Overexpression of *AtWRKY30* enhances abiotic stress tolerance during early growth stages in *Arabidopsis thaliana*

Telma E. Scarpeci[1], María I. Zanor[1], Bernd Mueller-Roeber[2,3], Estela M. Valle[1*]

[1]Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, S2002LRK Rosario, Argentina.

[2]University of Potsdam, Institute of Biochemistry and Biology, Karl-Liebknecht-Straße 24-25, Haus 20, 14476 Potsdam-Golm, Germany.

[3]Max-Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Potsdam-Golm, Germany.

*corresponding author

Dr. Estela M. Valle

e-mail: valle@ibr-conicet.gov.ar

Phone: +54-341-4237070/4237500 Ext. 643. Fax: +54-341-4390465

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Abstract

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Keywords: antioxidant response, chloroplast, germination, oxidative stress, stress signaling

INTRODUCTION

Several environmental stresses lead to the production of excess reactive oxygen species (ROS). The site of ROS formation and scavenging depends on the nature of the adverse condition. Expression profiling of Arabidopsis leaves subjected to diverse abiotic stresses revealed an induction of different sets of genes involved in antioxidant defence, signalling pathways and transcriptional regulation. The WRKY family of transcription factors represents one of the most frequently induced transcription factors under stress conditions, particularly during biotic stress (Kalde et al., 2003;Eulgem et al., 2000;Dong et al., 2003). Although the function of several members of the WRKY family has been elucidated, the role of a large number of them remains to be determined. In a previous study we have identified several *WRKY* genes that are rapidly and highly induced by ROS generated in the chloroplast after methyl viologen (MV) treatment, a known superoxide anion propagator generated during photosynthesis (Scarpeci et al., 2008).

WRKY proteins belong to a large family of transcription factors in plants, which encompasses 74 members in Arabidopsis. All known WRKY proteins contain either one or two DNA-binding domains harbouring the strictly conserved amino acid sequence WRKY, which binds to the W-box binding motif (T/CTGACC/T) in target gene promoters. They can be classified on the basis of both, the number of WRKY domains and the features of their zinc-finger-like motif. WRKY proteins with two WRKY domains belong to group I, whereas most proteins with one WRKY domain (C-C-H-H zinc finger) belong to group II. The single C-C-H-C zinc finger motif of a small subset of WRKY proteins distinct from that of group I and II members is defined as group III (Eulgem et al., 2000).

Among the *WRKY* genes up-regulated by superoxide anion generated in the chloroplast (Scarpeci et al., 2008) two members of group III, i.e. *AtWRKY30* and *AtWRKY46,* were strongly induced by MV treatment. The WRKY proteins encoded by these genes have similar molecular masses and primary structures (Eulgem et al., 2000) with conserved basic residues at the same position of the WRKY domain (Yamasaki et al., 2005). In addition, expression of *AtWRKY30* was increased in *Arabidopsis thaliana* overexpressing a glycolate oxidase in chloroplasts after 6 h of high light exposure, which caused accumulation of H_2O_2 (Fahnenstich et al., 2008). Generally, the rapid accumulation of *WRKY* mRNA appears not to require *de novo* synthesis of regulatory factors (Eulgem et al., 2000).

WRKY transcription factors are crucial regulators of the defence transcriptome and disease resistance (Eulgem and Somssich, 2007). As suggested by different transcriptome analyses, W-boxes are over-represented in the promoters of Arabidopsis genes that are up-regulated during basal defence, elicitor responses, and systemic acquired resistance. In one expression profiling study, 49 of 72 tested Arabidopsis *WRKY* genes were induced in response to treatment with salicylic acid (SA) or *Pseudomonas syringae* pv. *tomato* (Dong et al., 2003). An example for a gene controlled by WRKY factors is *NPR1,* which encodes a key transcription co-activator of genes involved in resistance signalling pathways in Arabidopsis (Yu et al., 2001) and which is expressed at low levels in healthy uninfected plants. W-box sequences are recognized in the promoter of *NPR1*. Another likely target gene of WRKY transcription factors is *ICS1,* encoding an isochorismate synthase necessary for SA synthesis (Wildermuth et al., 2001). The *ICS1* promoter shows an enrichment of W-boxes. However, the identities of the specific WRKY factors controlling *ICS1* and *NPR1* expression are still unknown. Four members of the WRKY II-d subfamily (*AtWRKY11*,

AtWRKY17, *AtWRKY7* and *AtWRKY15*) were strongly induced in response to *P. syringae* infection, but only a weak induction was observed after treatment with various abiotic stresses, including wounding, cold, high salt concentration and drought, as well as to treatment with the stress hormone abscisic acid (ABA) (Journot-Catalino et al., 2006). The authors therefore concluded that they are not general stress response genes.

Previously, a sequence resembling the W-box consensus was found in promoters of genes specifically regulated by MV including the *AtWRKY30* promoter (Scarpeci et al., 2008). In addition, the binding of proteins to W-boxes was increased by MV treatment of Arabidopsis leaves in the light, suggesting a role of WRKY proteins in the oxidative stress response to ROS generated in the chloroplasts (Scarpeci et al., 2008).

In this paper, we report the expression pattern of *AtWRKY30* under several stress conditions, including salt and oxidative stress. We generated transgenic Arabidopsis plants constitutively overexpressing *AtWRKY30*, subjected them to different treatments and analyzed their response to stress. Our results indicate that *AtWRKY30* is a general stress response gene playing an important role in the plant's defence against various stresses, especially during early growth stages.

MATERIALS AND METHODS

General methods

Standard molecular techniques were performed as described (Sambrook and Russell, 2001). DNA sequencing was performed by the University of Maine DNA sequencing facility (USA, Orono, ME). For sequence analyses the tools provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), MIPS (http://mips.gsf.de/), the Arabidopsis Information Resource (TAIR;

http://www.arabidopsis.org/), and the Plant Transcription Factor Database (http://plntfdb.bio.uni-potsdam.de) were used.

Plant material

Arabidopsis seeds were sown on soil in 10-cm pots or 0.5x Murashige and Skoog (MS) medium (Sigma, St Louis, USA) containing 0.8% (w/v) agar and grown in a controlled environment chamber under a regime of 16-h day length provided by fluorescent light at 120 µmol quanta m^{-2} s^{-1} at 23 ± 2°C, and relative humidity of 65-70%. *Agrobacterium tumefaciens* strain GV3101 (pMP90) was used to transform *Arabidopsis thaliana* (L.) Heynh. wild-type (Col-0) by floral dip infiltration (Clough and Bent, 1998).

Promoter-GUS fusions

To generate the promoter-GUS (*uidA* from *Escherichia coli*) fusion lines, 1.96-kb and a 0.28-kb 5' genomic fragments upstream of the ATG initiation codon of *AtWRKY30* (At5g24110) were amplified by polymerase chain reaction (PCR) using the same reverse primer (5'-CGGGATCCTACGTTCAAAGAGTGG-3') (added *Bam*HI restriction site underlined) and the following forward primers (added *Sal*I restriction sites underlined): 5'-CGTCGACGACCGGTATCGCAACAC-3' and 5´-CGGTCGACTCAAAATCCAATAAATC-3´ for the long promoter (LP) and short promoter (SP) fragment, respectively. All other procedures were as previously described (Scarpeci et al., 2008). Homozygous transgenic lines were selected for these studies.

AtWRKY30 promoter sequence analysis

Predicted *cis* elements in the *AtWRKY30* promoter were searched for by using the PLACE database (Higo et al., 1999).

Overexpression of AtWRKY30

PCR was used to amplify the *AtWRKY30* coding region using Arabidopsis Col-0 flower cDNA as template and two specific primers: UnkF 5'-CGGGATCCATGGAGAAGAACCATAGTAG-3' (added *Bam*HI restriction site underlined) and UnkR 5'-TGCGGTCGACCTAAGAATAGAACCCACC-3' (added *Sal*I restriction site underlined). To generate the 35S::W30 construct, the entire *AtWRKY30* cDNA was inserted downstream of the Cauliflower Mosaic Virus (CaMV) 35S promoter in the binary expression vector pBinAR (Röber et al., 1996). Homozygous T_3 transgenic lines were selected for these studies.

Stress and hormone treatments of soil-grown plants for GUS staining

For abiotic treatments, three-week-old Arabidopsis plants were carefully removed from soil and incubated with 0.005% (v/v) Silwet L-77 (Lehle Seeds, Round Rock, TX, USA) solution (control) or solution supplemented with either 50 µM MV (Sigma, St Louis, USA), 20 mM H_2O_2, 100 mM NaCl, 200 mM mannitol, 100 µM sodium arsenate, 2 mM SA, 100 µM ABA, 100 µM 1-aminocyclopropane carboxylic acid (ACC), 100 µM methyl jasmonate (MeJA), or 5 mM ethephon (Sigma, St Louis, USA). For drought stress treatment, plants grown on soil were maintained under optimal irrigation during 10 days. Watering was interrupted for the following two weeks and then plants were re-watered. For biotic stress treatments Arabidopsis leaves were infiltrated using a syringe with a suspension (10^8 cfu ml^{-1}) of *Xanthomonas axonopodis* pv. *citri* or *Pseudomonas syringae* pv. *tomato* DC3000, supplemented with 10 mM

$MgCl_2$. For elicitor treatments, Arabidopsis leaves were vacuum-infiltrated and then floated for different periods of time in a solution containing 1.3 mg ml^{-1} autoclaved cellulase Onozuka R-10 (Yakult Honsha, Tokio, Japan) (Salinas-Mondragon et al., 1999), or in *A. tumefaciens* suspension (OD_{600}: 0.5-0.8) previously sonicated and autoclaved.

