OsSFR6 is a functional orthologue of Arabidopsis SENSITIVE TO FREEZING-6 and can act as a regulator of *COR* gene expression, osmotic stress and freezing tolerance in Arabidopsis

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Abbreviations:

SFR6: sensitive to freezing 6

COR: cold-on regulated

DREB: DRE-binding

CBF: C-repeat binding factor

Summary

- The Arabidopsis protein SENSITIVE TO FREEZING 6 (AtSFR6) is required for cold- and drought-inducible expression of *COLD-ON REGULATED (COR)* genes and as a consequence, AtSFR6 is essential for osmotic stress and freezing tolerance in Arabidopsis. Therefore, orthologues of AtSFR6 in crop species represent important candidate targets for future manipulation of stress tolerance. We identified and cloned a homologue of AtSFR6 from rice, OsSFR6, and confirmed its orthology in Arabidopsis.
- OsSFR6 was identified by homology searches, and a full-length coding region isolated using RT-PCR from *Oryza sativa* cDNA. To test for orthology, OsSFR6 was expressed in an Arabidopsis *sfr6* loss-of-function mutant background, and restoration of wild type phenotypes assessed.
- Searching the rice genome revealed a single homologue of *AtSFR6*. Cloning and sequencing the *OsSFR6* coding region showed OsSFR6 to have 69.8% identity and 80.7% similarity to AtSFR6 at the predicted protein sequence level. Expression of OsSFR6 in the *Atsfr6* mutant background restored the wild-type visible phenotype, as well as restoring wild type levels of *COR* gene expression and tolerance of osmotic and freezing stresses.
- OsSFR6 is an orthologue of AtSFR6, and thus a target for future manipulation to improve tolerance to osmotic and other abiotic stresses.

Keywords:

50 SFR6, freezing tolerance, cold acclimation, drought, osmotic stress, germination,
51 rice, Arabidopsis, *COR* genes

Introduction

Freezing of plants in the field can cause significant damage, a major part of which is due to cellular dehydration as a result of water loss from the cell protoplast when extracellular ice forms (Levitt, 1960; Thomashow, 1999). It is perhaps not surprising, therefore, that of the numerous genes whose expression increases in response to low temperature, many are inducible by drought also (Hughes & Dunn, 1996; Thomashow, 1999). In Arabidopsis the *COLD ON-REGULATED* (*COR*) genes represent a major cold-inducible gene regulon (Fowler & Thomashow, 2002); their expression is activated via the C-repeat (CRT) promoter motif or drought-inducible element (DRE) (Yamaguchi-Shinozaki & Shinozaki, 1994). Two distinct families of transcription factors activate *COR* gene expression via the CRT/DRE in Arabidopsis; the C-box binding factors (CBFs) 1-3 (Gilmour *et al.*, 1998), also known as DRE-binding proteins 1A-C (DREB1A-C; (Shinwari *et al.*, 1998)) in response to cold and DREB2A and 2B in response to drought (Liu *et al.*, 1998). A further less closely related member of the CBF family, CBF4, is also involved in drought-, but not cold-inducible *COR* gene expression (Haake *et al.*, 2002). Overexpression of active forms of both families of transcription factor in Arabidopsis leads to tolerance of both drought and frost (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Sakuma *et al.*, 2006).

The CRT/DRE motif is utilised in the control of gene expression in response to cold and drought in several crop species, including rice (Dubouzet *et al.*, 2003; Ito *et al.*, 2006). Overexpression of CBF/DREB1 transcription factors, both native and heterologous, has been shown to induce native crop *COR* genes, and lead to osmotic stress tolerance in these species (Jaglo *et al.*, 2001; Dubouzet

et al., 2003; Gao *et al.*, 2009). Interestingly, CBF transcription factors have been identified in chilling-sensitive species such as tomato, which are not able to achieve freezing tolerance (Jaglo *et al.*, 2001; Hsieh *et al.*, 2002a; Hsieh *et al.*, 2002b; Zhang *et al.*, 2004). In these cases it appears that CBF transcription factors, and the CRT/DRE motif are involved in inducing genes required for both drought and chilling tolerance (Jaglo *et al.*, 2001; Hsieh *et al.*, 2002a; Hsieh *et al.*, 2002b; Zhang *et al.*, 2004). Manipulating the expression and function of these transcription factors, therefore, has led to the possibility of engineering altered tolerance not only to desiccation stresses such as freezing and drought, but also to chilling.

