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RESEARCH PAPER

Virus-induced gene silencing of *Arabidopsis thaliana* gene homologues in wheat identifies genes conferring improved drought tolerance

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Abstract

In a non-model staple crop like wheat (*Triticum aestivuml* L.), functional validation of potential drought stress responsive genes identified in *Arabidopsis* could provide gene targets for breeding. Virus-induced gene silencing (VIGS) of genes of interest can overcome the inherent problems of polyploidy and limited transformation potential that hamper functional validation studies in wheat. In this study, three potential candidate genes shown to be involved in abiotic stress response pathways in *Arabidopsis thaliana* were selected for VIGS experiments in wheat. These include *Era1* (enhanced response to abscisic acid), *Cyp707a* (ABA 8'-hydroxylase), and *Sal1* (inositol polyphosphate 1-phosphatase). Gene homologues for these three genes were identified in wheat and cloned in the viral vector barley stripe mosaic virus (BSMV) in the antisense direction, followed by rub inoculation of BSMV viral RNA transcripts onto wheat plants. Quantitative real-time PCR showed that VIGS-treated wheat plants had significant reductions in target gene transcripts. When VIGS-treated plants generated for *Era1* and *Sal1* were subjected to limiting water conditions, they showed increased relative water content, improved water use efficiency, reduced gas exchange, and better vigour compared to water-stressed control plants inoculated with RNA from the empty viral vector ($BSMV_0$). In comparison, the *Cyp707a*-silenced plants showed no improvement over $BSMV_0$-inoculated plants under limited water condition. These results indicate that *Era1* and *Sal1* play important roles in conferring drought tolerance in wheat. Other traits affected by *Era1* silencing were also studied. Delayed seed germination in *Era1*-silenced plants suggests this gene may be a useful target for developing resistance to pre-harvest sprouting.

Key words: *Arabidopsis*, drought, *Era1*, *Sal1*, virus-induced gene silencing, wheat.

Introduction

Drought is a major problem that affects wheat production worldwide. Tolerance to water stress is a quantitative trait with a complex phenotype that is often confounded by plant phenology (Fleury *et al*., 2010). Because of the complexity of water stress tolerance and the effect of other harsh environmental factors in the field, improvement of this trait has largely relied on direct phenotypic selection for improved performance under drought conditions. On the other hand, the application of genomic tools in *Arabidopsis* has produced large datasets of gene expression profiles that result from exposure to water stress (Seki *et al*., 2001). Experimental validation of candidate genes identified from such transcript profiling studies has identified genes that appear to contribute to plant performance under limiting water conditions in

greenhouse studies. Two such genes are *Era1* and *Cyp707a*, which are both involved in abscisic acid (ABA) regulation in the abiotic stress pathway. *Era1* is a gene that encodes the β-subunit of farnesyltransferase (Cutler *et al*., 1996), whose loss of function in *Arabidopsis thaliana* caused greater ABA-induced guard cell S-type anion-channel activation and a rapid increase in the cytosolic Ca^{2+} concentration. *Era1* mutation resulted in significantly tighter stomatal closure at a wide range of physiologically relevant ABA concentrations (Pei et al., 1998; Allen *et al*., 2002). Transgenic *Brassica napus* silenced for *Era1* showed enhanced ABA sensitivity as well as significant reduction in stomatal conductance and water transpiration under drought stress conditions (Wang *et al*., 2005). *Cyp707a* is a catabolic gene encoding ABA 8'-hydroxylase, an enzyme playing a key role in ABA catabolism and in controlling ABA levels in various aspects of the plant life cycle (Kushiro *et al*., 2004). The *Cyp707a* gene reportedly degrades ABA during seed imbibition and dehydration stress (Kushiro *et al*., 2004; Saito *et al*., 2004). A T-DNA insertion mutant of *Cyp707a3*, which is the most abundantly expressed gene among the four *Cyp707a* members under stress conditions, exhibited elevated drought tolerance with a concomitant reduction in transpiration rate (Umezawa *et al*., 2006). A third gene is *Sal1*, an inositol polyphosphate1-phosphatase-encoding gene. *Sal1* was originally isolated from *Arabidopsis* for its ability to complement a salt-sensitive yeast strain (Quintero *et al*., 1996). In *Arabidopsis*, mutation of this gene has shown increased drought tolerance (Wilson *et al*., 2009). *Sal1* acts as a negative regulator of predominantly ABA-independent and also ABA-dependent stress response pathways. As far as is known, none of these three genes have been experimentally proven to have a role in drought tolerance in wheat. Furthermore, wheat homologues for *Sal1* have not been identified prior to this study.

Homologous genes in different species may not have the same function due to the different course of evolution that each species has taken. The high frequencies of duplication and deletion events in the wheat genome make it difficult to predict the function of a gene in one species based on the function of the homologous gene in the other species (Dubcovsky and Dvorak, 2007). Furthermore, it has been shown that while common regulatory mechanisms exist across species in response to abiotic stress, the conservation of the molecular response to dehydration across experiments (Mohammadi *et al*., 2007; Aprile *et al*., 2009) is low due to variation in stress dynamics, stage of development and tissue analysed.

Functional validation of the role of genes in stress can be studied either by overexpression or downregulation. For gene overexpression studies, full-length sequence information and an efficient genetic transformation protocol are required. However, wheat has been the most recalcitrant cereal species to culture *in vitro* (Shah *et al*., 2009). Transformation in wheat is still confined mainly to a few responsive varieties with quite different transformation frequencies such as the model spring genotype 'Bobwhite' (Cheng *et al*., 1997, 2003; Hu *et al*., 2003). For downregulation studies, knockout mutants are used. Not only will this require a transformation step, but the functional redundancy of homeologous genes present in the other wheat genomes could complement the absence of gene expression resulting from a single gene knockout (Lawrence and Pikaard, 2003). This limitation can be overcome by generating double and triple mutants, although this process is cumbersome and time consuming. Therefore, faster alternatives are required for functional gene analysis in polyploid wheat.

Virus-induced gene silencing (VIGS) provides an alternative strategy for gene functional analysis through the simultaneous knockdown of expression of multiple related gene copies. This is a technique that was first developed in dicots (Burch-Smith *et al*., 2006) and is based on post-transcriptional gene silencing of host sequences that are complementary to host-derived sequences contained in a recombinant viral vector inoculated on plants. VIGS was developed in barley using barley stripe mosaic virus (BSMV), a natural pathogen in this species and consisting of a tripartite genome (Holzberg *et al*., 2002; Lu *et al*., 2003). The usefulness of VIGS in wheat was first demonstrated by Scofield *et al*. (2005) and has now been used to study genes for resistance to pathogens and the Russian wheat aphid (Scofield *et al*., 2005; Cloutier *et al*., 2007; Zhou *et al*., 2007; Van Eck *et al*, 2010). VIGS has not been used for characterization of water stress response genes in wheat.

The main objective of this study was to evaluate the roles of *Era1*, *Cyp707a*, and *Sal1* in the response of wheat to limiting water conditions using VIGS. Optimization of the VIGS protocol was required in order to accomplish this.

Materials and methods

Plant material

All experiments were conducted using hexaploid spring wheat (*Triticum aestivum* L.) cv UC1041, a breeding line (Yecora Rojo/'Tadinia') from the University of California-Davis Wheat Breeding Program, which was kindly provided by Dr Jorge Dubcovsky.