GUS assay

After the treatments, tissues were collected and rinsed in 50 mM sodium phosphate (pH 7.2), 10 mM EDTA, 0.33 mg ml^{-1} potassium ferricyanide, and then transferred to the same solution containing in addition 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc; Gold Biotechnology, St Louis, MO, USA). Tissues were vacuum-infiltrated for 3 min and then incubated at 37ºC for 24 h. Tissues were destained by soaking in 70% (v/v) ethanol.

Treatments for germination assays

Age-matched Arabidopsis seeds used for assays were harvested from plants grown in parallel in the same growth chamber and seeds were harvested on the same day and ripened for six weeks at room temperature. Seeds were surface sterilized in a 1.4% bleach, 0.05% Tween-20 solution for 15 min, followed by four washes with sterile distilled water. Sterilized seeds were plated on 0.5x MS solution supplemented or not with 0-2 μM MV or 0-300 mM NaCl. Each plate was divided into four parts: Col-0 or empty vector control line, 35S::W30-1, 35S::W30-8 and 35S::W30-21. Thus, the different seeds were placed side by side on the same plate. Seeds were stratified on plates at 4ºC for two days, placed in the growth chamber and scored for percentage of seedlings at stage 0.7 (Boyes et al., 2001).

Oxidative stress treatment, extraction of soluble proteins and enzyme activities

Ten-day-old Arabidopsis seedlings were carefully removed from soil and floated in 0.005 % (v/v) Silwet L-77 solution (control) or solution supplemented with 50 µM MV for 4 h, harvested, and the tissue was homogenized with 50 mM potassium phosphate (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 5 mM ascorbate, 15% (v/v) glycerol and 2% (w/v) polyvinyl polypyrrolidone for ascorbate peroxidase (APX) and catalase (CAT) activity assays. In all cases the supernatant was recovered at 4ºC by centrifugation (20,000 ***g***, 20 min) and used for enzyme activity assay. Unless otherwise stated, APX and CAT were assayed as described (Scarpeci et al., 2008).

Production of recombinant AtWRKY30 protein and electrophoretic mobility shift assays

For generation of recombinant AtWRKY30 protein, its full-length cDNA was cloned into pET32 (Novagen, Madison, WI, USA) and transformed into *E. coli* strain BL21 (DE3 pLys). Induction of expression was performed according to the protocol provided by the manufacturer. Antibodies against recombinant AtWRKY30 protein were produced according to (Scarpeci et al., 2007). For the band shift experiments, soluble proteins from the *E. coli* strain expressing *AtWRKY30* were concentrated by ultrafiltration using Vivaspin 2 (Vivascience, Hannover, Germany). The control extract consisted of *E. coli* cells transformed with the pET32 plasmid lacking the *AtWRKY30* cDNA. Double-stranded DNA probes were formed by mixing equal amount of oligonucleotides W-box (5'-CGTTCTTCAG**TCA**AAAAG**TCA**AACTATCTC-3') or mW-box (5'-CGTTCTTCAG**GTC**AAAAG**GTC**AACTATCTC-3') and their corresponding complementary oligonucleotides. The mix was kept at 80ºC for 5 min

and then left to reach room temperature. Double-stranded DNA was radio-labelled at its 5´-end using T4 polynucleotide kinase to achieve specific activities of approximately 10^3 Bq ng^{-1}. The ^{32}P-labelled probes were purified from unincorporated nucleotides with Sephadex-G50 spin columns. Binding buffer contained 3 µg poly(dIdC) (Sigma, St Louis, MO, USA), 1 U heparin, 20 mM Hepes, 40 mM KCl, 10% (v/v) glycerol, 4 mM $MgCl_2$, 1 mM DTT and protein extract in a final volume of 20 µl. The binding reaction was carried out using 0.1 pmol double-stranded oligonucleotides (10^3 Bq) and 0.4-40 µg of *E. coli* extract containing recombinant AtWRKY30 protein. Inhibition of W-box binding activity was assayed after pre-incubation for 30 min at room temperature with 1 µg of antibodies raised against AtWRKY30 and, as control, 1 µg of pre-immune serum or heat denatured anti-AtWRKY30 antiserum. The requirement of zinc ions for the binding of AtWRKY30 to DNA was tested by the addition of the zinc chelator 1,10-*o*-phenanthroline at 5 mM concentration. DNA-protein complexes were allowed to form at room temperature for 10 min and then at 4ºC for 30 min, and were resolved on a 5% polyacrylamide gel in 0.5x TBE, 4 mM $MgCl_2$ and 5 % (v/v) glycerol. The gels were pre-run for 90 min at 100 V using 0.5x TBE and 4 mM $MgCl_2$. After sample loading the gels were run until the bromophenol dye had migrated three-fourth through the gel. Following electrophoresis, the gel was fixed with 10 % (v/v) acetic acid for 5 min, dried onto 3 MM Whatman paper at 80ºC for 30 min and radioactivity was detected using a Storm 840 apparatus (Amersham Biosciences, Buenos Aires, Argentina).

Stress and hormone treatments of soil-grown plants for qPCR

Arabidopsis plants grown for three weeks were carefully removed from soil and vacuum infiltrated and then floated in 0.005 % (v/v) Silwet L-77 solution (control plants) or solution supplemented with fungal elicitor (1.3 mg ml^{-1} autoclaved cellulase),

50 µM MV, 100 µM ABA and 2 mM SA. Leaves were collected after 2 h of treatment for transcript profile analysis by qPCR.

RNA isolation and blot analysis

Total RNA was prepared from leaves of a wild-type plant (Col-0) and 35S::W30 lines using TRIzol (Invitrogen Life Technologies, Karlsruhe, Germany) following the manufacturer's procedure. Quality and quantity of RNA were monitored spectrophotometrically at 260 and 280 nm. RNA with a A_{260}/A_{280} ratio higher than 1.2 was used for further analysis. Twenty µg of total RNA from aerial tissue were further processed for RNA gel blot analysis as previously described (Scarpeci et al., 2008).

qPCR analysis of gene expression

Total RNA (2 µg) from cotyledons and stress or hormones treated and control leaves was isolated using TRIzol and then digested with DNAse (Sigma) and reverse-transcribed with Superscript III reverse transcriptase (Invitrogen) in a reaction volume of 20 µl to generate first-strand cDNA.

qPCR analyses of *AtWRKY30* transcripts were performed using protein phosphatase 2A gene (At1g13320) as the reference gene according to (Czechowski et al. 2005). The following primers were used: W30L (5'-CGCTGGACGATGGATTCAGTTGGAGA-3') and W30R (5'-TCGGTTCGAGGTTTTGTATCGGCATTG-3'); PP2AF (5'-CCTGCGGTAATAACTGCATCT-3') and PP2AR (5'-CTTCACTTAGCTCCACCAAGCA-3'). cDNAs were amplified using Mastercycler ep Realplex^2 thermocycler (Eppendorf, Westbury, USA). PCR conditions were 1 min at 95°C and 40 cycles of 15 s at 95°C, 30 s at 55°C and 40 s at 72°C. Following

amplification, products were denatured by heating from 60 to 95°C to check amplification specificity. qPCR was performed using a SYBR Green fluorescence-based assay. Gene-specific cDNA amounts were calculated from threshold cycle (Ct) values, expressed as relative to controls, and normalized with respect to *PP2A* cDNA, used as internal reference. Values were normalized by an internal reference (Ct_r) according to the equation $\Delta Ct = Ct - Ct_r$ and quantified as $2^{-\Delta Ct}$. A second normalization by a control (Ct_c) $\Delta\Delta Ct = Ct - Ct_c$ produces a relative quantification: $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Statistical analysis

Experimental data were subjected to statistical analysis of variance (ANOVA) with lines (One-way ANOVA) and lines and treatments (Two-way ANOVA) as factors, followed by Holm-Sidak or Fisher's least significant difference *post hoc* test. A *P* value less than 0.05 was considered statistically significant. Model assumptions were tested by analysis of residuals. All experiments were carried out at least three times.

RESULTS

Analysis of the *AtWRKY30* promoter

The promoter region of a eukaryotic protein-coding gene is arbitrarily divided into two segments: a core promoter region of around 50 nucleotides adjacent to the transcription start site and a more distant control region to which specific transcription factors bind. There are two key genetic elements within a core promoter: the TATA box and/or an initiator (Inr) element. The Inr element was defined as a discrete core promoter element that is functionally similar to the TATA box and can function independently of it (Smale and Kadonaga, 2003). A survey carried out in Arabidopsis

(Yamamoto et al., 2007) showed that a limited number of promoters (less than 10%) have the Inr motif around the transcription start site and that the most frequently observed sequence at the -1/+1 position among Arabidopsis transcription start sites was CA, and TA was the second. No TATA-box sequence was identified in the *AtWRKY30* core promoter. Instead, the dimeric sequence CA was found at the -77 position from ATG which was determined using its longest transcript sequence found in GenBank (Accession number AK117885).