We have recently described the cloning of *SENSITIVE TO FREEZING-6* (*AtSFR6*); a protein that regulates CBF/DREB-dependent *COR* gene expression in Arabidopsis (Knight *et al.*, 2009). Our previous work has shown that AtSFR6 is needed for induction of *COR* genes in response to both cold and osmotic stresses and that is it required for tolerance to osmotic stress and the acquisition of freezing tolerance (Knight *et al.*, 1999; Boyce *et al.*, 2003; Knight *et al.*, 2009). In the case of cold at least, SFR6 acts post-translationally of the transcription factors that activate *COR* genes via the CRT/DRE motif (Knight *et al.*, 2009). Orthologues of AtSFR6 in crop species are therefore obvious candidate targets for manipulation of osmotic stress tolerance. The first step towards such a long-term goal is to demonstrate that functional orthologues of AtSFR6 exist in crop plants. Here we describe the identification of a homologue of AtSFR6 in rice, its cloning and sequencing, and demonstrate orthology through genetic complementation.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. (*A. thaliana*) ecotype Columbia (Col-0) was obtained from Lehle Seeds (Round Rock, Texas, USA). The Arabidopsis mutant, *sfr6-1*, also in Col-0 background has been described previously (Knight *et al.*, 1999; Boyce *et al.*, 2003; Knight *et al.*, 2009). Rice (*Oryza sativa* L.) seedlings of *cv*. Japonica *var*. Lemont (Herbiseed, Twyford, UK) was used for extraction of mRNA for cloning OsSFR6. Plants were grown in a SANYO MLR351 growth chamber (Sanyo E&E Europe BV, Loughborough, UK) under a 16:8 h light:dark cycle at 150 μmol m^{-2} s^{-1} at 20°C +/- 1°C unless stated otherwise. The cold treatments used in gene expression experiments were carried out in the growth chambers described above set to 4°C. Osmotic stress-induced gene expression was measured in plants floated on 350 mM mannitol solutions in transparent plastic cell culture dishes in the same growth chambers set to 20°C. All samples were harvested after 6h of treatment.

Cloning OsSFR6 and production of overexpression construct

Total RNA was extracted from rice leaf tissue using RNeasy Plant Total RNA Kit (Qiagen, Hildon, UK), following the manufacturer's instructions. Total plant RNA (5 μg) was annealed to 0.5 μg oligo dT primer (Fermentas, York, UK) and reverse-transcribed at 42°C for 60 min using 200 units of H minus M-MuLV Reverse Transcriptase (Fermentas) according to the manufacturer's instructions. The full-length OsSFR6 coding sequence (3510 bp) was PCR amplified from the cDNA produced using the following primers: 5'-CCGGTACCCCCGGGGATGCGCGTGCCCGAGCTCTGCAGGAACTT-3'

(Forward) and 5'-GGGCGGGGGCGGCCGATCCCGTCAAATTCAAACGACTTTCAC-3' (Reverse). Amplification was performed with Phusion DNA polymerase (Finnzymes, Keilaranta, Finland) according to the manufacturer's instructions. The *OsSFR6* coding sequence was cloned into the pENTR1A gateway entry vector (Invitrogen, Paisley, UK) using the *Kpn*1 and *Not*1 sites and sequenced. The full-length *OsSFR6* coding sequence was transferred by LR recombination from pENTR1A into the pB7WG2 gateway binary destination vector (Karimi *et al.*, 2002), which contains the cauliflower mosaic (CaMV) 35S promoter upstream. For comparison, the full-length *AtSFR6* genomic coding sequence (Knight *et al.*, 2009) was cloned into the same binary vector.

Plant transformation

Binary vectors containing *35S::AtSFR6* and *35S::OsSFR6* were introduced into *A. tumefaciens* C58C1 and transformed into Col-0 and *sfr6-1* mutant using the floral dip method (Clough & Bent, 1998). Primary T_1 transformants were identified by glufosinate ammonium (Basta; 250 mg/l) selection (Bayer Crop Science, Cambridge, UK) on soil. Subsequent analyses were performed on the T_2 generation.

Quantitative Real Time PCR

A High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA) was used to reverse transcribe cDNA from 1.5 μg total RNA extracted using Qiagen RNeasy plant mini kit (described above) in conjunction with RNAse-free DNase (Qiagen) to remove any genomic DNA contamination.

Quantitative real time PCR (qRT-PCR) was performed on 10 µl of 1:50 diluted cDNA reaction in a 25-µl reaction using an Applied Biosystems 7300 system. Relative transcript levels were measured using gene-specific TAQMAN© probes purchased from Applied Biosystems for *AtSFR6* (At4g04920; probe identifier At02209654_g1), *KIN2* (At5g15970; At02354775_s1) and *LTI78* (At5g52310; At02320470_g1) and expression levels were normalised to the expression of *PEX4* (At4g25760; At02304594_g1), an endogenous control gene. A custom-made TaqMan Probe was prepared for *OsSFR6* by Applied Biosystems to the following specifications: Forward primer, CGGTGGTGACTAAGTGGTTGTC; reverse primer, GTACTAGAGTTTGCAGGAAGCCAT; FAM-labelled probe, CTATACCGGAGAAATTC. Reactions were performed in an optical 96-well plate (Applied Biosystems) with 3 technical replicates for each sample. In all cases, relative quantitation was performed by the $\Delta\Delta C_T$ (comparative C_T) method (Livak & Schmittgen, 2001) and Relative Quantitation (RQ) values and estimates of statistical variation (SV) for each sample calculated as described previously (Knight *et al.*, 2009). The algorithm used is described in Relative Quantitation (RQ) algorithms, Applied Biosystems Real-Time PCR Systems Software, July 2007.