Silencing construct development

PCR products used in the construction of silencing vectors were amplified from wheat cDNA using the VIGS primers listed in Table 1. Primers were designed using Primer3 (Rozen and Skaletsky, 2000) and used to amplify a 297-bp fragment of *Cyp707a* (accession EU430344.1) and a 316-bp fragment of *Era1* (accession ABI74692). For identification of *Sal1* sequences in wheat, annotated *Arabidopsis Sal1* (TAIR accession 4010745380) sequences were screened against publicly available wheat expressed sequence tag sequences (http://wheat.pw.usda.gov/wEST/) which were then assembled into one contig using CAP3 at a stringency level of 95% similarity over a 20-bp overlap (Huang and Madan, 1999). The assembled sequences were validated by PCR amplification of wheat cDNA followed by sequencing, resulting in a 1077-bp region. A PCR product of 275 bp was generated from this 1077 bp using primers listed in Table1.

Vector constructs were made by the method described by Cakir and Scofield (2008). The orientation of the cloned inserts was determined via PCR using a combination of vector-specific (Cakir and Scofield, 2008) and fragment-specific primers (Table 1). Clones putatively containing the fragments in the antisense orientation relative to the γ genes were sequenced to confirm their identity and subsequently used for gene silencing.

Table 1. Wheat primers used in this study.

Name/ Accession	Type	Target region	Primer (5'–3')
Era1	VIGS	57–372	AATGGCAGGGTCTGATGAAC
			TGCGCTGTACTGGCTAACTG
	qRT-PCR	216–307	TGAAGCTCATGGTGGGTACA
			AGCCAATCAAGCTAGGCAAA
Sal1	VIGS	219–494	TCTATGGTGGCCGAAGAGGA
			CCAACACGCCCAAAACAACT
	qRT-PCR	104–201	ACAATTGGTTGTGGTGCTGA
			AAAAACGAGGCATTCACTGG
Cyp707a	VIGS	368–664	GTCCCCAGGCCATCTTCTTC
			GTCCCCAGGCCATCTTCTTC
	qRT-PCR	375–469	GGGTGATCCAGGAGACGAT
			GGGAATCAGGTACCCTTGGT

Viral inoculation

The α, β, and γ RNAs of the BSMV genome were synthesized from linearized plasmids containing cloned cDNA genome segments (Petty *et al*., 1989), using the mMessage mMachine T7 kit (Ambion, Austin, TX, USA). Capped *in vitro* transcripts of each RNA segment were combined in an equimolar ratio and added to an abrasive FES buffer (0.1 M glycine, 0.06 M K_2HPO_4, 1% w/v tetrasodium pyrophosphate, 1% w/v bentonite, 1% w/v celite, pH 8.5) according to the procedures of Scofield *et al*. (2005). Each silencing construct consisted of BSMV α, β, and γ with the target gene insert. The original BSMV, $BSMV_0$, was used as the viral control, and was constituted from α, β, and γ RNA derived from the original empty pSL038-1 vector. A volume corresponding to 3 µg viral RNA was rub inoculated onto the second leaf of silenced seedlings at the 3–4 leaf stage. For *Era1*, another silencing experiment was done at the booting stage by rub inoculating the stem just underneath the growing spike. Seeds were collected at 23 days post-inoculation (dpi).

Quantitative PCR

Eleven days after viral inoculation of plants, leaf tissue was collected to determine the efficiency of silencing. Quantitative real-time PCR (qRT-PCR) was performed to determine changes in *Era1*, *Sal1*, and *Cyp707a* transcript abundance in each treatment group. All the qRT-PCR primers are listed in Table 1. The distal 5 cm of the third leaf from four experimental plants per treatment was collected into liquid nitrogen. For *Era1* inflorescence silencing, heads from silenced spikes were harvested on day 17 after viral inoculation. First-strand cDNA synthesis was conducted using the Retroscript reagent (Ambion) on purified RNA for each sample and primed with a mix of poly A and random decamers. All amplifications were performed on the iCycler iQ instrument (Biorad, Hercules, CA, USA) using the Perfecta SYBR Green Supermix (Quanta Biosciences, Gaithersburg, MD, USA) and input cDNA equivalent to 2.5 ng total RNA. The following cycling parameters were used: initial denaturation at 95 °C for 2 min, 30 cycles consisting of denaturation at 95 °C for 15 s and annealing and extension at 57 °C for 45 s. Single-fragment amplification was verified by dissociation curve analysis. Gene expression values were normalized as previously described (Willems *et al*., 2008). Relative transcript abundance was calibrated to the mean expression of the viral control (water-stressed $BSMV_0$-treated plants) treatment group and normalized against the level of housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) in each sample (Pfaffl, 2001).

Imposition of water stress

Each of the five treatment groups consisted of 10 plants. Plants were grown in plastic pots holding 500 g of potting mixture. Plants were maintained in a temperature-controlled growth room at 22–25 °C and relative humidity of 60% with a 12 h photoperiod with light intensity ranging from 300 to 400 µE m^{-2} s^{-1}. Prophylactic measures were taken to maintain the plants disease and pest free. Samples from plants were not pooled, and each plant was observed as an independent biological replicate for a total of 10 biological replicates per treatment. For each experiment, two subsets of plants were maintained. One set of plants was maintained at 100% field capacity (FC) and stress was imposed on the other set of plants by withholding water until 50% FC, which was achieved by withholding water continuously for 3 days. Withholding water up to 50% FC was found to induce the water stress phenotype in the experimental plants placed in 500 g of potting mix within the time frame of the experiment (24 days). The plants were maintained at 100% FC until imposition of moisture stress. Withholding of water started 3 days after rub inoculation of BSMV. Plants were maintained at 50% FC during the entire duration of the study. Soil moisture regimes were monitored gravimetrically by weighing the pots every day.

Estimation of plant water status

To assess the drought stress effects, leaf relative water content (RWC) was estimated according to the method of Ekanayake *et al*. (1993). Water use efficiency (WUE, seedling dry weight increment per weight of water used) was also estimated. The soil evaporation across all pots was assumed to be similar. Water used by the plant was estimated by the amount of water required to maintain the weight for each experimental pot with plants. At day 11 after initiation of water stress moisture, stress responses were assessed by taking leaf samples from the uppermost fully expanded leaves of both stressed and non-stressed plants. Samples were collected at midday, quickly sealed, and kept on ice. After determining the fresh weight, the leaf segments were floated on deionized water for 24 h to determine their turgid weight. The dry weight was determined after oven drying at 70 °C to a constant weight. The RWC was calculated using the formula RWC (%) = [(W – DW) ÷ (TW – DW)] × 100, where W is sample fresh weight, TW is turgid weight after rehydrating plant sample for 24 hours, and DW is sample dry weight (Ekanayake *et al*., 1993).

Gas exchange

Plants in all treatment groups (water-stressed silenced plants, water-stressed viral control, non-silenced well-watered (WW), and non-silenced water-stressed (WS) plants) were tested for variation in photosynthetic gas exchange since the genes under consideration may have direct or indirect effects on stomatal opening/closure (Wang *et al*., 2009). The level of water stress used was selected to cause immediate visible phenotypes to accommodate the transient nature of the VIGS assay. This led to all the water-stressed wheat seedlings looking wilted and chlorotic compared to well-watered plants. To carry out the gas exchange studies, this study used a higher light intensity compared to normal greenhouse light conditions to ensure that photosynthesis and transpiration were not light limited and that the difference in gas exchange among treatment plants is due to the effect of silencing stomatal conductance-regulating genes. The light intensity was set at 800 µmol m^{-2} s^{-1} with a red/blue light source and the CO_2 level was set at 400 µmol mol^{-1}. A portable, open flow, gas-exchange system (LCpro+, Opti-Sciences, Hudson, NH, USA) was used to record the gas exchange on the fifth fully expanded leaf. One day prior to the gas exchange reading, the pots were watered to maintain the exact 50 or 100% FC depending on the treatment. Leaf gas-exchange rates were measured at 7, 14, and 20 dpi.