Using the PLACE database (Higo et al., 1999) the non-coding 5' upstream flanking sequence, 2,842 bp between At5g24110 and At5g24120.1, was analyzed to identify potential *cis*-regulatory elements. Most notably, six putative W-boxes (T/CTGACC/T), the binding sites for WRKY factors, were identified at distances of -132, -140, -195, -223, -1162, and -1895 bp upstream of the ATG initiation codon (Fig. 1). Two of the identified putative W-boxes (-195, -1162) contain the TTGACC sequence, known as ELRE (Elicitor Responsive Element) sequence, which was reported as a fungal elicitor responsive element found in the promoter of the parsley *PR1* gene (Rushton et al., 1996).

Many *WRKY* gene promoters are statistically enriched for W-boxes, suggesting an auto-regulation or cross-regulation. For example, ChIP-qPCR assay demonstrated that AtWRKY33 could regulate its own expression by interacting with the W-boxes in its own promoter (Mao et al., 2011). As several W-boxes are present in the *AtWRKY30* 5´ upstream region, we were interested to know whether AtWRKY30 binds to its own promoter, using electrophoretic mobility shift assay (EMSA). More specifically, we analysed the binding of recombinant AtWRKY30 protein to the SP DNA fragment (0.28 kb) containing four densely spaced W-boxes (-132, -140, -195 and -223; Fig. 1). One retarded band was observed, which did not form when total protein extract from

E.coli cell lacking AtWRKY30 protein was used (Supplementary Fig. 1), suggesting the formation of a complex between the recombinant AtWRKY30 protein and the SP DNA fragment. Functional W-boxes frequently cluster within short promoter stretches (Du and Chen 2000; Lippok et al. 2007), as observed here for the *AtWRKY30* promoter (W-boxes found at -132 and -140, Fig. 1). We therefore tested the binding activity of purified AtWRKY30 protein to a 30-mer DNA probe containing these two W-boxes (Fig. 2). One of the W-boxes of the probe is part of motif 7, GAAAA**GTCA**AAC, which was found in the promoters of MV induced genes such as cinnamoyl CoA reductase (At1g80820), receptor like protein kinase 3 (*RLK3*; CRK11, CYSTEINE-RICH RLK11, At4g23190), peroxidase ATP24a (At5g39580), UDP-glycosyltransferase 73B4 (At2g15490) and putative protein phosphatase 2C (At1g07160) (Scarpeci et al., 2008). Binding reactions containing AtWRKY30 protein and W-box probe produced one DNA/protein complex (Fig. 2a). The binding specificity of AtWRKY30 to the W-box was confirmed by competition experiments, which were carried out with unlabelled W-box DNA (Fig. 2c). The binding signal of recombinant AtWRKY30 to W-box DNA diminished when unlabelled W-box or anti-AtWRKY30 antiserum was added to the binding mixture (Fig. 2b, c). On the other hand, AtWRKY30 binding activity was unaltered when pre-immune serum, denatured anti-AtWRKY30 antiserum or unlabelled mutated W (mW) box was used (Fig. 2b, c). Heat denatured recombinant AtWRKY30 protein did not show any binding activity to W-box DNA (Fig. 2b). As the WRKY domains contain a zinc-finger-like motif we decided to test whether AtWRKY30 is a zinc-dependent DNA-binding protein. The zinc chelator 1,10-*o*-phenanthroline (Phe) was added to the binding reaction. The amount of AtWRKY30-W-box complex clearly diminished when Phe was added to the reaction mixture, and completely disappeared

when heat denatured protein was used as a control (Fig. 2b). The results indicate zinc ion as a cofactor required for formation of the DNA-protein complex.

Responses of the *AtWRKY30* promoter to phytohormones, abiotic and biotic treatments

In order to study the *AtWRKY30* expression pattern in more detail, transgenic Arabidopsis plants were generated expressing the *GUS* reporter gene under the control of two fragments of different length of the *AtWRKY30* promoter, encompassing either 1.96 kb (LP) or 0.28 kb (SP) long 5´-upstream regions (relative to the ATG initiation codon), including the 5´-untranslated region. It is worth mentioning that all six W-boxes identified in the 5´ upstream region were included in the LP DNA fragment, with four of them being also present in the SP fragment (Fig. 1). Furthermore, the SP fragment probe was used in the band shift experiments and showed binding to recombinant AtWRKY30 protein (Supplementary Fig. 1).

GUS staining was not detected in tissues from SP lines grown under normal conditions (data not shown). In contrast, LP seedlings showed a blue staining in cotyledons, roots and leaves of ten-day-old seedlings (Fig. 3b). Additionally, according to qPCR data, *AtWRKY30* gene expression level is 2.2 ± 0.3 fold higher in cotyledon with respect to twenty-day-old Col-0 leaves. This result is in accordance with *AtWRKY30* transcript level measured in other labs (AtGenExpress, AtGE development database). In adult LP plants, GUS activity was very low in roots and leaves under control conditions (Fig. 3b). In flowers, GUS staining was observed in the locules of the anthers and, at later stages of flower development, it was detected in the residual stigmatic papillae and the abscission zone of siliques (Fig. 3c).

SP and LP lines were subjected to several abiotic and biotic challenges and the GUS staining pattern was recorded. In LP lines, *GUS* expression was induced by most of the stress conditions known to trigger ROS production, such as treatment with MV, NaCl, mannitol, arsenic and drought (Fig. 4a and Supplementary Fig. 2). In SP lines, treatments with NaCl and mannitol produce a low expression of the reporter gene visible ~24 h after onset of the treatment (Supplementary Fig. 3). SP leaves treated during 24 h with MV were severely damaged and GUS activity could not be detected.

In LP leaves, GUS activity was clearly evident when plants were treated with fungal elicitor (autoclaved cellulase, Fig. 4a) or at sites of *X. axonopodis* pv. *citri* and *P. syringae* pv *tomato* DC3000 infiltration, but was not detected in the absence of bacteria or when the tissue was wounded (see *A. tumefaciens*; Supplementary Fig. 2). As *X. axonopodis* pv. *citri* is not a pathogen of Arabidopsis, the response of *AtWRKY30* expression to bacterial infiltration is probably due to an interaction of the plant with components of the bacterial surface. This conclusion is strengthened by the observation that strong GUS staining was observed when leaf tissue was challenged with bacterial elicitor (boiled *A. tumefaciens* suspension, Supplementary Fig. 2). It is worth mentioning that, in LP plants, biotic elicitors rapidly enhanced *AtWRKY30* expression to the highest level. In addition, the fungal elicitor was more effective when assayed in the LP lines than in the SP lines in all the organs tested (Fig. 5). Known intermediates of the pathogenic response such as SA, ethylene (ethephon and ACC) or MeJA were also tested in LP GUS lines. These compounds had no or only slight (in the case of SA) effect on the activity of the *AtWRKY30* promoter (Fig. 4a and Supplementary Fig. 2), indicating that they are probably not involved in the *AtWRKY30* activation signalling pathway. We tested the effect of ABA on LP lines and verified that *AtWRKY30* is responsive to this hormone (Fig. 4a). ABA is a phytohormone that plays a major role in

abiotic stress responses (Fujita et al., 2006) and also affects a variety of growth and developmental processes.

In line with these observations, qPCR results using RNA isolated from twenty-day-old Col-0 plants treated with MV, fungal elicitor, SA and ABA showed an increment of *AtWRKY30* transcripts when compared with untreated plants (Fig. 4b).

Characterization of transgenic Arabidopsis plants overexpressing *AtWRKY30*

The enhancement of *AtWRKY30* promoter-mediated GUS activity after abiotic and biotic treatments indicated that AtWRKY30 has a role in plant defence signalling. To further address this possibility, we searched for a *wrky30* mutant in the publically available transposon databases/germplasm collections and identified T-DNA insertion mutants only upstream or downstream of the coding region, but not within the transcribed region itself. As the available mutant that contained a T-DNA insertion at -339 bp upstream the ATG initiation codon (Salk_090390) did not efficiently silence *AtWRKY30* expression (qPCR data not shown), we decided to characterize this transcription factor using an overexpression strategy. We generated transgenic plants that constitutively express *AtWRKY30* under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter (35S::W30) and several independent lines were obtained that under control conditions accumulated *AtWRKY30* transcript to levels that were comparable to or higher than those detected in wild-type plants subjected to MV treatment (Fig. 6a) (Scarpeci et al., 2008). Transgenic lines that constitutively expressed *AtWRKY30* at elevated levels were chosen for further studies. Analysis of T_3 homozygous 35S::W30 plants showed that they were similar to wild-type plants with respect to growth and development (Fig. 6b). Furthermore, when adult transgenic plants

were subjected to biotic stress (leaves infiltrated with *P. syringae* pv. *tomato* DC3000) or abiotic stresses (plants sprayed with 25 μM MV or irrigated three times a week with 200 mM NaCl during three weeks), no difference was observed in comparison to wild-type plants (data not shown). For this reason and taking into consideration the expression pattern of *AtWRKY30* (Fig. 3 and 4), we decided to test the behaviour of 35S::W30 plants under different conditions at early growth stages. Transgenic plants overexpressing *AtWRKY30*, the empty vector control line and wild-type plants were germinated in parallel on MS agar plates supplemented with different concentrations of MV (0, 0.5, 1, 2 μM) or NaCl (0, 100, 150, 200, 300 mM). From all tested concentrations only 1 μM MV or 150 mM NaCl showed statistically significant difference between the lines in ten-day-old plants (Fig. 7). It is worthwhile to mention that the empty vector control line showed no statistically significant differences from Col-0 plants under all treatments. Col-0 plants, the empty vector control line and 35S::W30 lines germinated at the same time on 0.5x MS agar plates. Additionally, measurements of fresh weight of ten-day-old plants after 1 μM MV or 150 mM NaCl treatment showed statistically significant differences of 35S::W30 plants to Col-0 or empty vector control lines (Supplementary Fig. 4). Furthermore, we followed AtWRKY30::GUS activities in the LP #1 line during germination in the presence of 0.5 μM MV or 100 mM NaCl (concentrations that allow increased germination rates). This assay showed that the level of GUS activity was slightly higher (especially in the cotyledons and root tips) than in the control seedlings (Supplementary Fig. 5). Overall, the results showed that the 35S::W30 plants were more tolerant to MV and salinity stress ($P < 0.05$) than wild-type plants (Col-0) or the empty vector control line.

