Stress tolerance to stress escape in plants: role of the OXS2 zinc-finger transcription factor family

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During dire conditions, the channelling of resources into reproduction ensures species preservation. This strategy of survival through the next generation is particularly important for plants that are unable to escape their environment but can produce hardy seeds. Here, we describe the multiple roles of OXIDATIVE STRESS 2 (OXS2) in maintaining vegetative growth, activating stress tolerance, or entering into stress-induced reproduction. In the absence of stress, OXS2 is cytoplasmic and is needed for vegetative growth; in its absence, the plant flowers earlier. Upon stress, OXS2 is nuclear and is needed for stress tolerance; in its absence, the plant is stress sensitive. OXS2 can activate its own gene and those of floral integrator genes, with direct binding to the floral integrator promoter *SOC1*. Stress-induced *SOC1* expression and stress-induced flowering are impaired in mutants with defects in OXS2 and three of the four OXS2-like paralogues. The autoactivation of OXS2 may be a commensurate response to the stress intensity, stepping up from a strategy based on tolerating the effects of stress to one of escaping the stress via reproduction.

The EMBO Journal (2011) 30, 3812–3822. doi:10.1038/emboj.2011.270; Published online 9 August 2011

Subject Categories: plant biology

Keywords: fitness; flowering; reproduction; ROS; XPOI

Introduction

Reproductive success in plants depends on the proper timing of the floral transition (Mouradov *et al*, 2002). At the transition from vegetative to reproductive phase, the shoot apical meristem of *Arabidopsis* ceases to produce rosette leaves and becomes an inflorescence meristem competent to produce flowers. Four major pathways regulate this transition: the

autonomous and the gibberellin pathways are endogenous, while the photoperiod and vernalization pathways respond to environmental cues (Araki, 2001; Cerdan and Chory, 2003; Komeda, 2004; Searle and Coupland, 2004). The floral integrators *SOC1*, *LFY*, and *FT* (Figure 1A) perceive signals from all flowering pathways and set the temporal outcome for flowering (reviewed by Parcy, 2005). *Arabidopsis* is a facultative long-day (LD) plant that flowers more rapidly in LDs than in short days (SDs). In the photoperiod pathway, the zinc-finger (ZF) protein *CONSTANS* (*CO*) accumulates in LD via transcriptional activation by the circadian clock and protein stabilization by light (Putterill *et al*, 1995; Suarez-Lopez *et al*, 2001; Imaizumi *et al*, 2003; Valverde *et al*, 2004). In turn, *CO* activates the transcription of *FT* that encodes a protein similar to Raf kinase inhibitors (Kardailsky *et al*, 1999; Kobayashi *et al*, 1999). While *FT* transcripts are produced in the phloem companion cells (Takada and Goto, 2003; An *et al*, 2004), *FT* proteins migrate to the apex (Corbesier *et al*, 2007), and interact with the flowering transcription factor *FD* to activate three floral identity genes that encode MADS-box transcription factors *APETALA1* (*AP1*) in floral meristems (Abe *et al*, 2005; Wigge *et al*, 2005), and *FRUITFULL* (*FUL*) and *SEPALLATA3* (*SEP3*) in leaves (Teper-Bamnolker and Samach, 2005). As opposed to *FT*, *TERMINAL FLOWER 1* (*TFL1*) represses floral identity all along the life cycle and is a determinant factor of the inflorescence architecture (Shannon and Meeks-Wagner, 1991; Bradley *et al*, 1997).

The vernalization pathway, mostly relevant to winter annual accessions, promotes flowering in response to week-long exposure to cold temperature by preventing transcription of *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino, 1999, 2001; Sheldon *et al*, 1999, 2000) through chromatin remodelling of its locus (Sung and Amasino, 2004; He and Amasino, 2005). *FLC* encodes an MADS-box transcription factor that delays flowering through direct repression of *FT* and *SOC1* (Hepworth *et al*, 2002; Michaels *et al*, 2005; Searle *et al*, 2006). The *SOC1* promoter is regulated negatively by *FLC* binding to a *CArG* box, and positively at an unspecified *cis* element in a *CO*-dependent process (Hepworth *et al*, 2002).