Freezing assays

To test complementation with AtSFR6 seven-day-old seedlings (grown as described above) were transferred to peat plugs and maintained for 5 weeks in a growth chamber (Arctic plant growth chamber A3655, Weiss Gallenkamp Ltd., Loughborough, UK) programmed for short day conditions (8:16 h light: dark cycle), 20^{o}C+/-0.5^{o}C, 60 % relative humidity and 150 µmol m^{-2} s^{-1} light level.

Experiments to test complementation with OsSFR6 were performed on plants grown under comparable conditions using a SANYO MLR351 chamber. Cold acclimation in both cases was performed under the same day length and light levels at 4^{o}C for 11 days. The temperature was subsequently reduced to below freezing (-6.5, -7.5 and -8.5^{o}C) for 24 h, then returned to ambient levels. The temperature increases and decreases were achieved by ramping over 3 h.

Osmotic stress tolerance

Osmotic stress tolerance was assessed in seedlings as we have described previously (Knight *et al.*, 1998; Boyce *et al.*, 2003). Eight-day old seedlings grown under the conditions described above were floated on 2 ml of water, 330 mM, 440 mM or 550 mM mannitol (BDH, Poole, UK), in a transparent 24-well culture plate. Five seedlings were added to each well. The plate was sealed with micropore tape and returned to the growth chamber for 72 h before photographing.

Sensitivity of germination to osmotic stress was assessed as described previously (Boyce *et al.*, 2003). Seeds were sown on solid MS medium containing different levels of osmoticum (0, 200, 300 or 400 mM mannitol) and 0.8% agar at pH5.8. Seeds of each line to be tested were sown at a density of approximately 30-80 seeds per 55-cm diameter petri dish, with 6 replicate petri dishes for each line/ treatment. Seeds were stratified on the agar plates at 4°C for 4 days and transferred to standard growth chamber conditions for 7 days. Germination was scored on the basis of radicle emergence.

Statistical Inference

For each osmoticum treatment and plant line pairing we estimated the probability of seed germination using maximum likelihood. To account correctly for potential unknown variation among plates (e.g. subtle variations in the dryness of agar) and differing numbers of seeds per plate, we assumed that the variation in our data between plates for each treatment could be well described by a beta-binomial distribution. The log-likelihood equation we maximised when estimating each probability and details regarding fitting can be found in Richards (Richards, 2008). Uncertainty in these probabilities was estimated using the profile-likelihood approach (Venzon & Moolgavkar, 1988).

To explain any potential patterns in our wild type Col-0 and *sfr6*-1 data (i.e. variation in germination among osmoticum treatments), we proposed that the relationship between the probability of seed germination (p) and the osmoticum concentration (x) could be described by

$$p(x) = \frac{\exp(\beta_0 + \beta_1 x^{\alpha_1})}{1 + \exp(\beta_0 + \beta_1 x^{\alpha_1})}.$$

This relationship is a modified form of the commonly adopted logistic equation; however, the x-axis has also been scaled by the positive parameter α_1. The parameter β_1 describes the strength at which osmoticum concentration affects germination success; here a negative value indicates that germination declines with increasing osmoticum strength. We proposed that germination success for *sfr6-1* was potentially affected by osmoticum concentration in a similar manner, but also allowed germination to be affected by the level of *OsSFR6* transcript in each complemented line. In this case, if *OsSFR6* transcript level was y, then germination success of the mutant was predicted to be

$$p(x,y) = \frac{\exp(\beta_0 + \beta_1 x^{\alpha_1} + \beta_2 y^{\alpha_2})}{1 + \exp(\beta_0 + \beta_1 x^{\alpha_1} + \beta_2 y^{\alpha_2})}. \quad (1)$$

For this model, a positive value of β_2 indicates that an increase in *OsSFR6* transcript level increased germination success. Model parameters (i.e., the α_i and the β_i) were fitted to the data using maximum likelihood, and we again assumed that our data were consistent with a beta-binomial distribution. In all cases, when we checked our fits we found that our residuals were as expected.

To look for evidence that *sfr6-1* and Col-0 wild type differed in their response to elevated levels of osmoticum, we performed a likelihood ratio test (LRT; (Sokal & Rohlf, 1994)). In this case, the null model assumed that model parameters β_0, β_1, and α_1 were identical for both lines; whereas, the alternative model assumed that the β_0, β_1, and α_1 had to be estimated separately for each line. Evidence that *OsSFR6* transcript level affected the complemented mutant's response to osmoticum was also investigated using a LRT. Specifically, the null model assumed that transcript level did not affect germination success (by setting $\beta_2 = 0$ and fitting β_0, β_1, and α_1); whereas, the alternative model also allowed α_2 and β_2 to vary). Finally, we used a LRT to look for evidence that the highest *OsSFR6*-expressing line differed in its response to osmoticum with respect to the wild type. This last test was identical to the first mentioned LRT test, except that we replaced the non-complemented mutant with the highest *OsSFR6*-expressing line ($y = 6.85$).