The antioxidant response of ten-day-old Arabidopsis seedlings under MV treatment was followed by determining APX and CAT activities. To this end, Col-0

plants and 35S::W30 lines were exposed to 50 µM MV for 4 h. We observed a significant drop in APX activity in MV-treated Col-0 plants in comparison to the control whilst activity was not affected in the transgenic lines (P <0.05) (Fig. 8a). On the other hand, MV treatment significantly enhanced CAT activity in relation to non-stressed plants in particular in 35S::W30-8 and 35S::W30-21 lines (P <0.05) (Fig. 8b).

DISCUSSION

AtWRKY30 promoter analysis

Several DNA-protein interaction assays have shown that the sequence 5'-T/CTGACC/T-3' is the minimal consensus specifically recognized by WRKY proteins (Rushton et al., 1996; Chen and Chen, 2000; Cormack et al., 2002). The W-box TTGACT found at -132 bp in the *AtWRKY30* promoter is part of motif 7 (GAAAAGTCAAAC), which was found to be overrepresented among the promoter regions of genes highly up-regulated by superoxide anion (Scarpeci et al., 2008). It is worth noting that AtWRKY26, AtWRKY38 and AtWRKY43, which do not bind to the TTGACT sequence (Ciolkowski et al., 2008), are also not induced at the transcript level by oxidative stress (Scarpeci et al., 2008). In contrast, *AtWRKY6* and *AtWRKY11* are both induced under oxidative stress condition and their proteins bind to the TTGACT *cis*-element (Scarpeci et al., 2008; Ciolkowski et al., 2008). Additionally, a *cis*-regulatory element containing this sequence (TTGAC) was found in several stress-responsive genes in *A. thaliana* (Zou et al., 2011).

Recently, Koschmann et al. (2012) discovered new sequences which are responsive to elicitors. One of these sequences is part of motif 7 (AAAAAGTC) and it is superimposed with two W-boxes found in the promoter of *AtWRKY30*, situated at a distance of -132 and -140 bp upstream of the ATG initiation codon. The sequence

GGACTTTCC was also reported as elicitor responsive element and it was found at position -208 of the *AtWRKY30* promoter.

The *AtWRKY30* promoter contains several W-boxes in its own promoter (Fig. 1), suggesting that WRKY transcription factors regulate its expression. Moreover, band shift analysis have provided evidence that recombinant AtWRKY30 protein could bind alone and specifically to the W-box sequences (TTGACT) present in its own promoter and which are part of the reported motif 7 (Fig. 2; (Scarpeci et al., 2008)).

The expression of *AtWRKY30* in different growth stages, tissues and treatments was investigated by generating Arabidopsis plants carrying the *AtWRKY30* promoter fused to the *GUS* reporter gene (SP and LP lines). Under non-stress condition *AtWRKY30* expression is higher in cotyledons than in leaves as revealed from GUS activity and qPCR experiments. GUS staining was also visible in anthers, residual stigmatic papillae and in the floral organ abscission zone of siliques (Fig. 3c). It is well known that pollen grains undergo desiccation to a level of as low as 10% water content during late stages of maturation (Heslop-Harrison and Heslop-Harrison, 1985). The high level of GUS activity in mature pollen grains of LP transgenic plants suggests that the activity of the *AtWRKY30* promoter is related to changes in water potential. High amounts of ROS/H_2O_2 were shown in stigmatic papillae of *A. thaliana* where pollen produces nitric oxide (NO) (McInnis et al., 2006). ROS/H_2O_2 have a variety of roles in cell metabolism but also act as signalling molecules mediating a range of cellular processes from development to defence, often in association with NO, so it is not unreasonable to speculate that ROS/H_2O_2 might be involved in pollen–stigma interactions. These data suggest that *AtWRKY30* could be induced by ROS generated during initial stages of pollen-stigma recognition, having a signalling role in these processes (McInnis et al., 2006).

AtWRKY30 expression under stressful conditions

GUS reporter gene expression driven by the *AtWRKY30* promoter showed inducibility by treatment of different organs with pathogen-derived elicitors termed pathogen-associated molecular patterns (Fig. 4a and 5, Supplementary Fig. 2). For this reason, AtWRKY30 could be part of the innate immune system, which is the first line of inducible defence against infectious micro-organisms. In line with this, *AtWRKY30* expression was enhanced in twelve-day-old Arabidopsis seedlings treated with 10 nM of the 22–amino acid peptide of flagellin for 0.5 hours (Lu et al., 2011), or 5 μM of the hairpin protein Hpa1 of *X. axonopodis* pv. *citri* for 5 hours (Sgro et al. 2012), indicative of an involvement of *AtWRKY30* in the induction of the immune response in Arabidopsis.

The two novel elicitor-responsive elements found by Koschmann et al (2012) (-132, -140), together with the ELRE sequence (-195) and the -223 W-box could be enough for *GUS* expression in SP lines after treatment with elicitors. The higher level of GUS activity after elicitor treatment in LP in comparison to SP lines could be due to other *cis*-regulatory elements which are only present in the longer promoter version, such as another ELRE sequence (-1162) and two W-boxes (-1162, -1895) (Fig. 4a and 5, Supplementary Fig. 2).

AtWRKY30 was clearly induced by different sources of oxidative stress including treatment with MV, H_2O_2, arsenic, drought, NaCl and mannitol (Fig. 4, Supplementary Fig. 2, 3 and 5). It is interesting to note that even when *AtWRKY30* was induced during germination under normal conditions (Supplementary Fig. 5) this induction was higher in the presence of MV or, to a lower extent, NaCl, especially in the cotyledons and root tips. These data suggest an involvement of *AtWRKY30* in early phases of plant development, which involve production and signalling of ROS (Gapper

and Dolan, 2006). In SP and LP leaves treated with NaCl, GUS activity was detected in most cells, although those within and surrounding the vascular tissue showed more intense staining. This fact could be related in part to the way ions are transported into the leaf. Furthermore, it is known that especially the vascular tissue is sensitive against abiotic stressors such as low temperature or high salt, as it was shown for dehydrins, which accumulate in the vascular tissue and bordering parenchymal cells (Nylander et al., 2001). It is worth noting that analyses of the *AtWRKY30* promoter revealed the presence of a GT-1 *cis*-element, which was found in salt-induced promoters (Park et al., 2004).

Phytohormones such as SA, MeJA, ethylene, and ABA are endogenous, low-molecular-weight molecules that primarily regulate the plants´ protective responses against both biotic and abiotic stresses via synergistic and antagonistic actions, representing signalling crosstalk (Fujita et al., 2006). *AtWRKY30* showed a low level of expression after SA treatment and was unresponsive to MeJA and ethylene, hormones playing central roles in biotic stress signalling upon pathogen infection (Fig. 4, Supplementary Fig. 2). However, ABA enhanced *AtWRKY30* promoter-GUS activity (Fig. 4a). ABA directly stimulates the generation of ROS such as $O_2^{\cdot -}$ and H_2O_2 probably by a mechanism involving activation of a plasma membrane-bound NADPH oxidase (Pei et al., 2000;Murata et al., 2001;Zhang et al., 2001;Jiang and Zhang, 2003). This oxidative burst could be responsible for *AtWRKY30* induction after ABA treatment.

Phenotype of *AtWRKY30* overexpression lines

Plants overexpressing *AtWRKY30* showed an advantage to germinate under high saline concentration and MV treatment (Fig. 7a, b). Furthermore, CAT activity was enhanced by MV in 35S::W30-8 and 35S::W30-21 lines, but not in Col-0 plants. In the

case of APX activity, there was a general drop after MV treatment and it was only significant in Col-0 but not in transgenic plants. The observed enhancement of *AtWRKY30* gene expression and tolerance under oxidative treatment in transgenic 35S::W30 plants in younger but not in mature stages (data not shown) brings up an interesting question about the mode of action of this transcription factor. It is possible that, in order to activate genes involved in the antioxidant defence, AtWRKY30 may require coordination with developmentally regulated components to activate defence responses. These components may modify or interact with AtWRKY30 protein to activate plant defence gene expression. Further studies of WRKY transcription factors and their relationship with biotic and abiotic stress could be of importance to generate transgenic crops with improved performance under environmental stresses.