Stresses are known to impact flowering in a wide range of plants (Blazquez *et al*, 2003; Yang *et al*, 2004), but both delayed and early flowering have been observed (Magome *et al*, 2004; Martinez *et al*, 2004). The disparate responses may reflect differences in the interactions of the genotype with the type and the intensity of the stress, though the genetic control of stress-induced flowering conceptually converges to the floral integrators. Many types of stress lead to the accumulation of reactive oxygen species (ROS) that are known, for example, to trigger sexual induction in *Chlamydomonas reihardii* and *Volvox carteri* (Nedelcu, 2005). In higher plants, different ROS act as signalling molecules that can specify the response to each particular stress (Mahalingam and Fedoroff, 2003; Mittler *et al*, 2004; Wagner

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Received: 4 November 2010; accepted: 7 July 2011; published online: 9 August 2011

Figure 1 OXS2 is needed to delay floral transition. **(A)** Abbreviated model for the regulation of floral transition. **(B)** Structure of OXS2 gene (At2g41900) and partial OXS2 cDNA (AT3, black rectangle). Blue boxes: ANKYRIN repeats; red box: ZF domain; green boxes: polyglutamine stretches; grey boxes: untranslated regions; line: intron; red lollipop: T-DNA insertion in *oxs2* alleles. **(C, D)** Flowering time of growth chamber plants measured by total number of primary leaves at bolting. **(C)** Homozygous plants subjected to long days at 21°C without or with a drought stress (water withheld after 7 days of growth); s.e. from >12 plants. Lower panel: RT-PCR analysis on 12-day-old seedlings grown on plates; g, genomic DNA. **(D)** Mutant rescue of *oxs2-1* with a 35S-OXS2 construct. A, G, and H represent three independent lines homozygous for the transgene; Lower panel: northern blot analysis of OXS2 expression.

et al., 2004). For instance, H₂O₂ induces a mitogen-activated protein kinase cascade (Kovtun *et al.*, 2000), and also serves as an intermediate in abscisic acid (ABA)-induced stomatal closure (Zhang *et al.*, 2001).

When stressed, a sessile organism with a finite life cycle has few options: (i) ignore the stress and maintain vegetative growth, which may delay reproduction; (ii) alleviate the stress through tolerance mechanisms; (iii) or escape the stress via reproduction, an altruistic response least favourable for an individual but essential for species preservation. Here, we report on the characterization of *Arabidopsis* OXIDATIVE STRESS 2 (OXS2), which encodes a ZF transcription factor that is cytoplasmic until nuclear translocation is induced by stress. The cytoplasmic form is needed for vegetative growth, while the nuclear form is necessary for

stress tolerance. In conjunction with other members of its protein family, OXS2 is also necessary for stress-induced reproduction and activation of *SOC1*. Because OXS2 can also autoactivate its own gene, we propose a model in which the regulation of the compartmentalization and abundance of OXS2 provides commensurate responses to the stress intensity—to grow, to fight the stress, or to hand the task to the next generation.

Results and discussion

OXS2 confers stress tolerance

To identify candidate genes that participate in cellular stress tolerance, we screened an *Arabidopsis* expression library in the fission yeast *Schizosaccharomyces pombe*. A cDNA that can enhance oxidative stress tolerance was found effective against the heavy metal cadmium, the thiol oxidizing agent diamide, or the organic peroxide *tert*-Butyl hydroperoxide (*t*-BOOH). This cDNA (AT3) corresponds to the C-terminus of At2g41900, predicted to encode a protein of 716 aa (Figure 1B; Supplementary Figure S1). The full-length ORF also enhanced tolerance to these chemicals, and strains expressing either cDNA showed lower levels of thiobarbituric acid reactive substances during *t*-BOOH treatment, indicating less lipid peroxidation and less oxidative stress (Supplementary Figure S2). Homozygotes of four T-DNA insertion mutants in At2g41900 all lacked detectable transcripts by RT-PCR or northern blotting (Figure 1C and D) and showed reduced tolerance to the same set of chemicals (Figure 2C and D). Since this gene is necessary to alleviate oxidative stress in *Arabidopsis*, we named it OXS2.

OXS2 belongs to a family of five ZF proteins with a canonical C2-H2 ZF, two C3-H ZFs, and two ANKYRIN repeat motifs (Mosavi *et al.*, 2004; Figures 1B and 2B; Supplementary Figure S1A). However, loss-of-function alleles in each of these paralogous OXS2-Like (O2L) genes were not sensitive to stress (Figure 2C and D) unless examined under high stress (Figure 2D: 300 μM *t*-BOOH). Given the wider range of stress tolerance observed with OXS2, we focused on this member of the family. The *Arabidopsis* microarray database Genevestigator (Zimmermann *et al.*, 2004) describes OXS2 transcripts as moderately elevated by cold, salt, ABA, osmotic stress and during senescence, to which we confirmed by northern analysis for exposure to cold, Cd, or ABA (Supplementary Figure S3J). The ABA induction, along with a slight ABA insensitivity in germination of *oxs2-1* hints at a possible involvement in the water stress response (Supplementary Figure S3K). A promoter-reporter gene fusion also revealed activity during ovule development and embryogenesis, in the shoot apical meristem and in roots with induced response to Cd (Supplementary Figure S3A–I).