Results

We have recently cloned the *AtSFR6* gene (At4g04920) from Arabidopsis (Knight *et al.*, 2009). This gene controls freezing and osmotic stress tolerance in Arabidopsis (Knight *et al.*, 1999; Boyce *et al.*, 2003; Knight *et al.*, 2009). We

sought, therefore, to identify orthologues of *AtSFR6* from crop species, as potential targets for future manipulation of crop stress tolerance. Using homology searches, we found a single gene in the rice genome (Os10g35560) that showed strong homology to *AtSFR6*. We named this gene *OsSFR6*. Having identified the gene, we cloned and sequenced the full length coding region from cDNA derived from rice mRNA. Fig. 1 shows a line up of the predicted protein sequence of OsSFR6 with AtSFR6. When comparing the whole sequences, there is 72% protein identity between AtSFR6 and OsSFR6. *OsSFR6* encodes a predicted protein of 1170 amino acids (the length of AtSFR6 protein is 1268; (Knight *et al.*, 2009)).

To establish whether *OsSFR6* is an orthologue of *AtSFR6*, we tested complementation of the Arabidopsis *sfr6-1* mutant (Knight *et al.*, 2009). Previously, we had used three mutant alleles of *AtSFR6* to prove linkage of AtSFR6 to the phenotypes of freezing-sensitivity, pale cotyledons and leaves and large cotyledons but complementation had not been attempted. Therefore before testing the effect of *OsSFR6* expression in an *sfr6* mutant background, we tested whether *AtSFR6* itself expressed under the control of the 35S promoter was capable of complementing the visible *sfr6-1* mutant phenotype. Fig. 2a shows four independent 35S::AtSFR6 lines in the *sfr6-1* background (lower row). These all showed complementation of the visible pale leaf and cotyledon phenotype. This complementation was not apparent in 35S::GUS controls in the *sfr6-1* background (Fig. 2a,b). Similarly, expression of *35S::OsSFR6* in the *sfr6-1* background resulted in complementation of the visible phenotype (Fig. 3). However, in contrast to complementation with *AtSFR6*, *OsSFR6* complemented to different extents in different lines. Fig. 3b shows one line, #8, with relatively

276 weak complementation compared to another line, #10, which showed strong
277 complementation.

278 We have previously shown that *sfr6* mutants of Arabidopsis are unable to
279 acclimate to freezing, as a result of reduced cold-induced *COR* gene expression
280 (Knight *et al.*, 1999; Boyce *et al.*, 2003; Knight *et al.*, 2009). Therefore to test if
281 the reduced *COR* gene expression phenotype could also be complemented with
282 *AtSFR6* we tested expression of *AtKIN2*, a typical *COR* gene, which shows
283 reduced expression in *sfr6* mutants following cold treatment (Knight *et al.*, 1999;
284 Boyce *et al.*, 2003; Knight *et al.*, 2009). As can be seen in Fig. 4, whilst the *sfr6-*
285 *1* mutant showed low levels of *AtKIN2* expression in the cold compared to wild
286 type Columbia (as reported previously; (Knight *et al.*, 1999; Boyce *et al.*, 2003;
287 Knight *et al.*, 2009)), three lines complemented with *AtSFR6* showed levels of
288 *KIN2* expression comparable to wild type (Fig. 4a). These three lines, #1, #2 and
289 #6 were chosen as they showed medium, low and high levels of *AtSFR6*
290 expression, respectively (Fig. 4b). Interestingly, differences in *AtSFR6* expression
291 did not result in different levels of *AtKIN2* expression (Fig. 4a).

292 To test whether *OsSFR6* also was capable of complementing the low
293 *AtKIN2* expression phenotype, we tested six *sfr6-1* lines complemented with
294 *OsSFR6*. As can be seen in Fig. 5, these six lines showed a range of *OsSFR6*
295 expression levels: there was an approximately six-fold difference between the
296 lowest (line #8) and the highest level (line #10). We therefore tested both of these
297 lines, and a third line expressing *OsSFR6* to intermediate levels (line #19) for
298 *COR* gene expression in the cold. Fig. 6 shows the expression of *COR* genes
299 *AtKIN2* and *AtLTI78* in these three lines. *AtKIN2* and *AtLTI7*8 expression was

300 significantly lower in line #8 than line #10. Line #19 showed slightly reduced
301 *COR* gene expression, but not significantly, when compared to line #10 (Fig. 6).

302 Given the complementation of the *COR* gene expression phenotype, we
303 tested whether this would also lead to restoration of freezing tolerance. Fig. 7
304 shows that the three lines of *sfr6-1* complemented with AtSFR6 that were tested
305 for *COR* gene expression all showed freezing tolerance comparable to that of wild
306 type (Fig. 7a). In a separate experiment, the three lines of *sfr6-1* complemented
307 with *OsSFR6* showed visible symptoms consistent with variable levels of freezing
308 tolerance: line #8 appearing indistinguishable from the original *sfr6-1* mutant, and
309 lines #10 and #19 showing tolerance comparable to wild type (Fig. 7b).