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FIGURE LEGENDS

Fig. 1 Scheme representing the *AtWRKY30* promoter. The positions of the six W-box elements (black boxes, W) are indicated. LP (1.96 kb) and SP (0.28 kb) represent the promoter fragments fused to the GUS reporter gene. The W-box probe sequence used for DNA binding assays is shown in the diagram.

Fig. 2 DNA binding activity of recombinant AtWRKY30 protein. (a) Different amounts of recombinant AtWRKY30 protein produced in *E. coli* were used for DNA binding assays with ^{32}P-labelled double-stranded oligonucleotide encompassing two W-boxes (d). (b) Recombinant AtWRKY30 protein (W) (0.4 µg) was assayed for DNA binding activity after adding anti-AtWRKY30 antiserum (Ab), pre-immune control serum (CS), heat denatured anti-AtWRKY30 antiserum (D-Ab), heat denatured recombinant AtWRKY30 protein (D-W) or 1,10-*o*-phenanthroline (Phe). (c) Competition experiments were carried out with recombinant AtWRKY30 and ^{32}P-labelled W-box probe. As competitors, unlabelled W-box or mW-box probes were added in 100- and 1000-fold molar excess. (d) Nucleotide sequences of probes used for DNA binding assays. W-box probe contains a double W-box (AGTCAA), which is part of motif 7 (GAAAAGTCAAAC) previously found in the promoters of MV-induced genes (Scarpeci et al., 2008). The mW-box indicates mutation to AGGTCA in the mW-box probe. FP: free probe.

Fig. 3 (a) Scheme of plasmid pBI101 with the 1.96 kb (LP) DNA fragment from the *AtWRKY30* promoter cloned upstream of the *β-glucuronidase* reporter gene. (b) GUS enzymatic activity in six, ten and twenty-day-old plants or (c) flower and young silique

of LP #1 transgenic plants. In (c), the arrow shows blue staining in the locules of the anthers. Representative images are shown.

Fig. 4 Response of *AtWRKY30* expression to different treatments in Arabidopsis leaves. (a) Leaves of LP #1 and #2 GUS lines were treated with the following solutions: 50 µM MV, fungal elicitor, 2 mM SA or 100 µM ABA all in 0.005 % (v/v) Silwet L-77, and incubated for 4 h. Representative images are shown. (b) Expression of *AtWRKY30* in response to different treatments in Arabidopsis leaves. Col-0 plants were treated with 50 µM MV, fungal elicitor, 2 mM SA or 100 µM ABA. Leaves were collected after 2 h of treatment for transcript profile analysis by qPCR. Inductions are the ratios of transcript levels in treated leaves to those in control leaves. Data are the means ± SE of three individual experiments.

Fig. 5 Detection of GUS activity in SP #1 and LP #1 plants that were treated with the fungal elicitor or not (control). Schemes of plasmid pBI101 with either the 0.28 kb (SP) or the 1.96 kb (LP) DNA fragment from the *AtWRKY30* promoter cloned upstream of the *β-glucuronidase* gene are shown in (a) or (f), respectively. Time-dependent study of GUS activity in SP#1 (b) or LP#1 (g) seedlings treated with fungal elicitor for 0-24 h. GUS activity in different organs of adult SP#1 and LP#1 lines treated with fungal elicitor for 24 or 4 h respectively: (c) and (h) roots, (d) and (i) flowers, (e) and (j) cauline leaves. Representative images are shown.

Fig. 6 Transgenic plants constitutively overexpressing *AtWRKY30*. (a) RNA blot analysis: RNA samples were prepared from leaves of a wild-type plant (Col-0), leaves from plants transformed with the empty vector (EV) and eight independent lines

overexpressing *AtWRKY30* (35S::W30). Twenty micrograms of total RNA from each sample were fractionated on a formaldehyde–agarose gel, transferred to a nylon membrane and hybridized with ^{32}P-labelled *AtWRKY30*-specific DNA probe. (b) Representative three-week-old *AtWRKY30* overexpressing (35S::W30-1) and Col-0 plants.

Fig. 7 Germination and seedling growth of 35S::W30-1, 35S::W30-8, 35S::W30-21 and wild-type (Col-0) plants subjected to different treatments. Transgenic lines and Col-0 age-matched seeds were germinated in parallel on 0.5x MS agar plates containing 1 μM MV (a) or 150 mM NaCl (b) and scoring was carried out 10 days after treatment. Data represent per cent seedlings at growth stage 0.7. Error bars represent the SE over three replicate experiments, each containing at least 30 plants of each line. Experimental data were subjected to One-way ANOVA test. Significant difference between Col-0 and 35S::W30 plants (P <0.05) is indicated by an asterisk.

Fig. 8 Effect of MV on antioxidant enzymes in Col-0 and 35S::W30 lines. Soluble proteins were extracted from ten-day-old Arabidopsis seedlings treated with 50 μM MV for 4 h and used for activity quantification of APX (a) and CAT (b). Error bars represent the SE over three replicate experiments. Experimental data were subjected to two-way ANOVA test (P <0.05). Different capital letters (A or B) indicate significant differences between treatments within a given line. Different lower-case letters (a, b or c) indicate significant differences between lines within a given treatment.

Supplementary Fig. 1 DNA binding assay of recombinant AtWRKY30 protein to ^{32}P-labelled SP promoter fragment (0.28 kb) encompassing four W-boxes (Fig. 1). Total recombinant AtWRKY30 protein extract (40 μg) from *E. coli* was used for DNA

binding assays with 0.28 kb ^{32}P-labelled promoter fragment (+). As a control, total protein extract (40 µg) from *E. coli* that did not produce recombinant AtWRKY30 protein was used (-). FP: free probe.

Supplementary Fig. 2 GUS activity in LP #1 transgenic plants. (a) Scheme of plasmid pBI101 with 1.96 kb (LP) DNA fragment from the *AtWRKY30* promoter cloned upstream of the *β-glucuronidase* gene. (b) Leaves were subjected to biotic, abiotic and hormone treatments. LP #1 plants were treated with a solution containing: 20 mM H_2O_2, 100 mM NaCl, 200 mM mannitol or 100 µM sodium arsenate all in 0.005 % (v/v) Silwet L-77, and incubated for 4 h, or kept without watering for 15 days and then re-watered (indicated as drought). Biotic treatments were assayed by syringe infiltration with *X. axonopodis* or *P. syringae* pv. *tomato* DC3000 (10^8 cfu ml^{-1}) or by vacuum infiltrating leaves with bacterial (*A. tumefaciens*) elicitor. The left half of a leaf infiltrated with a solution without *A. tumefaciens* is also shown. The arrows indicate the site of infiltration using a syringe with the *X. axonopodis* or *P. syringae* bacterial suspension or with $MgCl_2$ (control) and the wounded tissue in the *A. tumefaciens* treated leaf. For hormone treatments, leaves were incubated with 5 mM ethephon, 100 µM ACC or 100 µM MeJA. Representative images are shown.

Supplementary Fig. 3 GUS staining detected in SP #1 line. (a) Scheme of plasmid pBI101 with the 0.28 kb (SP) DNA fragment from the *AtWRKY30* promoter cloned upstream of *β-glucuronidase* gene. (b) *AtWRKY30* promoter activity was followed by histochemical localization of GUS activity in leaves or cauline leaves after 24 h of 200 mM mannitol or 100 mM NaCl treatment. Representative images are shown.

Supplementary Fig. 4 Fresh weight (in mg) of seedlings after growth on (a) 1 μM MV or (b) 150 mM NaCl; shown are data for 35S::W30-1, 35S::W30-8, 35S::W30-21, empty vector control (EV) and wild type (Col-0) plants. Age-matched seeds of all lines were germinated in parallel on 0.5x MS agar plates containing either MV or NaCl and scoring was carried out after 10 days of treatment. Error bars represent the SE over three replicate experiments, each including at least 30 plants of each line. Experimental data were subjected to One-way ANOVA test. Significant difference between Col-0 and 35S::W30 plants ($P < 0.05$) is indicated by an asterisk.

Supplementary Fig. 5 GUS staining detected in LP #1 line during germination. Seeds from LP #1 line were germinated and grown in MS 0.5x (a), MS 0.5x supplemented with 0.5 μM MV (b) or 100 mM NaCl (c). GUS staining was carried out when seedlings were four, six and eight-day-old. Representative images are shown.