Stress-induced nuclear accumulation

OXS2 was fused to GFP or DsRed and expressed from the CaMV 35S RNA (35S) promoter transiently in onion epidermal cells, and stably in *Arabidopsis* (Figure 3). In the absence of stress, the OXS2 fusion, encoded by fragment RB39, was restricted to the cytoplasm (Figure 3A–C). However, when treated with cold, ABA or leptomycin B (LMB), it appeared in the nucleus (Figures 2E and 3B). Because LMB inhibits exportin1 (XPO1) (Kudo *et al.*, 1998), the LMB-induced nuclear localization indicates that OXS2 is an XPO1-regulated

Figure 2 OXS2 paralogous genes display differences and similarities in their regulation and function relative to stress. (A) Chromosome map of OXS2 and O2L paralogues; genetic linkage of OXS2 and O2L4 was broken once in 400 F2 siblings of a cross between *oxs2-1* and *o2l4-1*. (B) Gene structure and T-DNA insertions as in Figure 1B. Polyglutamine stretches are specific features of OXS2 but not other members of the family, and hence not indicated. RT-PCR data show transcripts not detected in homozygotes of mutant alleles. (C, D) Root length of Arabidopsis seedlings grown vertically for 10 days on plates containing growth medium without or with diamide (1 mM result chosen for illustration) or a range of t-BOOH concentrations; error bars = s.d. from ≥ 10 root measurements; * indicates statistical difference between wild type and the mean value of the four *oxs2* alleles combined ($\alpha < 5\%$). (E). Subcellular localization of OXS2 and paralogous proteins tagged with GFP and transiently expressed in onion epidermal cells before and after 1 h exposure at 4°C.

protein. This deduction is consistent with the mapping of an XPO1-type nuclear export signal (NES) within the OXS2 C-terminus (aa 699–711, LEAWIEQMQLDQL; L⁷⁰⁸ in bold). Deletions encompassing this sequence (Figure 3A: RB40, RB50, RB492; Figure 3C: RB40) and substitution of the leucine at aa 708 with proline (L708P, Figure 3A: RB206)

were associated with nuclear localization whereas removal of the contiguous segment containing the two polyglutamine stretches (Q domain, Figure 3A: RB51) did not show a similar effect. Reintroduction into RB40 of the NES from either OXS2 or the HIV-1 Rev protein (NES-rev) restored nuclear exclusion (Figure 3A: RB54, RB46).

Although the protein enters the nucleus, a canonical NLS was not found in OXS2. Deletions that encompass both or just one of the ankyrin motifs reduced nuclear accumulation (Figure 3A: RB43, RB47, RB507; Figure 3C: RB43), similar to adding a HIV-1 Rev NES to a fragment lacking one of the ankyrin motifs (Figure 3A and C: RB44). Since the OXS2-NES could mask the NLS activity, we combined the deletions covered by RB507 (presumptive NES⁺, NLS⁻) and RB492 (presumptive NES⁻, NLS⁺). In contrast to its progenitors that are either constitutively cytoplasmic or nuclear, the resultant construct (presumptive NES⁻, NLS⁻) produced a protein that clustered outside the nucleus (Figure 3A: RB509). This indicates that the segment covered by the RB507 deletion is necessary for OXS2 nuclear entry. It seems possible that the ankyrin motifs have a role, such as by interacting with a yet to be identified carrier. Removal of the segment containing the ZF domain (Figure 3A: RB41) did not affect nuclear accumulation. Insertion of a synthetic NLS from SV40 (Haasen *et al*, 1999) resulted in strong nuclear accumulation, regardless of the presence (Figure 3A: RB45) or absence of the C-terminal NES (Figure 3A: RB493). This shows that a strong NLS can override the OXS2 NES activity, and also implies that the native OXS2 NLS activity is weaker than its NES in the absence of stress, hence ensuring a tight nuclear exclusion.