310 We have shown previously that AtSFR6 is a regulator of both osmotic
311 stress and low temperature responses (Knight *et al.*, 1999; Boyce *et al.*, 2003). To
312 assess whether OsSFR6 is a potential regulator of osmotic stress responses also,
313 we examined the ability of OsSFR6 to restore osmotic stress responsiveness and
314 tolerance in the three complemented lines. Transcript levels of the *COR* genes
315 *AtKIN2* and *AtLTI78* were measured in response to a 6-h treatment with 350 mM
316 mannitol. As expected, the treatment strongly induced both genes in Col-0 wild
317 type plants, with a reduced response seen in *sfr6-1* (Fig. 8). Varying degrees of
318 restoration of the response were seen in the three complemented lines; little or no
319 effect was observed with the lowest expresser, line #8, whilst *AtLTI78* and
320 *AtKIN2* transcript levels in lines #10 and #19 were restored almost to wild type
321 levels (Fig. 8).

322 To examine whether this restoration of osmotically-induced *COR* gene
323 expression was accompanied by a return to wild type levels of osmotic stress
324 tolerance, we performed two assessments. We showed previously that *sfr6-1* is

325 sensitive to osmotic stress at both the germination and seedling stages (Boyce *et*
326 *al.*, 2003). Therefore we tested the ability of the three OsSFR6 complemented
327 lines to tolerate a range of mannitol concentrations. Seedlings were floated on
328 mannitol (0, 330, 440 and 550 mM) for 72 h in a standard 16h:8h light:dark cycle
329 and examined for signs of osmotic stress-induced chlorosis after this time.
330 Seedlings of each line maintained in water showed no signs of damage (Fig. 9).
331 Wild type plants showed slight signs of chlorosis with the 330 mM treatment,
332 becoming more severe at 440 mM, whilst *sfr6-1* was clearly more susceptible,
333 showing some signs of chlorosis even at 220 mM and becoming severe at 330
334 mM. In complemented line #8, only very minor improvements in osmotic stress
335 tolerance were observed; in lines #10 and #19, tolerance was restored to levels
336 similar to wild type (Fig. 9).

337 Seeds sown on agar plates containing 0, 200, 300 or 400 mM mannitol
338 were used to assess the effects of elevated levels of osmoticum on germination
339 success. This assay allowed us to make a quantitative assessment of the effects of
340 expressing OsSFR6 to different levels in *sfr6-1*. Small reductions in the
341 percentage of wild type Col-0 seeds germinating were observed with each
342 increase in mannitol concentration; germination rate fell from close to 100% to
343 approximately 70% in wild type plants when mannitol concentration was raised
344 from 0 to 400 mM. As reported previously, *sfr6-1* seed germination was more
345 sensitive to the high osmoticum levels; germination fell to only 38% at 300 mM
346 and to below 20% at 400 mM (Fig. 10a). Our analysis confirmed that germination
347 success on elevated levels of osmoticum differed significantly between Col-0 wild
348 type and *sfr6-1* (LRT; $G_4 = 73.2$, $P < 0.001$). For both lines germination success
349 was reduced as osmoticum concentration increased; however, for any given level

of osmoticum, germination frequency was always higher for the wild type (Fig. 10a).

When comparing the behaviour of the 3 complemented lines with non-complemented *sfr6-1* we also found significant evidence that the level of *OsSFR6* transcripts (see Fig. 5) affected germination success in *sfr6-1* lines transformed with 35S::OsSFR6 (LRT; $G_2 = 53.7$, $P < 0.001$). Specifically, an increase in *OsSFR6* transcript level increased germination success across all levels of osmoticum investigated (Fig. 10b). The complemented line associated with the highest *OsSFR6* transcript level (line #10; $y = 6.85$) exhibited a significantly higher germination success rate compared with wild type Col-0 (LRT; $G_4 = 40.9$, $P < 0.001$). In fact, for all four levels of osmoticum, this line showed higher germination success than the wild type (c.f. Figs. 10a and 10b). Interestingly, the fits suggest that the rate of reduction in germination success with increased osmoticum may be less for the wild type (Fig.10).

Discussion

Identification of plant genes that contribute to environmental stress tolerance is vital for crop breeding if food security is to be maintained for a rapidly growing human population in an increasingly unpredictable climate. Previous work has identified a number of genes that contribute to these traits in plants, but arguably the most significant discoveries have been key regulators, for instance, transcription factors. Such genes encode master-regulators that control the expression of many other genes involved in a particular trait, and thus their effect individually is profound. Good examples of these are the CBF/DREB1

375 (Stockinger *et al.*, 1997; Jaglo-Ottosen *et al.*, 1998) and DREB2 (Liu *et al.*, 1998;
376 Sakuma *et al.*, 2006) transcription factors, originally identified in Arabidopsis but
377 which exist in rice also (Dubouzet *et al.*, 2003; Ito *et al.*, 2006; Matsukura *et al.*,
378 2010). The CBF/DREB1 and DREB2 transcription factors regulate the
379 expression of so-called *COLD ON-REGULATED (COR*) genes via a single
380 promoter motif, the DRE/CRT (Yamaguchi-Shinozaki & Shinozaki, 1994), in
381 response to low temperature and osmotic stress, respectively.