Figure 1
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W-box	Sequence
-132	AGTCAA
-140	AGTCAA
-195	TTGACC
-223	GGTCAG
-1162	TTGACC
-1895	TTGACT

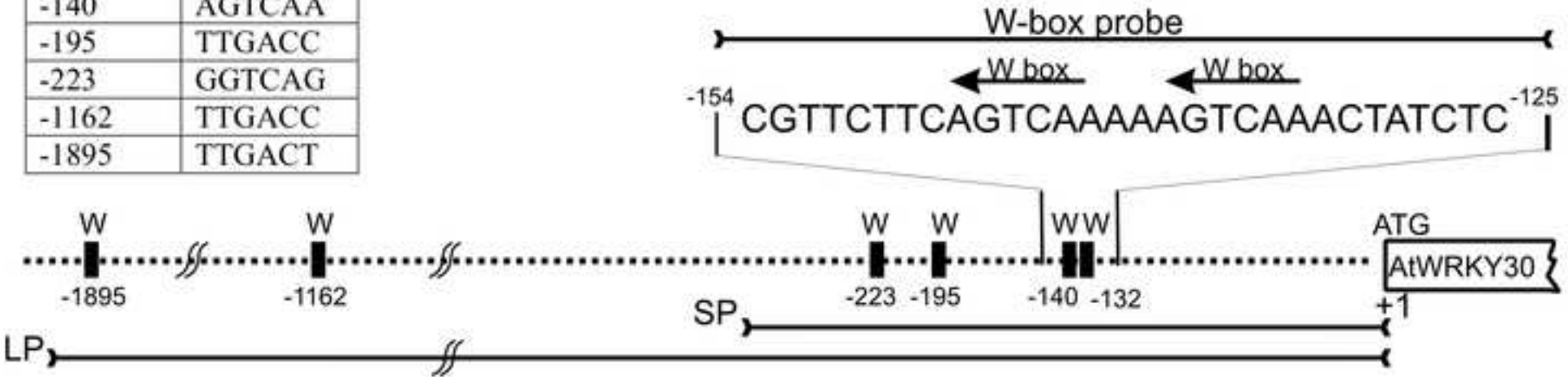

Figure 2
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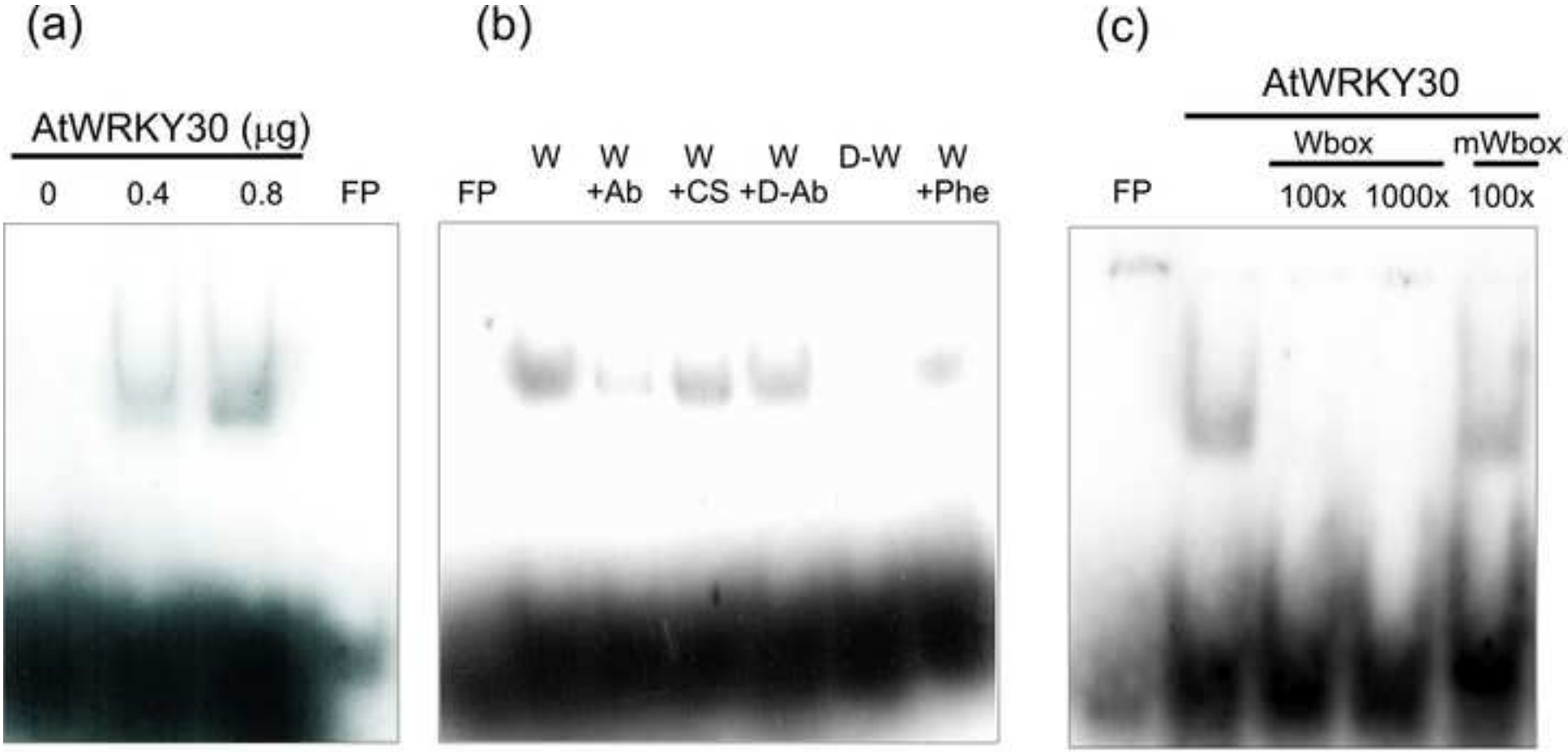

(d)

W box CGTTCTTCAGTCAAAAAGTCAAACTATCTC
mW box CGTTCTTCAGg t cAAAAGg t c AACTATCTC

Figure 3
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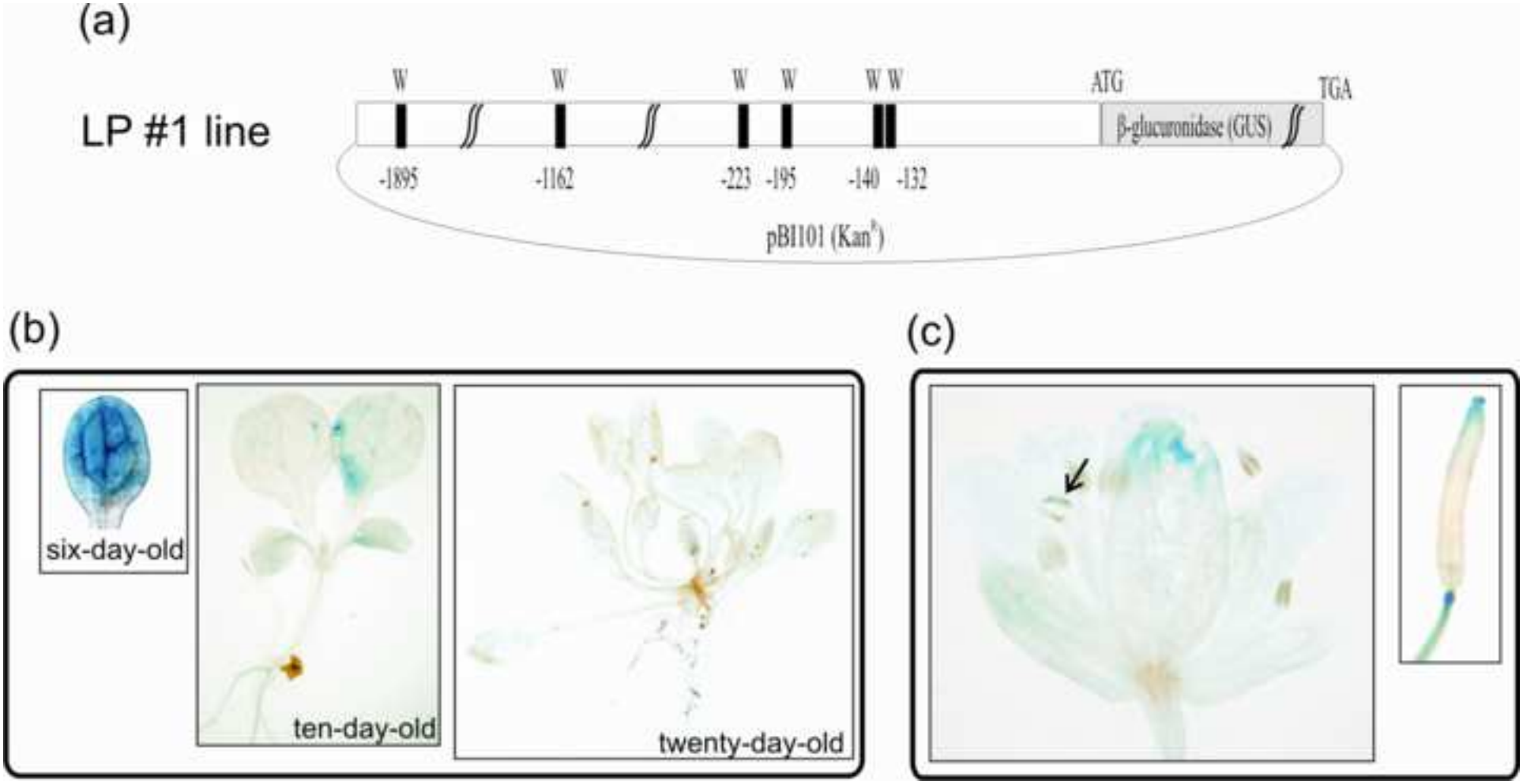