Germination study

Seeds from plants inoculated with $BSMV_{Era1}$ during the booting stage were collected 23 days post- inoculation. Seeds from the control

$BSMV_0$-inoculated plants were also collected. Seeds from silenced and control plants of similar size were surface sterilized with 5% (w/v) calcium hypochlorite for 15 min and washed four times with sterile deionized water. These seeds were then transferred to sterile Petri dishes (five seeds per dish) containing moist Whatman No. 1 filter paper. Four replicates of five seeds per dish were used in each treatment. Seeds were allowed to germinate at room temperature in the dark and germination percentages were recorded daily up to 6 days using radicle extrusion as a criterion. A seed was considered germinated if the radicle length was ≥2 mm.

Bacterial pathogen study on Era1-silenced plants under drought

Xanthomonas translucens pv. *undulosa* (denoted as strain B75) and *Xanthomonas translucens* pv. *translucens* (denoted as strain B74) were incubated at 28 °C for 72 h on modified Wilbrink's media and a single colony was selected and grown in nutrient broth for 16 hours at 28 °C. An aqueous suspension of 10^9 colony-forming units ml^{-1} were inoculated by syringe infiltration on the flag leaf at the 3-leaf stage, and the plants were incubated in the greenhouse. Sterilized water was used as the negative control for inoculation treatments. The bacterial pathogen inoculation was done at 7 dpi with $BSMV_{Era1}$ or $BSMV_0$. In a pilot study, it was determined that the strains used exhibit a compatible interaction with wheat cv. UC1041 (data not shown). Seven days after inoculation with *X. translucens*, the leaf lesion area was quantified using digital image analysis using ASSESS software (American Phytopathological Society, St. Paul, MN, USA). The experiment was arranged in a completely random design. There were 24 individual treatments (3 bacterial inoculants × 2 water stress conditions × 3 silencing types) and each of these treatments was replicated 24 times. Statistical analysis was carried out using the general linear model of the SAS 9.1 statistical package (SAS Institute, Cary, NC, USA). Data were subjected to two-way analysis of variance (ANOVA) for finding the effect of drought and *Era1* silencing on disease, followed by a comparison of the means according to a Duncan's multiple range test at $P < 0.05$.

Results

Reduction in the transcript levels of silenced genes

qRT-PCR was performed to determine the transcript levels of *Era1*, *Sal1*, and *Cyp707a* in water-stressed silenced plants relative to the water-stressed viral controls at 11 dpi. As Fig. 1 shows, the transcript levels of the genes were reduced in silenced plants compared to transcript levels of the genes in plants inoculated only with $BSMV_0$. Plants that were inoculated with $BSMV_{Era1}$ showed average 2.3-fold and 2.6-fold reductions in *Era1* at the inflorescence and seedling stages, respectively, while the mean *Sal1* transcript level was reduced 14.2-fold in $BSMV_{Sal1}$-inoculated plants ($P < 0.0001$, Fig. 1A and B). The largest reduction was observed in $BSMV_{Cyp707a}$ plants with an average 22-fold reduction in transcript level compared to water-stressed $BSMV_0$-treated plants ($P < 0.0001$, Fig. 1C).

Transcript levels were also determined in non-silenced WW and WS plants. The transcript levels of *Sal1* and *Cyp707a* in WW and WS plants were not statistically different from water-stressed viral control plants ($P = 0.1550$ and $P = 0.2700$, for *Sal1* in WW and WS, respectively; $P = 0.3250$ and $P = 0.3300$ for *Cyp707a* in WW and WS, respectively, Fig. 1B and C). Interestingly, the mean transcript levels of *Era1* in WW and WS plants were statistically higher compared to the viral controls ($P < 0.0350$ and $P < 0.0175$, Fig. 1A). This suggests that *Era1* was downregulated as a result of virus infection.

Silencing alters the rate of gas exchange

Era1 and *Sal1* function to modulate a plant's ABA-mediated stomatal response to control the rate of transpiration (Pei *et al*., 1998; Wilson *et al*., 2009). *Cyp707a* is involved in the catabolism of ABA and also has the potential to regulate stomatal response under drought (Umezawa *et al*., 2006). The present study therefore tested whether and how the rate of gas exchange would be affected by silencing of these genes. Water-stressed $BSMV_0$-treated plants and non-silenced WS plants did not show statistically significant differences in stomatal conductance and transpiration rate (Fig. 2). The water-stressed $BSMV_{Era1}$- and $BSMV_{Sal1}$-treated lines showed statistically significant reductions in stomatal conductance and transpiration rate under limiting water conditions compared to the water-stressed viral control plants at both 7 and 14 dpi. This is consistent with previous reports of reduced stomatal conductance in loss-of-function mutants in *Arabidopsis* (Pei *et al*., 1998; Rossel *et al*., 2004; Wilson *et al*., 2009). These loss-of-function mutant plants also showed increased tolerance in drought stress conditions in greenhouse experiments. At 14 dpi, the transpiration level of $BSMV_{Sal1}$-treated plants showed further reduction in stomatal conductance relative to that at 7 dpi. A slight reduction was also observed in $BSMV_{Era1}$-treated plants at 14 dpi compared to 7 dpi (Fig. 2). On the other hand, $BSMV_{Cyp707a}$-treated plants did not show a significant reduction in stomatal conductance compared to viral control plants. Photosynthesis measurements taken during this study were inconclusive due to the total shut down of photosynthesis across all water-stressed treatment groups.

Impact of silencing on plant water status under water limitation

Water stress was examined in terms of RWC and WUE at 14 dpi in all the treatment groups (Fig. 3). The RWC and WUE measurements indicate potential photosynthetic gain during a water stress period. The WUE values obtained were lower than typically observed in potted plants (usually in the range 0.6–0.9 g kg^{-1}). This is most likely a result of the plants not getting sufficient light in the greenhouse during the winter when these experiments were carried out. This would have resulted in reduced photosynthesis and consequently low biomass gain. However, since all plants were grown under the same conditions, it was possible to compare plants in the different treatments. The non-silenced plants showed a drastic reduction in both WUE and RWC in the WS plants relative to the WW plants, which was expected. The WS plants also did not differ significantly from the water-stressed viral control, indicating that virus inoculation had no effect on these two measurements. Similarly, plants silenced for *Cyp707a* did not differ significantly from the viral control in both WUE and RWC. On the other hand, plants silenced for *Era1* and *Sal1* showed significant gains in RWC and WUE over viral control and WS plants.