382 Our previous work showed that CBF/DREB1- and DREB2-dependent
383 stress gene expression in Arabidopsis requires AtSFR6 (Knight *et al.*, 1999;
384 Boyce *et al.*, 2003; Knight *et al.*, 2009). Loss of function *sfr6* mutants of
385 Arabidopsis show reduced expression of genes controlled by the DREB
386 transcription factors in response to either osmotic stress or cold. As a result, *atsfr6*
387 mutants are unable to mount the correct defence against these conditions and are
388 sensitive to both dehydration and freezing. Thus, SFR6 is a hub regulating at least
389 2 transcription factor systems in Arabidopsis, affecting two overlapping gene
390 regulons leading to freezing and osmotic stress tolerance. Orthologues of AtSFR6
391 in crop species, therefore, represent good targets for future breeding or
392 manipulation. With this in mind, we identified a rice homologue of AtSFR6 and,
393 through testing its function, confirmed it as a orthologue.

394 Examination of the rice genome revealed a sole gene (Os10g35560)
395 showing any significant homology to *AtSFR6*. We named this gene *OsSFR6*.
396 AtSFR6 also exists as a single copy gene in Arabidopsis. Empirical determination
397 of the coding region of *OsSFR6* showed that the predicted coding region had high
398 homology to AtSFR6 (69.8% identity and 80.7% similarity at the predicted
399 protein sequence level: Fig. 1). Interestingly, the N-terminal half of the predicted

OsSFR6 protein sequence was more highly conserved than the C-terminal half. In Arabidopsis, 3 mutations in the N-terminal third of AtSFR6 strongly affect phenotype (Knight *et al.*, 2009). Thus is seems likely that the N-terminal part of AtSFR6 and OsSFR6 are important for their function.

Having identified a potential orthologue of AtSFR6, we sought to test for orthology by functional complementation of an Arabidopsis *atsfr6* mutant. Firstly it was necessary to demonstrate that this was a viable approach, therefore we tested complementation with Arabidopsis AtSFR6 itself. Expressing AtSFR6 using a 35S CaMV constitutive promoter in an *atsfr6* background fully restored the ability to induce *COR* gene expression in response to cold, and also to allow cold acclimation and acquisition of freezing tolerance (Figs. 4 and 7). It is most likely that the restoration of cold acclimation is as a direct consequence of the restoration of full levels of *COR* gene expression: up-regulation of *COR* gene expression by overexpression of CBF/DREB1 transcription factors at ambient temperature is sufficient to induce freezing tolerance (Jaglo-Ottosen *et al.*, 1998).

Having established a system for functional testing of SFR6 orthologues by complementation, we used this approach with the coding region of *OsSFR6*. OsSFR6, like AtSFR6, was able to restore both cold-induced *COR* gene expression and acquisition of freezing tolerance (Figs. 6 and 7). However, wild type levels of *COR* gene expression and freezing tolerance were only achieved in the highest OsSFR6 expressing lines (lines #10 and #19); poor levels of complementation were observed in the low (line #8) level expresser (Figs. 6 and 7).

The experiments above demonstrated that OsSFR6 (from rice, a species incapable of freezing tolerance) can act as a functional orthologue of AtSFR6 in

425 the acquisition of freezing tolerance in Arabidopsis. Osmotic stress is a major
426 component of freezing stress, and in accordance with this, the targets of
427 CBF/DREB1 and DREB2 transcription factors overlap substantially.
428 Overexpression of both CBF/DREB1 (Jaglo-Ottosen *et al.*, 1998) and
429 constitutively active forms of DREB2 (Sakuma *et al.*, 2006) lead to elevated
430 levels of *COR* gene expression and to both freezing and osmotic stress tolerance.
431 It appeared likely, therefore, that the role of OsSFR6 in rice is to facilitate
432 tolerance to osmotic rather than freezing stress. To test this possibility, we
433 examined *COR* gene expression and sensitivity to osmotic stress conditions in
434 *sfr6-1* lines overexpressing OsSFR6. Osmotic stress-inducible *COR* gene
435 expression and tolerance of elevated osmoticum levels at seedling and
436 germination stages were all complemented in *sfr6-1* lines expressing
437 35S::OsSFR6 (Figs. 8-10).

438 When we modelled our quantitative germination data, our best fitting
439 model demonstrated a significant increase in germination success with increasing
440 transcript levels in *sfr6-1* lines expressing 35S::OsSFR6 (Fig. 10b). *OsSFR6*
441 transcript levels are likely to be a predictor of protein levels (although the
442 relationship between the two cannot be assumed to be linear). Therefore our data
443 strongly suggest that the degree of restoration of wild type phenotype in *sfr6-1* is
444 positively correlated with the level of OsSFR6 protein expression. This is similar
445 to the trend we saw in the qualitative assessments of freezing and osmotic stress
446 tolerance (Figs. 7 and 9), and our measurements of *COR* gene expression (Figs. 6
447 and 8). Interestingly, only in the case of germination did we observe indications
448 that expressing OsSFR6 to higher levels can actually supersede wild type levels of

449 tolerance (Fig. 10). This result might suggest a significant role for SFR6 in
450 osmotic stress tolerance in the germinating seed.

