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 aestivum 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 (BSMV0). In comparison, the Cyp707a-silenced plants showed no improvement over BSMV0-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 Ca2+ 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 Cyp707aβ, 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 Sall, an inositol polyphosphate1-phosphatase-encoding gene. Sall 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). Sall 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 Sall 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; Aprike 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.

Virused-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 Sall 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 Sall sequences in wheat, annotated Arabidopsis Sall (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 Table 1. 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        | AATGAGCGAATGTGAGTAAC  |
|                 |        |               | GTGCTGATCTCGGAACTTG   |
|                 |        |               | TGAAAGCTCTATGTTGGAACA |
| gRT-PCR         |        | 216–307       | GCACACTGAGCTGAGAAAAA  |
|                 |        |               | CCACAGGCGCCAAAAACACT  |
| Sali            | VIGS   | 219–494       | TCTATGTTGCGGAAGAGGA   |
|                 |        |               | GTTACCGGAGATTTCTCC    |
|                 |        |               | CAAAACGAGGATCCATCTGG  |
| gRT-PCR         |        | 104–201       | GTCCTCAACCAATGCTTC    |
|                 |        |               | GTCCCCCAAGGCAATTTTC   |
| Cyp707a         | VIGS   | 368–664       | GGTTGATCTCAAGGAGCAAGAT|
|                 |        |               | GGGGAATCGAAGTGCTTGGGT|
| gRT-PCR         |        | 375–469       | AGGTACCTGACTGGTTGGTT  |

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 transcriptions of each RNA segment were combined in an equimolar ratio and added to an abrasive FES buffer (0.1 M glycine, 0.06 M K2HPO4, 1% w/v tetrasodium pyrophosphate, 1% w/v bentonite, 1½ w/v/cellic, 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, BSMV0, was used as the viral control, and was α, construct consisted of BSMV according to the procedures of Scofield grown in plastic pots holding 500 g of potting mixture. Plants were

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, Sali, 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 (Bio-Rad, 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 BSMV0-treated plants) treatment group and normalized against the level of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample (Pfaffl, 2001).

Estimation of plant water status
To assess the drought stress effects, 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 WUE was calculated using the formula WUE (%) = [(W – DW) – (TW – DW)] × 100, where W is fresh sample weight (g), 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 mol−1 with a red/blue light source and the CO2 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.

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 planted 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 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.

Germination study
Seeds from plants inoculated with BSMV0 during the booting stage were collected 23 days post-inoculation (dpi).
BSMV<sub>v</sub>-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. *andulosa* (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<sup>6</sup> colony-forming units ml<sup>−1</sup> 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<sub>Era1</sub> or BSMV<sub>v</sub>. 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<sub>v</sub>. Plants that were inoculated with BSMV<sub>Era1</sub> 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<sub>Sal1</sub>-inoculated plants (P < 0.0001, Fig. 1A and B). The largest reduction was observed in BSMV<sub>Cyp707a</sub> transgenic lines with an average 22-fold reduction in transcript level compared to water-stressed BSMV<sub>v</sub>-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<sub>v</sub>-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<sub>Era1</sub>- and BSMV<sub>Sal1</sub>-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<sub>Sal1</sub>-treated plants showed further reduction in stomatal conductance relative to that at 7 dpi. A slight reduction was also observed in BSMV<sub>Era1</sub>-treated plants at 14 dpi compared to 7 dpi (Fig. 2). On the other hand, BSMV<sub>Cyp707a</sub>-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<sup>−1</sup>). 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\(\Phi\)-treated plants.
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 BSMV0-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 BSMV0-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 (BSMV0 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 ($P_{D75} = 0.52, P_{S75} = 0.46$).
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
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 plants.
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water-stressed BSMV<sub>0</sub>-inoculated plants, indicating better dry weight production under limiting water condition for BSMV<sub>Era1</sub> and BSMV<sub>Sal1</sub>-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.

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<sub>0</sub>, plants inoculated with empty BSMV viral vector; non-silenced, plants not inoculated with BSMV.
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 \textit{Sal1}.

Interestingly, the effect of silencing \textit{Cyp707a} homologues in wheat was different from previous findings in \textit{Arabidopsis}. Loss of function of \textit{Cyp707a} was reported to enhance drought tolerance in \textit{A. thaliana} (Umewaza \textit{et al.}, 2006). In contrast, \textit{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\textsubscript{t}treated control plants under water stress. The differences in response between \textit{Arabidopsis} and wheat plants to loss of function of \textit{Cyp707a} may be due to differences in stress adaptation pathways between wheat and \textit{Arabidopsis}. Another possible reason for the observed difference may have to do with the structure of the \textit{Cyp707a} gene family in the two species. In \textit{A. thaliana}, the \textit{Cyp707a} gene family consists of \textit{Cyp707a1–Cyp707a4}, all of which are upregulated in response to dehydration and subsequent rehydration, suggesting that the function of \textit{Cyp707a} genes is redundant (Kushiro \textit{et al.}, 2004). Although there is only one \textit{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 \textit{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 \textit{Arabidopsis} gene homologues in wheat.

Out of the three genes tested in this study, \textit{Era1} appeared to be the most promising as a potential target for drought tolerance breeding in wheat. Mutations in \textit{Era1} could be readily identified through TILLING, taking advantage of resources that already exist in wheat (Slade and Knauf, 2005; Uauy \textit{et al.}, 2009). Because of the role of ABA in germination and dormancy, the present study tested \textit{Era1}-silenced plants for germination rates. Indeed, \textit{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 \textit{Era1} has also been suggested to play a role in basal defence against bacteria and oomycetes (Goritschnig \textit{et al.}, 2008), the \textit{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 \textit{Era1}-silenced plants under favourable conditions. It would be interesting to further study the differences in the function of \textit{Era1} in pathogen susceptibility between wheat and \textit{Arabidopsis}.

In conclusion, this study showed the involvement of \textit{Era1} and \textit{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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