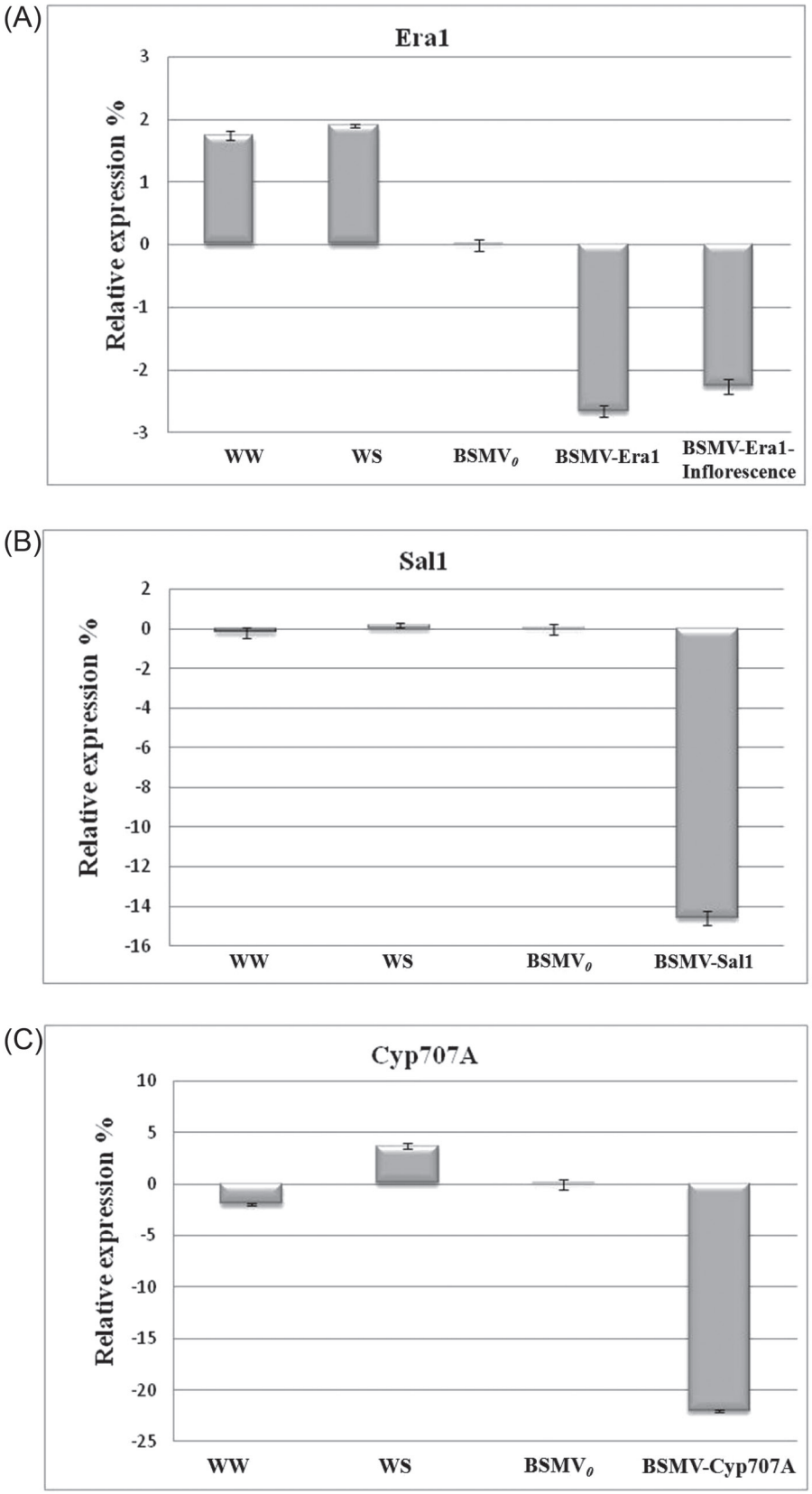

Fig. 1. Silencing efficiency as revealed by quantitative real-time PCR. Gene expression values were standardized across six independent biological replicates with three technical replicates. Expression of *Era1* (A), *Sal1* (B), and *Cyp707a* (C) in well-watered non-silenced (WW), water-stressed non-silenced (WS), and silenced plants (denoted by the gene name) at 11 dpi were calibrated to the mean level of expression of the respective gene in the water-stressed $BSMV_0$-treated plants.

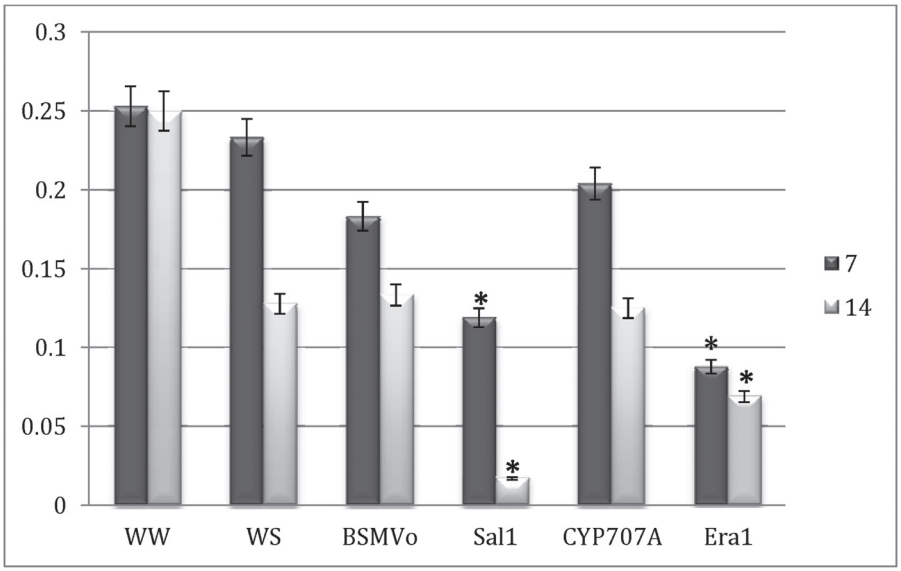

Fig. 2. Mean stomatal conductance (mmol m^{-2} s^{-1}) at 7 and 14 dpi. Measurements were taken daily from each treatment group for 14 days. Water-stressed $BSMV_0$-inoculated plants served as control. Values are means of six observations. Asterisks denote significant difference from the control WS ($P = 0.05$). WW, non-silenced well-watered; WS, non-silenced water-stressed; all others plants were water-stressed and inoculated with BSMV targeted to specific genes.

Plants silenced for Era1 and Sal1 showed improved vigour under water stress

Phenotypes of the plants were observed daily during the entire course of the experiment (27 days). Slight chlorosis was observed in all the silenced and viral control plants immediately after rub inoculation of the BSMV constructs. This has been reported in previous VIGS studies in wheat (Scofield *et al.*, 2005; Van Eck *et al.*, 2010) and is due to the plant's response to virus infection. Typical wilting symptoms and poor vigour were observed in all water-stressed plants, whether silenced for a gene or not (Fig. 4). The $BSMV_0$-treated control plants were visibly similar to the non-silenced WS plants under water stress. The water-stressed $BSMV_{Cyp707a}$-treated plants showed similar symptoms as the water-stressed viral control plants. On the other hand, water-stressed $BSMV_{Era1}$- and $BSMV_{Sal1}$-treated plants showed distinctly better turgidity and appeared more vigourous compared to plants in all the other water-stressed treatments. This improved vigour held up during the entire 24 day-stress period. Interestingly, more tillers were observed in $BSMV_{Sal1}$-treated plants compared to plants in all other treatments, including $BSMV_{Era1}$-treated plants. However, the $BSMV_{Sal1}$-treated plants were smaller compared to $BSMV_{Era1}$-treated plants under limiting water conditions during the initial 5–7 days of water stress. By day 24 of water stress, both $BSMV_{Era1}$ and $BSMV_{Sal1}$-treated plants were of similar size.

All in all, silencing of *Era1* and *Sal1* had similar effects on the plant. This includes reduced stomatal conductance, increased WUE, and increased RWC. These physiological measurements matched the observed improved vigour in $BSMV_{Era1}$- and $BSMV_{Sal1}$-treated plants under water stress. Decreased stomatal conductance under limiting water conditions may have contributed to improved WUE and RWC. Stomatal conductance was significantly and negatively correlated with the level of expression of *Era1* ($r = -0.80$, $P \leq 0.01$) and *Sal1* ($r = -0.89$, $P \leq 0.01$). In comparison, $BSMV_{Cyp707a}$-treated plants did not show significant changes in any of the physiological measurements or phenotypes relative to non-silenced or $BSMV_0$-treated plants, despite a significant reduction in expression level of *Cyp707a*.