Figure 4

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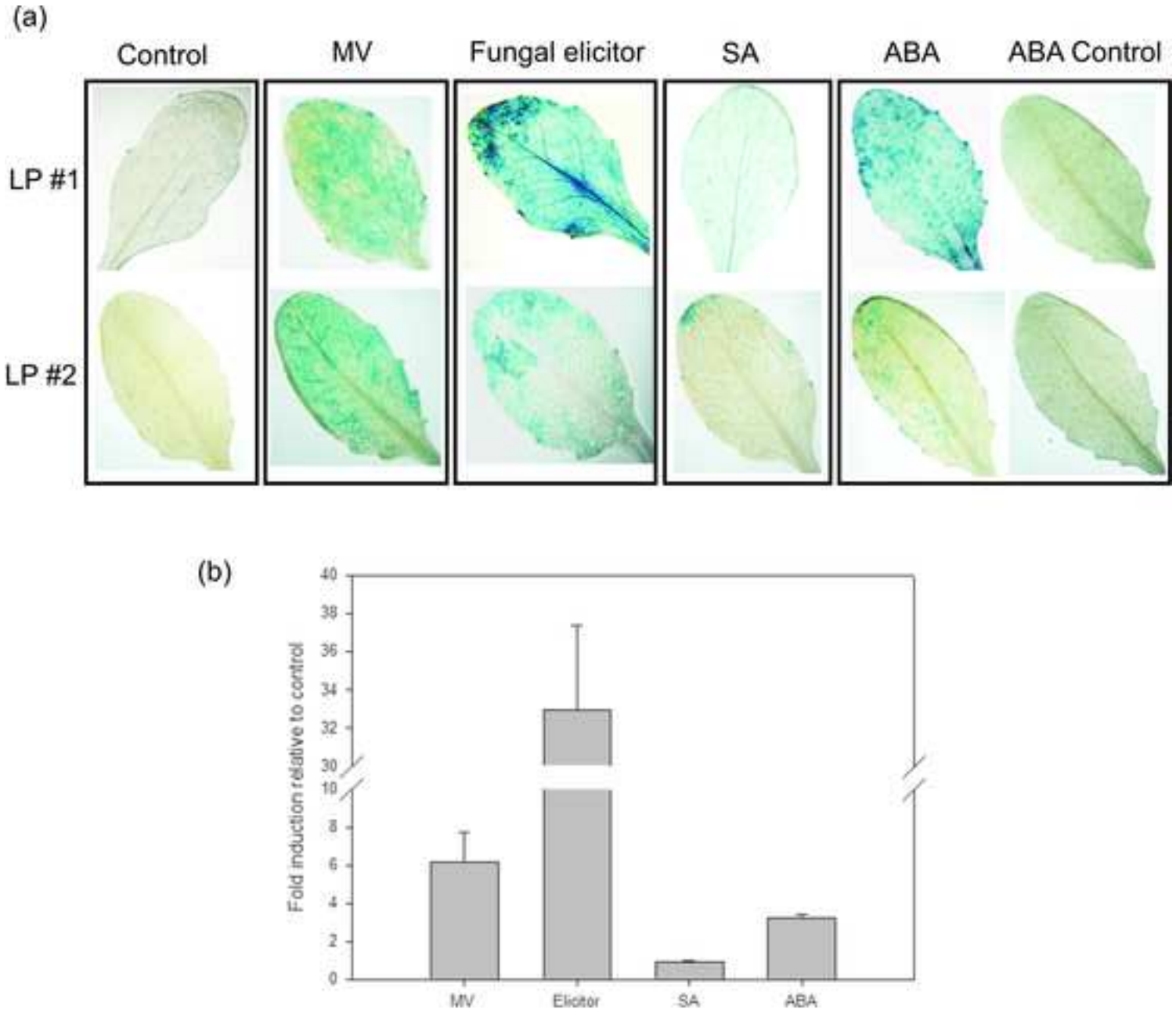

Figure 5
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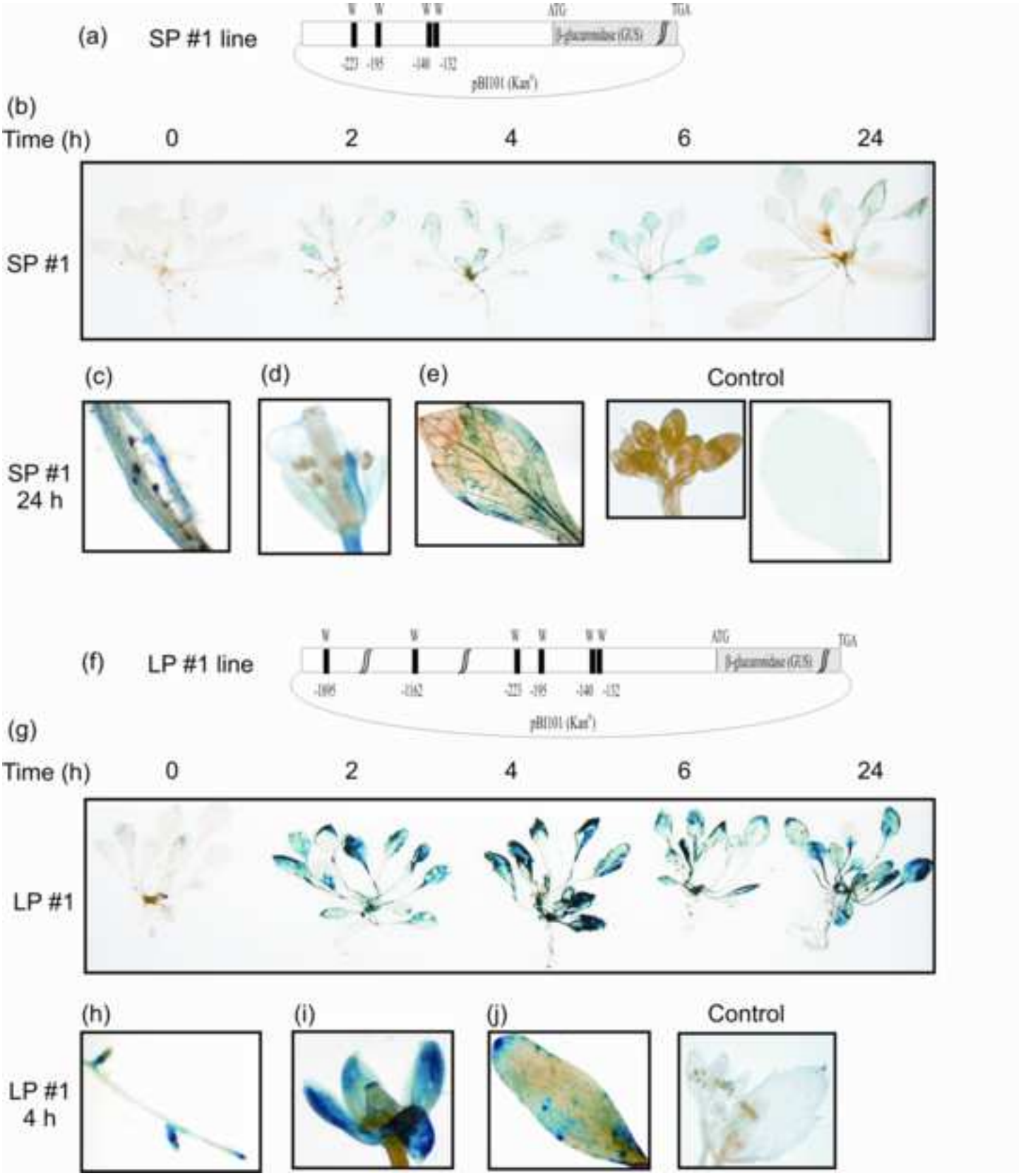

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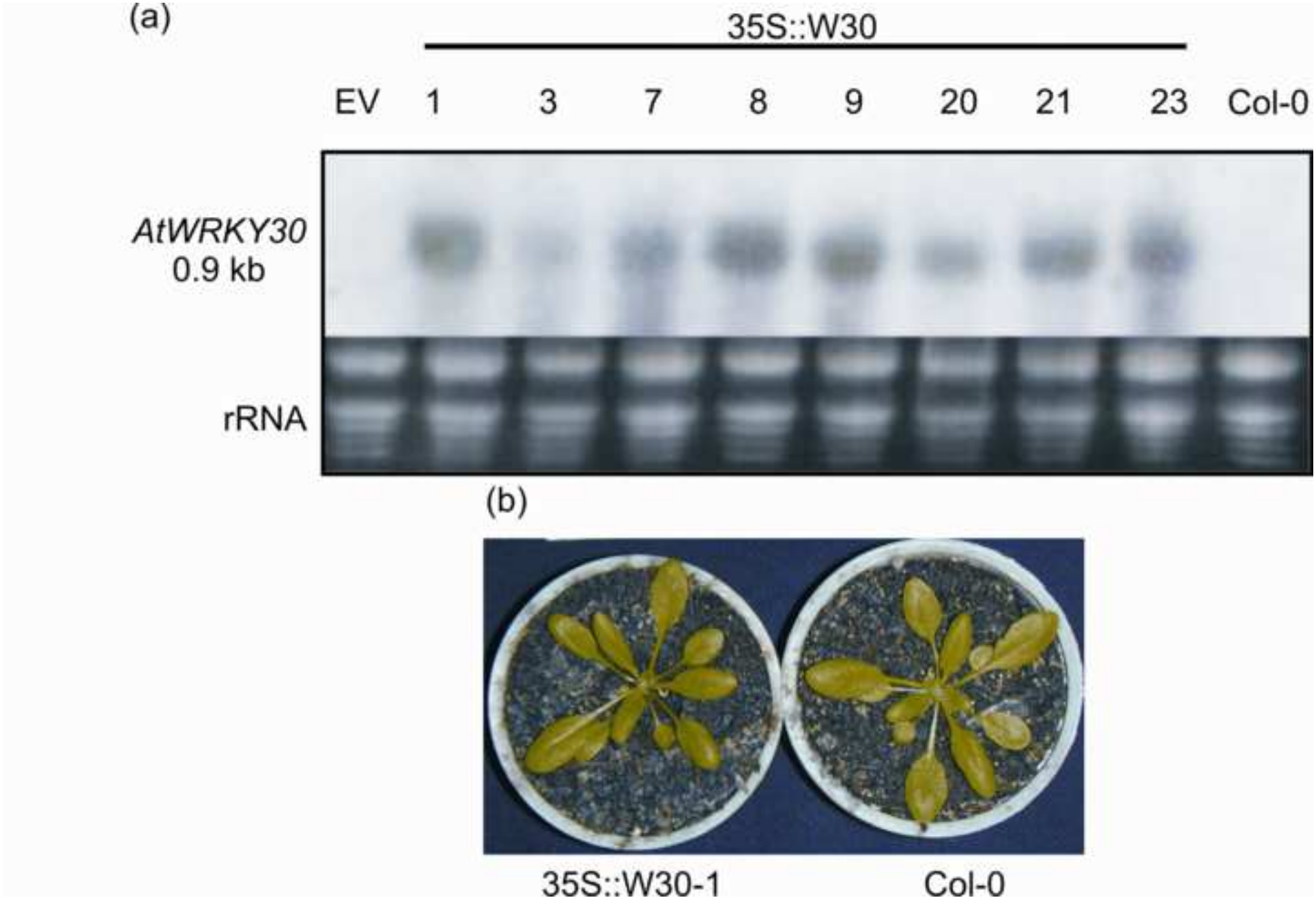