451 The fact that OsSFR6 appears to fully complement Arabidopsis *sfr6* loss
452 of function mutants only when expressed at relatively high levels, whilst all levels
453 of AtSFR6 overexpression resulted in complementation, could be interpreted as
454 differences in protein sequence between the two orthologues producing proteins
455 with different efficiencies. However, our quantitation of *SFR6* transcripts was
456 relative; comparison of absolute levels of *OsSFR6* with *AtSFR6* cannot be made
457 from our data. Furthermore, irrespective of whether or not *OsSFR6* and *AtSFR6*
458 transcripts were expressed to similar levels, we cannot rule out the possibility of
459 substantial differences in the levels of expressed OsSFR6 and AtSFR6 proteins in
460 our complemented lines and that these differences account for the dose-dependent
461 effect we see with OsSFR6 complementation. In either scenario, it can still be
462 concluded that OsSFR6 is a functional equivalent (orthologue) of AtSFR6. As
463 OsSFR6 affects osmotic stress-responsive *COR* gene expression and tolerance in
464 Arabidopsis it is very likely that OsSFR6 plays a role in tolerance of rice to
465 osmotic stress during periods of low water availability.

466 Our data demonstrate that OsSFR6 is a potential target for breeding or
467 manipulation to achieve increased abiotic stress tolerance in rice. Given that
468 OsSFR6 is functionally equivalent to AtSFR6, it is most likely that homologues
469 from other crops will be orthologues also, and thus be equally valuable targets.
470 The most obvious avenue to explore in the exploitation of SFR6 would be to
471 increase levels of its production in crop species; however, we have observed that
472 overexpression of AtSFR6 in wild type Arabidopsis does not lead to enhanced
473 expression of *COR* genes in response to cold (Supporting information Fig. S1), or

enhanced freezing tolerance (data not shown). In addition to the implications this has on the use of SFR6 in future crop protection strategies, this result gives an insight into the possible mode of action of the protein. Because increasing the titre of AtSFR6 protein has no effect *in vivo*, we surmised that SFR6 is likely to work in conjunction with other proteins in stoichiometric proportions, as part of a complex. If this were the case, elevating SFR6 levels in the absence of increases in the amounts of these other proteins would not be expected to enhance *COR* gene expression.

This hypothesis has now been proven correct, with the identification of At4g04920 (AtSFR6) as the gene that encodes the Arabidopsis homologue of yeast MED16, part of the mediator complex (Bäckström *et al.*, 2007). Mediator is a multi-subunit transcriptional co-activator complex that acts as a bridge between DNA-bound transcriptional regulators and the general RNA polymerase II transcriptional machinery. MED16 is one of the so-called "tail" subunits of mediator, whose functions are considered to be directly involved with transcription factor recruitment (Casamassimi & Napoli, 2007). Yeast MED16 (SIN4) (Li *et al.*, 1995) and drosophila MED16 orthologues (Kim *et al.*, 2004) have demonstrated roles in facilitating transcriptional activation by transcription factors. If the stoichiometry of mediator subunits remains constant, simple overexpression of OsSFR6 in rice or orthologues in other crop species is unlikely to result in enhanced stress tolerance. However, the ability of OsSFR6 to elevate *atsfr6-1* germination rates on high levels of osmoticum to above wild type levels does raise the possibility that orthologues from different species may have differing effectiveness in some cases. In the main, however, future exploitation of SFR6 in rice or other crop species is likely to necessitate engineering the protein

sequence to improve efficiency. Identification of transcription factor binding sites in SFR6 and tailoring these to optimise transcription factor binding may be one approach that could be adopted. This will be the focus for our future work in this area.

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Supporting information

Supporting information Fig. S1.

Expression of *AtSFR6* and *AtKIN2* in Arabidopsis lines transformed with 35S::SFR6.

Figure Legends

Figure 1

Alignment of predicted AtSFR6 and OsSFR6 protein sequences. Alignment was produced using Vector NTI software http://www.invitrogen.com. Identity and similarity of amino acid sequences are indicated in dark and light coloured boxes respectively. Gaps in the amino acid sequences are indicated by ".".

Figure 2

Visible phenotype of the *sfr6-1* mutant is restored by expression of 35S::AtSFR6. Col-0, *sfr6-1* and T_2 progeny of *sfr6-1* plants transformed to express either *35S::AtSFR6* (four independent transformed lines shown) or *35S::GUS* (two independent transformed lines shown) were grown on full-strength MS agar plates. **(a)** seedlings 10 days after germination. **(b)** Close-up of Col-0, *sfr6-1, sfr6-1* expressing *35S*::*GUS* and *sfr6-1* expressing *35S*::*AtSFR6.* Scale bars represent 10 mm throughout.