Effects of Era1 silencing on seed germination and response to fungal pathogens

Silencing of *Era1* potentially makes plants hypersensitive to ABA. Since ABA is involved in seed germination, a germination study was conducted on $BSMV_{Era1}$-treated plants. Seeds harvested from plants inoculated with $BSMV_{Era1}$ at the booting stage were tested for germination capacity. As Fig. 5A shows, the earliest germination of *Era1*-silenced seeds occurred 6 days later than those of control ($BSMV_0$ treated) plants, 6–8 days after 100% of seeds from control plants have germinated, only 60% of seeds from *Era1*-silenced plants had germinated, and 100% germination was achieved 10 days later than those of control plants.

Goritschnig *et al.* (2008) recently showed that loss of function in *Arabidopsis* results in enhanced susceptibility to virulent bacterial and oomycete pathogens. This led the authors to suggest that farnesylation is involved in basal defence. To study the potential for increased susceptibility in *Era1*-silenced wheat plants, two known wheat pathogenic bacterial strains (*X. translucens* B74 and B75) were tested for their increase or decrease in virulence against *Era1*-silenced wheat plants compared to non-silenced wheat plants under drought and well-watered conditions (Fig. 5B). Two-way ANOVA showed no significant difference in the lesion area of plants under drought and silencing treatments compared to control plants in both bacterial strains ($P_{D75} = 0.52$, $P_{s75} = 0.46$

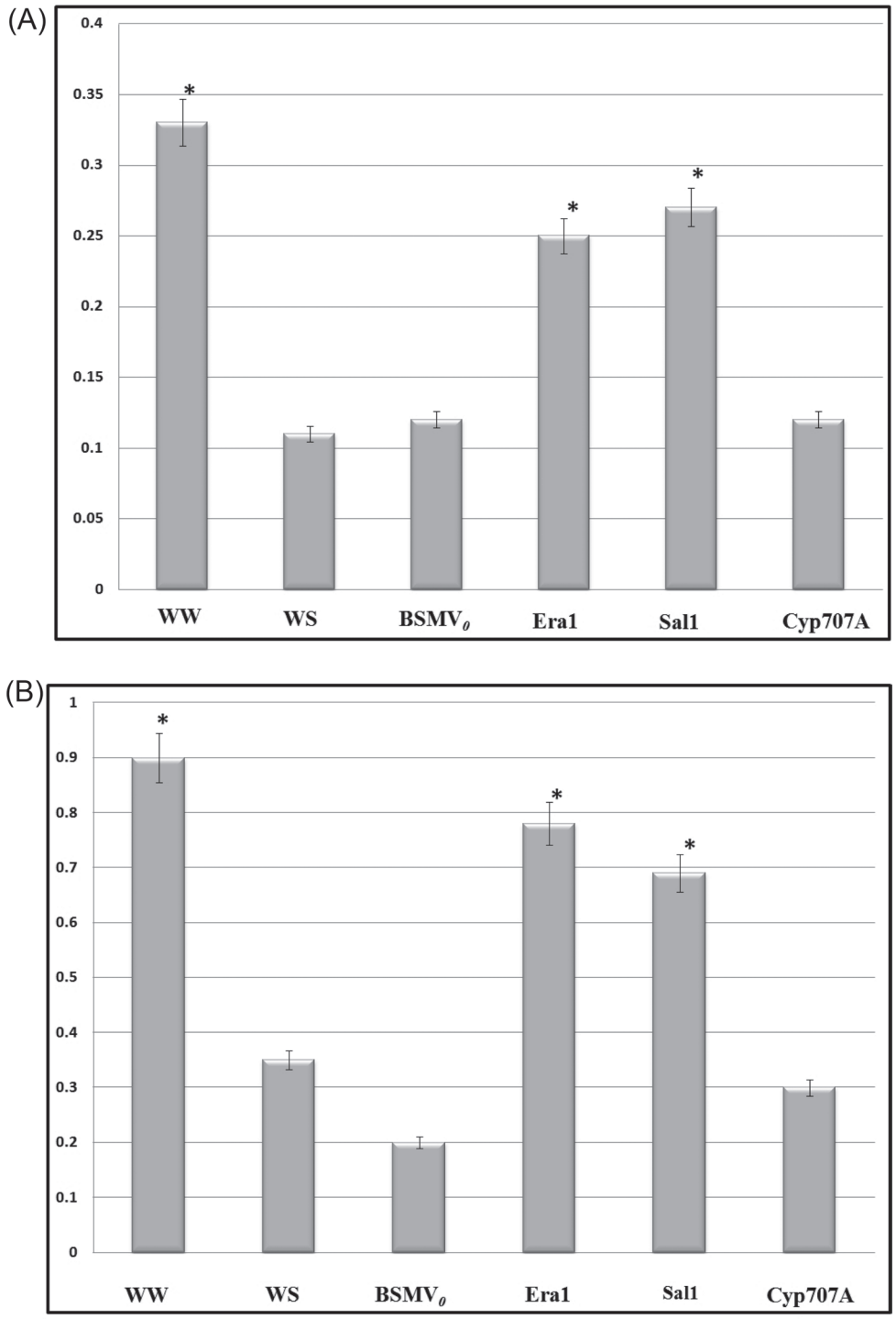

Fig. 3. (A) Water use efficiency by gravimetric determination (g kg^{-1}) at the end of treatment (24 dpi). (B) Mean relative water content (g) per treatment at 14 dpi. Water-stressed $BSMV_0$-inoculated plants served as control. Values are means of six observations. Asterisks denote significant difference from the control WS (P = 0.05). WW, non-silenced well-watered; WS, non-silenced water-stressed; all others plants were water-stressed and inoculated with BSMV targeted to specific genes.

and $P_{D73} = 0.51$, $P_{s73} = 0.304$). Also, there was no significant interaction between two treatments (drought X silencing) for both strains' lesion area ($P_{D75*S75} = 0.12$ and $P_{D73*S73} = 0.28$), suggesting that a combination of drought and *Era1* silencing does not affect bacterial growth in wheat individually and interactively.

Discussion

This study was undertaken to test whether single genes that have been shown to enhance drought tolerance in *Arabidopsis* when mutated would have similar effects in wheat. Three genes, namely *Era1*, *Cyp707a*, and *Sal1*, that represent

