was incubated with purified *GST::OsWRKY47* binding domain (BD). Binding reactions (20 µl) containing 20 mM of HEPES (pH 7.5), 50 mM of KCl, 2 mM of $MgCl_2$, 0.5 mM of EDTA, 1.0 mM of DTT, 0.5 % Triton X-100, 22 ng/ml of BSA, 1 mg of poly(dI-dC), and 10 % glycerol were incubated for 15 min at room temperature, supplemented with 2.5 % Ficoll, and immediately loaded onto a running gel (5 % acrylamide, 0.08 % bis-acrylamide in 0.53 TBE plus 2.5 % glycerol; 13 TBE is 90 mM of Tris–borate, pH 8.3, and 2 mM of EDTA). The gel was run in 0.53 TBE at 20 mA for 2 h and dried before autoradiography. Bound DNA molecules were separated by Electrophoretic mobility shift assay (EMSA) and eluted from gel slices with 0.5 ml of 0.5 M NH_4Ac, 10 mM $MgCl_2$, 1 mM EDTA, and 0.1 % (w/v) SDS. The selected DNA molecules were amplified using oligonucleotides SelexF and SelexR (Table S3). Amplification reactions were performed as follows: 18 cycles of 1 min at 94 °C, 1 min at 53 °C, and 1 min at 72 °C. The number of cycles was decreased to 12 after the fourth round. After purification through polyacrylamide gels, the amplified molecules were subjected to new cycles of labelling, binding, elution, and amplification. Enrichment in sequences bound specifically by *OsWRKY47* was monitored by binding and competition analysis in EMSA. After seven rounds of selection, the population of oligonucleotides was cloned into the pCR 2.1-TOPO vector (Invitrogen). Eighteen randomly picked clones were sequenced. DNA Binding Assays For EMSA performed with synthetic probes, aliquots of the purified proteins were incubated with double-stranded DNA (0.3–0.6 ng, 30,000 cpm, labelled with [α-^{32}P] dATP by filling in the 3′ends using the Klenow fragment of DNA polymerase) generated by hybridization of the complementary synthetic oligonucleotides named Oligo1, Oligo2, Oligo3 and Oligo4 (Table S3) with modifications within the binding sequence as described in the Results. When competition assays were performed, 100-fold unlabelled double-stranded oligonucleotides were included in the binding reaction mix previously described and incubated for 10 min before the addition of the selected labelled oligonucleotide. For competition EMSAs, fresh DTT was added in the binding reactions to avoid the formation of the double band typically caused by GST oligomerization.

For assays with promoter fragments containing W-boxes, DNA was labelled by annealing oligonucleotides CalShort-F or CysShort-F and filling in the 3′ end using Klenow fragment.

Quantitative PCR analysis

RNA were extracted from flag leaves of wild-type and transgenic plants under well-watered and water-stress conditions. Total RNA was extracted from plant tissue using RNeasy Mini Kit (Qiagen, Valencia, CA). The quality of RNA was measured by using a Nanodrop ND-1000. First strand cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative PCR were performed as described previously (Peleg et al. 2011b). The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) was used to normalize and calibrate expression values relative to the endogenous rice transcription elongation factor (*TEF*) gene. The different sets of primers used in qPCR are listed in Supplemental Table S3.

Identification of promoter fragments

The promoters of the selected genes were identified using the OSIRIS database (http://www.bioinformatics2.wsu.edu/cgi-bin/Osiris/cgi/home.pl). OSIRIS allows identification of promoter regions in genes maps as well as transcription initiation sites and translated regions. The selected promoters exhibit between 700 and 2000 bp upstream the +1 and correspond to the *japonica* variety.

In silico expression analysis of *OsWRKY47*

In silico co-expression analysis was carried out by using the following databases: HanaDB-Os (http://www.evolver.psc.riken.jp/seiken/OS/co-express.html); Rice Oligonucleotide Array Database (http://www.ricearray.org); Rice Genome Annotation Project (http://www.rice.plantbiology.msu.edu) and co-expressed biological processes (http://www.webs2.kazusa.or.jp/kagiana/cop0911).

Transient transformation of tobacco leaves

For agroinfiltration, *A. tumefaciens* strain LBA4404 containing individual constructs were grown overnight at 28 °C in LB supplemented with 100 μg/ml streptinomicin, 50 μg/ml rifampicin and 100 μg/ml kanamycin or 100 μg/ml spectinomycin, depending of the vector. Cells were collected by centrifugation and incubated in 10 ml *Agrobacterium* induction media (100 mM $MgCl_2$ and 100 μM acetosyringone). After further incubation, when cells reached a OD_{600} = 0.5, the *Agrobacterium* suspensions were mixed [half bearing the promoter construct and half bearing *35S::OsWRKY47* or empty vector (pBI101) as control]. The mixes were infiltrated into the abaxial side of fully expanded of 5-week-old young tobacco leaves using a needleless disposable syringe. After agro-infiltration, the leaves were kept in a growth chamber at 22 °C under a 16 h light regime for 48 h (Jung et al. 2006).

Stable transformation of rice plants

Rice (Oryza sativa L. ssp. Japonica cv. Kitaake) were transformed with *pH7m24GW-Ub:OsWRKY47* in the UC Davis Plant Transformation Facility (http://www.ucdptf.ucdavis.edu/). Transposon-DNA insertion was identified with PCR. The expression of *OsWRKY47* was examined by quantitative RT-PCR.

GUS activity assays

Specific GUS activity in protein extracts was measured using the fluorogenic substrate 4-Methylumbelliferyl-β-d-glucuronide (4-MUG) essentially as described by Welchen and Gonzalez (2005). Total protein extracts were prepared by grinding tissues in extraction buffer [50 mM $NaPO_4$ buffer, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1 % (w/v) sodium lauryl sarcosine, and 0.1 % (w/v) Triton X-100], followed by centrifugation at 13,000 g for 10 min. GUS activity was measured with 1 mM 4-methylumbelliferyl b-D-glucuronide and 20 % methanol. 20 μl of tobacco extracts were added to 180 μl of fluorometric solution and the reactions were carried out for 20 min at 37 °C, and finally stopped with 800 μl of 0.2 M Na_2CO_3. A fluorescence spectrophotometer (Hitachi, model F-2000, Hitachi, Tokyo, Japan) was used to quantify the amount of 4-methylumbelliferone (4-MU) cleaved from 4-MUG.

GUS histochemical staining

In situ assays of GUS activity were performed as described by Jefferson (1987). Leaf disks were immersed in a 1 mM 5-bromo-4-chloro-3-indolyl-b-glucuronic acid solution in 100 mM sodium phosphate pH 7.0 and 0.1 % Triton X-100 and, after applying vacuum for 5 min; and incubated at 37 °C overnight. Chlorophyll was cleared from green tissues by immersing them in 70 % ethanol.

Chlorophyll measurements

The flag leaves were weighed and ground in liquid N_2 and chlorophyll was extracted in 80 % acetone. The absorbance at 663 and 645 nm were measured and total chlorophyll contents were calculated as described elsewhere (Porra 2002).

Acknowledgments This research was supported by funds from the UC Office of the President, UC Discovery Grant # 212976 (EB). The research of the Argentinean group was supported by Agencia Nacional de Promoción Científica y Tecnológica (PICT 2011 0850

and PICT 2012 0955) and Universidad Nacional del Litoral (UNL). JR is a CONICET Ph. D. Fellow and RLC is a career member of the same institution.

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