Figure 3

Visible phenotype of the *sfr6-1* mutant is restored by expression of 35S::OsSFR6. T_2 progeny of *sfr6-1* plants expressing *35S::OsSFR6* (6 independent transformed lines shown) alongside Col-0 and *sfr6-1* mutant for comparison. **(a)** Seedlings ten days after germination. **(b)** Close up Col-0, *sfr6-1* and *sfr6-1* expressing *35S*::*OsSFR6.* Scale bars represent 10 mm throughout.

Figure 4

AtKIN2 expression is restored in the *sfr6-1* mutant by complementing with *AtSFR6*. Seven-day-old seedlings were subjected to cold treatments at 4^{o}C for 6

h. **(a)** Relative quantitation (RQ) values for *KIN2* expression in 3 complemented lines relative to Col-0. **(b)** Relative quantitation (RQ) values for *AtSFR6* expression in 3 complemented lines relative to Col-0. Expression of *AtKIN2* and *SFR6* was normalised to expression values for ß-*TUBULIN4* (endogenous control). Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

Figure 5

Expression levels of *OsSFR6* in *sfr6-1* transformed with *35S::OsSFR6*. Relative quantitation (RQ) values of *OsSFR6* expression in seven-day-old Arabidopsis seedlings from six independently transformed lines is presented relative to the level of *OsSFR6* expression in line #20. *OsSFR6* expression was normalised to expression values for *PEX4* (endogenous control). Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

Figure 6

Cold-inducible *AtKIN2* and *AtLTI78* expression is restored in the *sfr6-1* mutant by expressing 35S::*OsSFR6*. Seven-day-old seedlings from three independently transformed lines (low, medium and high levels of OsSFR6 expression) were subjected to cold treatments at $4^{o}C$ for 6 h alongside Col-0 and untransformed *sfr6-1*. Data represented here are relative quantitation (RQ) values of gene expression in the transformed lines relative to cold treated Col-0. *OsSFR6* expression was normalised to expression values for *PEX4* (endogenous control).

680 Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and
681 RQ_{MAX} constitute the acceptable error for a 95% confidence limit according to
682 Student's *t* test. **(a)** *AtKIN2*; **(b)** *AtLTI78*.
683
684 **Figure 7**
685 Freezing tolerance is restored in the *sfr6-1* mutant by expression of either
686 35S::*AtSFR6* or 35S::*OsSFR6*. Five-week-old plants were cold acclimated at 4°C
687 for 11 days under short day conditions before exposure to either -6.5, -7.5 or -
688 8.5°C for 24 h and then returned to 20°C. Photographs were taken 5 days after
689 returning to 20°C. **(a)** Freezing tolerance of Col-0, *sfr6-1*, *sfr6-1* expressing
690 *35S::GUS* and *sfr6-1* expressing *35S::AtSFR6* (Lines #1, #2 and #6). **(b)** Freezing
691 tolerance of Col-0, *sfr6-1* and *sfr6-1* expressing *35S::Os SFR6* (lines #8, #10 and
692 #19). Figures (a) and (b) depict 2 separate experiments and should not be
693 compared. Scale bars represent 50 mm throughout.
694
695 **Figure 8**
696 Osmotic stress-inducible *AtKIN2* and *AtLTI78* expression is restored in the *sfr6-1*
697 mutant by expression of 35S::OsSFR6. Seven-day-old seedlings from three
698 independently transformed lines (low, medium and high levels of OsSFR6
699 expression) were floated on water (white bars) or 350 mM mannitol (grey bars) at
700 20°C for 6 h alongside Col-0 and untransformed *sfr6-1*. Data represented here are
701 relative quantitation (RQ) values of gene expression in the transformed lines
702 relative to water-treated Col-0. *OsSFR6* expression was normalised to expression
703 values for *PEX4* (endogenous control). Each value is the mean of three technical

replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} constitute the acceptable error for a 95% confidence limit according to Student's *t* test. **(a)** *AtKIN2*; **(b)** *AtLTI78*.

Figure 9

Osmotic stress tolerance is restored in *sfr6-1* mutant seedlings by expression of 35S::*OsSFR6*. Five 8-day-old seedlings from three independently transformed lines (low, medium and high levels of *OsSFR6* expression) were transferred to a multi-well culture dish into wells containing water, 330, 440 or 550 mM mannitol and maintained at 20^{o}C for 72 h alongside Col-0 and *sfr6-1*. The scale bar represents 10 mm.

Figure 10

Expression of 35s::OsSFR6 in *sfr6-1* restores the ability to germinate on high concentrations of osmoticum (a) Observed and best-fit model predictions of seed germination over a range of mannitol (osmoticum) concentrations for wild type Col-0 and *sfr6-1*. (b) Observed and predicted effect of *OsSFR6* transcript expression level, using equation (1), on germination success of OsSFR6 complemented *sfr6-1* lines #8 (1), #19 (3.67) and #10 (6.85). Numbers in parentheses refer to relative *OsSFR6* expression levels in each line, and increasing levels of shading indicate increased transcript levels. *sfr6-1* is shown for comparison. In both panels the lines represent maximum-likelihood model fits to the data, and the error bars represent estimated 95% confidence intervals for the probability of seed germination, based on the data in each line-treatment pairing.