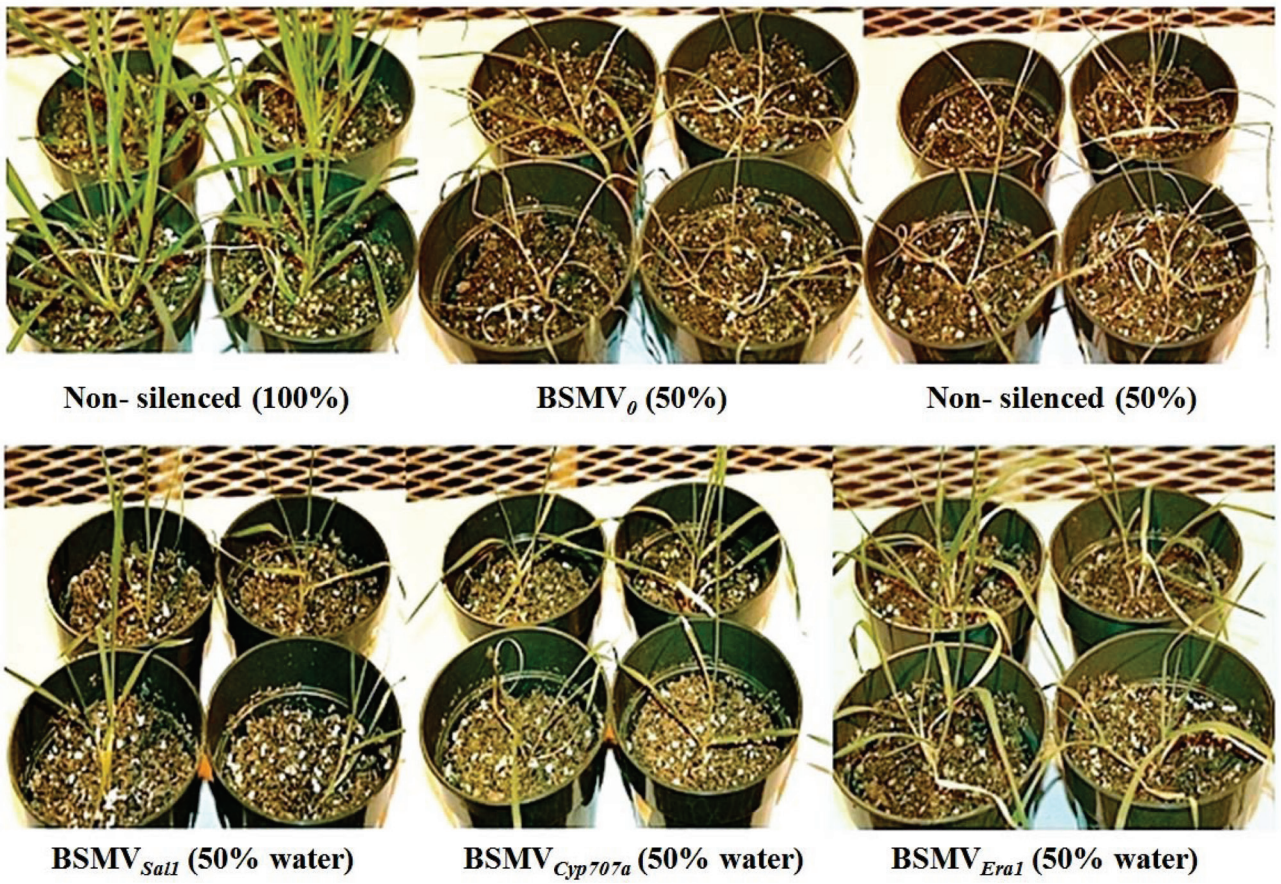

Fig. 4. Phenotypes of wheat plants at 24 dpi with BSMV RNA transcripts containing one of the following wheat genes: *Sal1*, *Cyp707A*, or *Era1*. Water stress was imposed on these plants by withholding water until 50% of field capacity. Water-stressed BSMV$_0$-inoculated plants served as control. Non-silenced well-watered (100%), non-silenced water-stressed (50%) plants, and water-stressed (50%) plants were included for comparison of phenotypes. Note the improved vigour of plants silenced for *Sal1* and *Era1* compared to the viral control plants.

different abiotic stress pathways were selected. The VIGS technique offered the best approach for testing the function of these genes in wheat for several reasons. The most important reason is that VIGS does not require a transformation step, which is still technically challenging in wheat. The potential of homologous copies of a gene to compensate for the absence of a homologue that has been silenced can be overcome in VIGS. All endogenous mRNA with at least 80% similarity to the host-derived sequence contained in the viral vector are targets for degradation. While this is an advantage of VIGS, it is also a disadvantage in that the specific gene responsible for the effect is not known and would require further experiments.

One concern of using VIGS for evaluating putative drought tolerance genes is the fact that environmental conditions used for stress induction might not favour viral replication leading to poor silencing. The stress protocol used in the present study involved initial exposure of silenced plants to an acclimation stress prior to exposure to severe stress. The viral inoculation was done at late evening on well-watered healthy plants and water stress was imposed gradually over 4 dpi by gradually withholding water. The initial adjustment time was important as the virus also imparts some level of stress on the plants. The protocol to characterize genes involved in the stress-recovery response involved inoculation of VIGS vector 4 days prior to water stress induction to coincide with higher transcript downregulation of targeted genes. The levels of silencing (from 2.6- to 22-fold) achieved for the targeted genes were comparable to other VIGS wheat studies (Scofield *et al.*, 2005; Bruun-Rasmussen *et al.*, 2007; Van Eck *et al.*, 2010). The consistent phenotype across the silenced plants also neutralized the concern of the stress response to BSMV masking the effect of water stress effects, suggesting that drought trait functional studies can be carried out by VIGS in wheat.

Of the three genes tested, only *Era1* and *Sal1* showed similar phenotypes in wheat upon loss of function as has been reported in *Arabidopsis* (Wang *et al.*, 2005; Wilson *et al.*, 2009). A possible role of regulation of stomatal conductance was indicated for the improved performance of *Era1*- and *Sal1*-silenced plants. Closed stomata has been suggested to lead to decreased photosynthesis and therefore decreased productivity for the plant in well-watered conditions (Farquhar and Sharkey, 1982; Schulze, 1986). The present experiment observed improved WUE compared to the viral control