Figure 7
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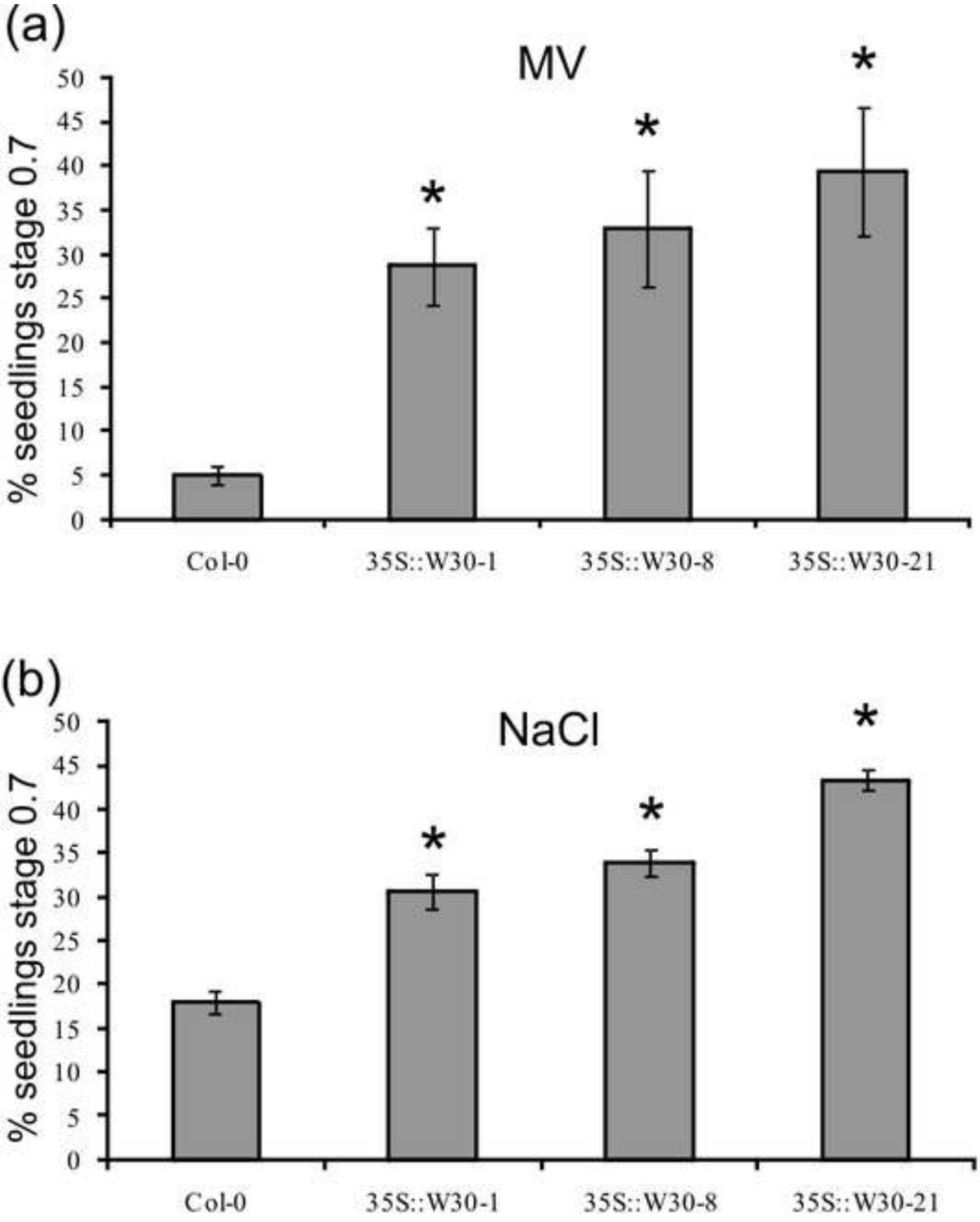

Figure 8
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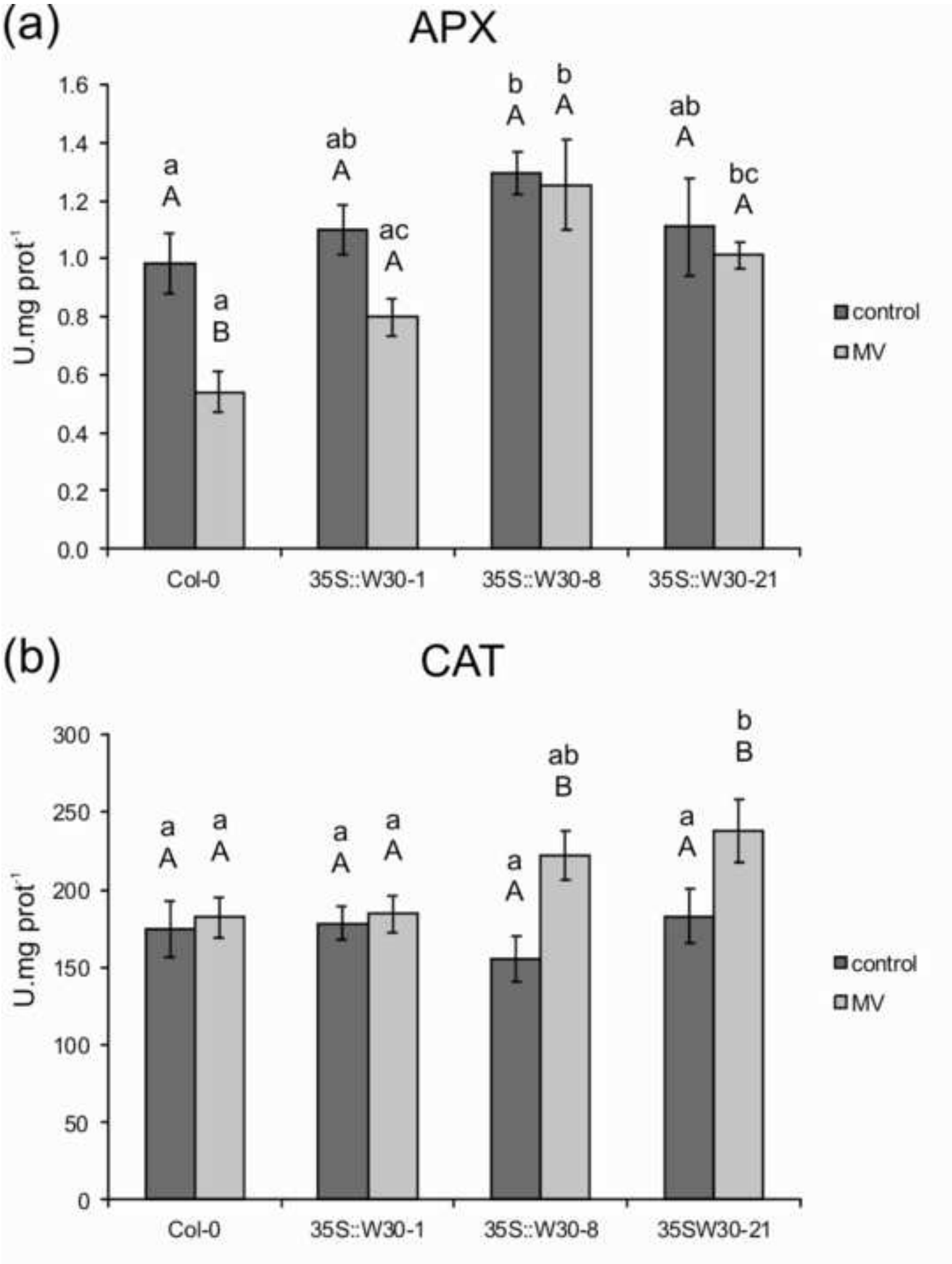

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