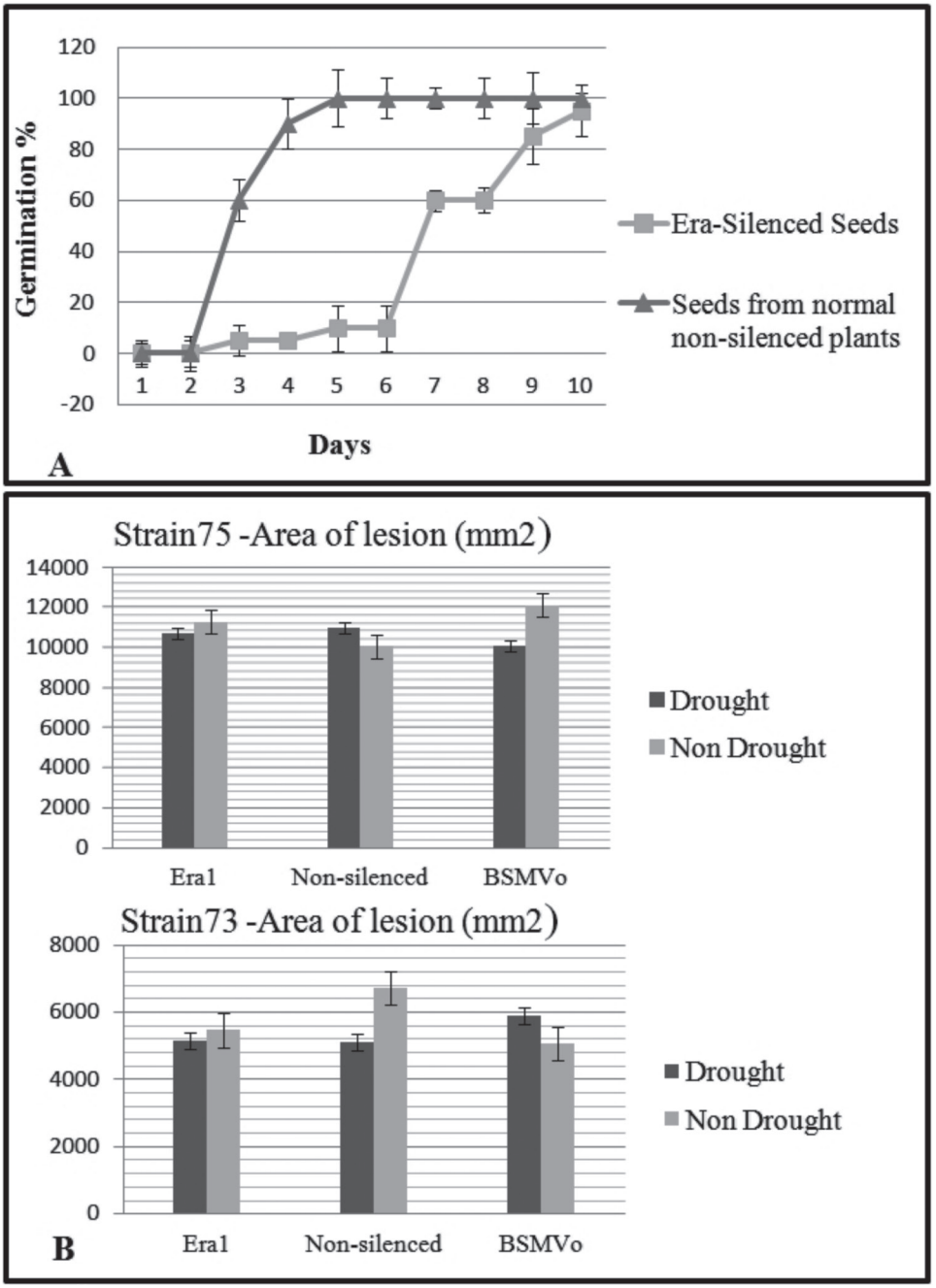

Fig. 5. Effect of *Era1* silencing on wheat seed germination and response to pathogens. (A) Number of germinated seeds in *Era1*-silenced and non-silenced plants, determined according to the 2-mm radicle extrusion criterion. (B) Cumulative lesion area observed after 7 days post infection with *Xanthomonas translucens* B74 and B75 on *Era1*-silenced or non-silenced plants under drought or non-drought conditions. *Era1*, plants silenced in *Era1*; $BSMV_0$, plants inoculated with empty BSMV viral vector; non-silenced, plants not inoculated with BSMV.

water-stressed $BSMV_0$-inoculated plants, indicating better dry weight production under limiting water condition for $BSMV_{Era1}$- and $BSMV_{Sal1}$-treated plants. A likely explanation is that plants which regulate the stomatal opening 'efficiently' and conserve water can achieve larger biomass per gram of water used (Farquhar and Richards 1984; Araus *et al.*, 2008).

In the case of *Sal1*, loss of function was reported to yield shorter and rounder leaves in *Arabidopsis* with an enhanced drought tolerance (Umezawa *et al.*, 2006). Down-regulation of *Sal1* leads to constitutively increased ABA content in *A. thaliana* (Wilson *et al.*, 2009). *Sal1* acts as a negative regulator of predominantly ABA-independent as well as ABA-dependent stress response pathways, such that its inactivation results in altered osmoprotectants, higher leaf RWC, and maintenance of viable tissues during prolonged water stress (Wilson *et al.*, 2009). This study also showed that reduced expression of *Sal1* in wheat resulted in increased RWC, and increased WUE, as well as improved vigour, suggesting a similar function of *Sal1*

in wheat. No other unusual phenotype was observed except increased/accelerated tillering, which is difficult to interpret at this point due to limited understanding of the mode of action of *Sal1*.

Interestingly, the effect of silencing *Cyp707a* homologues in wheat was different from previous findings in *Arabidopsis*. Loss of function of *Cyp707a* was reported to enhance drought tolerance in *A. thaliana* (Umezawa *et al*., 2006). In contrast, *Cyp707a*-silenced wheat plants did not show any improvements in terms of dry weight, WUE, or RWC of silenced plants compared to water-stressed $BSMV_0$-treated control plants under water stress. The differences in response between *Arabidopsis* and wheat plants to loss of function of *Cyp707a* may be due to differences in stress adaptation pathways between wheat and *Arabidopsis*. Another possible reason for the observed difference may have to do with the structure of the *Cyp707a* gene family in the two species. In *A. thaliana*, the *Cyp707a* gene family consists of *Cyp707a1–Cyp707a4*, all of which are upregulated in response to dehydration and subsequent rehydration, suggesting that the function of *Cyp707a* genes is redundant (Kushiro *et al*., 2004). Although there is only one *Cyp707a* cDNA sequence reported for wheat (accession EU430344.1), with three copies amplified by PCR in hexaploid wheat in this study, it is possible that other copies of *Cyp707a* exist in wheat. The DNA sequences of these other copies could potentially be different enough to cause ineffective silencing using the VIGS construct used in this study, leading to compensation of gene function by the remaining active genes. This emphasizes the importance of having the wheat genome sequence information in attempts to functionally test *Arabidopsis* gene homologues in wheat.

Out of the three genes tested in this study, *Era1* appeared to be the most promising as a potential target for drought tolerance breeding in wheat. Mutations in *Era1* could be readily identified through TILLING, taking advantage of resources that already exist in wheat (Slade and Knauf, 2005; Uauy *et al*., 2009). Because of the role of ABA in germination and dormancy, the present study tested *Era1*-silenced plants for germination rates. Indeed, *Era1*-silencing done at the booting stage resulted in a 6–10-day delay in germination compared to non-silenced plants. This trait may provide a solution for tackling pre-harvest sprouting in wheat. Pre-harvest sprouting is a consequence of ABA deficiency and leads to loss of grain weight and a reduction in end-use quality of wheat (Fang and Chu, 2008). While *Era1* has also been suggested to play a role in basal defence against bacteria and oomycytes (Goritschnig *et al*., 2008), the *Era1*-silenced wheat plants showed similar phenotypes in response to bacterial infection as non-silenced wheat plants under both water stress and well-watered conditions. This implies that there is no fitness cost to *Era1*-silenced plants under favourable conditions. It would be interesting to further study the differences in the function of *Era1* in pathogen susceptibility between wheat and *Arabidopsis*.

In conclusion, this study showed the involvement of *Era1* and *Sal1* in response to water deficit stress tolerance in wheat. This is the first report of VIGS being successfully used in wheat to functionally characterize genes thought to be involved in drought tolerance. The transient nature of VIGS did not deter from generating valuable observations for screening potential drought tolerant genes in wheat. One major strategy adopted by plants during drought tolerance is avoiding dehydration (Serraj and Atlin, 2009). Results from the present study indicate that downregulation of genes which reduce stomatal conductance may be a useful strategy for enhancing drought tolerance in wheat, if the same results hold in the field.

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References

Allen GJ, Murata Y, Chu SP, Nafisi M, Schroeder JI. 2002. Hypersensitivity of abscisic acid-induced cytosolic calcium increases in the *Arabidopsis* farnesyltransferase mutant *era1-2*. *The Plant Cell* **14,** 1649–1662.

Aprile A, Mastrangelo AM, De Leonardis AM, Galiba G, Roncaglia E, Ferrari F, De Bellis L, Turchi L, Giulliano G, Cattivelli L. 2009. Transcriptional profiling in response to terminal drought stress reveals differential responses along the wheat genome. *BioMed Central Genomics* **10,** 279.

Araus JL, Slafer GA, Royo C, Dolores SM. 2008. Breeding for yield potential and stress adaptation in cereals. *Critical Reviews in Plant Science* **27,** 377–412.

Bruun-Rasmussen M, Madsen CT, Jessing S, Albrechtsen M. 2007. Stability of barley stripe mosaic virus-induced gene silencing in barley. *Molecular Plant–Microbe Interactions* **20,** 1323–1331.

Burch-Smith TM, Schiff M, Liu YL, Dinesh-Kumar SP. 2006. Efficient virus-induced gene silencing in *Arabidopsis*. *Plant Physiology* **142,** 21–27.

Cakir C, Scofield SR. 2008. Evaluating the ability of the barley stripe mosaic virus-induced gene silencing system to simultaneously silence two wheat genes. *Cereal Research Communications* **36,** 217–222.

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiology* **115,** 971–980.

Cheng M, Hu TC, Layton J, Liu CN, Fry JE. 2003. Desiccation of plant tissues post-*Agrobacterium* infection enhances T-DNA delivery and increases stable transformation efficiency in wheat. *In Vitro Cellular and Developmental Biology – Plant* **39,** 595–604.

Cloutier S, McCallum BD, Loutre C, Banks TW, Wicker T, Feuillet C, Keller B, Jordan, MC. 2007. Leaf rust resistance gene Lr1, isolated from bread wheat (*Triticum aestivum L.*) is a member of the large *psr567* gene family. *Plant Molecular Biology* **65,** 93–106.

Cutler S, Ghassemian M, Bonetta D, Cooney S, McCourt P. 1996. A protein farnesyl transferase involved in abscisic acid signal transduction in *Arabidopsis*. *Science* **273,** 1239–1241.

Dubcovsky J, Dvorak J. 2007. Genome plasticity a key factor in the success of polyploid wheat under domestication. *Science* **316,** 1862–1866.

Ekanayake IJ, De Datta SK, Steponkus PL. 1993. Effect of water deficit stress on diffusive resistance, transpiration, and spikelet dessication of rice (*Oryza sativa L.*). *Annals of Botany* **72,** 73–80.

Fang J, Chu C. 2008. Abscisic acid and the pre-harvest sprouting in cereals. *Plant Signaling and Behavior* **3,** 1046–1048.

Farquhar GD, Richards RA. 1984. Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. *Australian Journal of Plant Physiology* **11,** 539–552.

Farquhar GD, Sharkey TD. 1982. Stomatal conductance and photosynthesis. *Annual Review of Plant Physiology* **33,** 317–345.

Fleury D, Jefferies S, Kuchel H, Langridge P. 2010. Genetic and genomic tools to improve drought tolerance in wheat. *Journal Experimental Botany* **61,** 3211–3222.

Goritschnig S, Weihmann T, Zhang Y, Fobert P, McCourt P, Li X. 2008. A novel role for protein farnesylation in plant innate immunity. *Plant Physiology* **148,** 348–357.

Holzberg S, Brosio P, Gross C, Pogue GP. 2002. Barley stripe mosaic virus-induced gene silencing in a monocot plant. *The Plant Journal* **30,** 315–327.

Hu T, Metz S, Chay C, *et al.* 2003. *Agrobacterium*-mediated large-scale transformation of wheat (*Triticum aestivum* L.) using glyphosate selection. *Plant Cell Reports* **21,** 1010–1019.

Huang X, Madan A. 1999. CAP3: a DNA sequence assembly program. *Genome Research* **9,** 868–877.

Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Hirai N, Koshiba T, Kamiya Y, Nambara E. 2004. The *Arabidopsis* cytochrome P450 CYP707A encodes ABA 8'-hydroxylases: key enzymes in ABA catabolism. *EMBO Journal* **23,** 1647–1656.

Lawrence RJ, Pikaard CS. 2003. Transgene-induced RNA interference: a strategy for overcoming gene redundancy in polyploids to generate loss-of-function mutations. *The Plant Journal* **36,** 114–121.

Lu R, Martin-Hernandez AM, Peart JR, Malcuit I, Baulcombe DC. 2003. Virus-induced gene silencing in plants. *Methods* **30,** 296–303.

Mohammadi M, Kav NNV, Deyholos MK. 2007. Transcriptional profiling of hexaploid wheat (*Triticum aestivum L.*) roots identifies novel, dehydration-responsive genes. *Plant, Cell and Environment* **30,** 630–645.

Pei Z-M, Ghassemian M, Kwak CM, McCourt P, Schroeder JI. 1998. Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. *Science* **282,** 287–290.

Petty IT, Hunter BG, Wei N, Jackson AO. 1989. Infectious barley stripe mosaic virus RNA transcribed in vitro from full-length genomic cDNA clones. *Virology* **171,** 342–349.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29,** e45.

Quintero FJ, Garciadeblas B, Rodriguez-Navarro A. 1996. The *SAL1* gene of *Arabidopsis*, encoding an enzyme with 3'(2'), 5'-bisphosphate nucleotide and inositol polyphosphate 1-phosphatase activities, increases salt tolerance in yeast. *The Plant Cell* **8,** 529–537.

Rossel JB, Cuttriss AJ. Pogson BJ. 2004. Identifying photoprotection mutants in *Arabidopsis. In:* R Carpentier, *ed, Photosynthesis research protocols* . Totowa: Humana Press, pp 287–300.

Rozen S, Skaletsky HJ. 2000. Primer3 on the WWW for general users and for biologist programmers. In: S Krawetz, S Misener, eds, *Bioinformatics methods and protocols: methods in molecular biology* . Totowa, NJ: Humana Press, 365–386.

Saito S, Hirai N, Matsumoto C, Ohigashi H, Ohta D, Sakata, K, Mizutani M. 2004. *Arabidopsis* CYP707As encode (+)-abscisic acid 80-hydroxylase, a key enzyme in the oxidative catabolism of abscisic acid. *Plant Physiology* **134,** 1439–1449.

Schulze ED. 1986. Carbon dioxide and water vapor exchange in response to drought in the atmosphere and in the soil. *Annual Reviews in Plant Physiology and Plant Molecular Biology* **37,** 247–274.

Scofield SR, Huang L, Brandt AS, Gill BS. 2005. Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the lr21-mediated leaf rust resistance pathway. *Plant Physiology* **138,** 2165–2173.

Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y, Shinozaki K. 2001. Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. *The Plant Cell* **13,** 61–72.

Serraj R, Atlin GN. 2009. Drought-resistant rice for increased rainfed production and poverty alleviation: a concept note. In: J Serraj, J Bennett, B Hardy, eds, *Drought frontiers in rice: crop improvement for increased rainfed production* . Singapore: World Scientific Publishing, pp 385–400.

Shah MM, Khalid Q, Khan UW, Shah SAH, Shah SH, Hassan A, Pervez A. 2009. Variation in genotypic responses and biochemical analysis of callus induction in cultivated wheat. *Genetics and Molecular Research* **8,** 783–793.

Slade AJ, Knauf VC. 2005. TILLING moves beyond functional genomics into crop improvement. *Transgenic Research* **14,** 109–115.

Uauy C, Paraiso F, Colasuonno P, Tran RK, Tsai H, Berardi S, Comai L Dubcovsky J. 2009. A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. *BMC Plant Biology* **9,** 115–128.

Umezawa T, Okamoto M, Kushiro T, Nambara E, Oono Y, Seki M, Kobayashi M, Koshiba T, Kamiya Y, Shinozaki K. 2006. CYP707A3, a major ABA 8'-hydroxylase involved in dehydration and rehydration response in *Arabidopsis* thaliana. *The Plant Journal* **46,** 171–182.

Van Eck L, Schultz T, Leach JE, Scofield SR, Peairs FB, Botha AM, Lapitan NL. 2010. Virus-induced gene silencing of *WRKY53* and an inducible phenylalanine ammonia-lyase in wheat reduces aphid resistance. *Plant Biotechnology Journal* **8,** 1023–1032.

Wang Y, Beaith M, Chalifoux M, Ying J, Uchacz T, Sarvas C, Griffiths R, Kuzma M, Wan J, Huang Y. 2009. Shoot-specific

down-regulation of protein farnesyltransferase (α-subunit) for yield protection against drought in canola. *Molecular Plant* **2,** 191–200.

Wang Y, Ying J, Kuzma M, *et al.* 2005. Molecular tailoring of farnesylation for plant drought tolerance and yield protection. *The Plant Journal* **43,** 413–424.

Willems E, **Leyns L**, **Vandesompele J.** 2008. Standardization of real-time PCR gene expression data from independent biological replicates. *Analytical Biochemistry* **379,** 127–129.

Wilson PB, Estavillo GM, Field KJ, *et al.* 2009. The nucleotidase/phosphatase SAL1 is a negative regulator of drought tolerance in *Arabidopsis*. *The Plant Journal* **58,** 299–317.

Zhou H, Li S, Deng Z, *et al.* 2007. Molecular analysis of three new receptor-like kinase genes from hexaploid wheat and evidence for their participation in the wheat hypersensitive response to stripe rust fungus infection. *The Plant Journal* **52,** 420–434.