Overproduction of cytoplasmic OXS2 suppresses floral transition

Some of the constructs used for mapping the regulatory elements were introduced into *Arabidopsis*. Differences in flowering time were observed in the greenhouse grown plants (Figure 3D). Under normal LD growth (16 h light 21°C/8 h dark 18°C), very late flowering was observed with a deletion that removed the N-terminus that included the ankyrin motifs (RB43). Since this construct directs a protein to the cytoplasm, we considered the possibility that the late flowering phenotype might be due to overaccumulation of a cytoplasmic form of OXS2. Consistent with this line of reasoning, late flowering also coincided with cytoplasmic accumulation when a heterologous NES (NES-rev) was added to OXS2 (RB44).

Overproduction of nuclear OXS2 promotes floral transition

In contrast to the late flowering observed with excess cytoplasmic accumulation of OXS2, a very early flowering phenotype was associated with excess nuclear accumulation of this protein, as in the NES point mutation L708P (RB206), in which leaf number at bolting was reduced to near half of the wild-type control (Figure 3D). Early flowering was also observed from overexpression of the OXS2 cDNA (pRB39), but not from overexpression of OXS2 variants that lack a mid-section including the ZF domain for presumptive DNA binding (RB41). These observations suggested that floral transition could be triggered by excess OXS2, but only if the protein is able to reach the nucleus and is active as a transcription factor. It seems that the nuclear action of OXS2 predominates over remaining pools of cytosolic OXS2.

To examine whether the OXS2 effect is dependent on the photoperiod or cold temperatures, selected transgenic lines were grown under SD or LD with cold treatments ranging from 1 to 6 weeks. Plants transgenic for RB41 exhibited flowering times that were similar to those of the wild type

(Supplementary Figure S4A). Regardless of the tested conditions, lines overproducing OXS2 (RB39) displayed a trend for early flowering while those producing its cytoplasmic forms (RB43 and RB44) showed late flowering times. These results indicate that the photoperiod and the exposure to cold temperature do not exert a significant effect on OXS2 action.

OXS2 is needed to delay floral transition during non-stress conditions

To examine how the loss of OXS2 function would affect flowering time, we tested the four *oxs2* null mutants (Figure 1C). Grown in environmental chambers, all flowered earlier than the wild type, suggesting that OXS2 is required to delay floral transition during non-stress conditions. However, the *oxs2* mutants make more branches. Overexpression of OXS2 in the *oxs2-1* background could restore the normal timing of floral transition (Figure 1D), and the higher expression from the 35S promoter did not cause flowering to occur later than the wild-type control. When stress was applied in the form of a drought treatment, the wild type flowered earlier. However, so did the *oxs2* mutants (Figure 1C), which would seem to contradict a hypothesis that OXS2 promotes stress-induced flowering, unless there is functional redundancy for this role. Hence, we sought to test whether OXS2 can indeed activate floral transition, and if so, whether other proteins can compensate for this role.

OXS2 is a transcription factor that targets floral integrator genes

In a transient expression assay, the *Gal4* promoter transcribing the firefly luciferase gene was monitored when co-introduced with potential activator constructs. OXS2 fused to the *Gal4* binding domain, with (RB39) or without (RB41) its ZF domain, produced nearly four times as much luciferase activity as the negative control (Figure 3E), comparable to the *Gal4* activation domain. Constructs that lack portions of the carboxyl terminus ($\Delta C1$, $\Delta C2$, ΔQ) showed basal activity. These results indicate that OXS2 has the capacity to activate transcription, and that this activity resides in the carboxyl terminus, including but not limited to the polyglutamine stretches.

To explore potential OXS2 targets related to flowering, we examined the expression of representative floral transition genes in the late and early flowering genotypes (Figure 3F). For the line that accumulates cytoplasmic OXS2 (RB43), lower expression of *FT*, *FUL*, *SOC1*, and *LFY* was observed, consistent with late flowering. For the NES mutant line (RB206) where OXS2 accumulates in the nucleus, upregulation was observed for *SOC1* and *LFY* by day 4 after germination, and for *AP1*, *FUL*, by day 7 after germination, consistent with early flowering. *FLC* and *TFL1* transcripts that encode negative regulators of the floral pathway also accumulated in these plants. The upregulation of these mRNAs has been previously reported in association with early flowering and *CO* overexpression (Simon *et al*, 1996; Hepworth *et al*, 2002). Interestingly, the L708P plants also showed higher accumulation of OXS2 mRNA, which hinted at the possibility that OXS2 may activate its own gene.

In a selection and amplification binding (SAAB) assay, His-tagged OXS2 purified from *E. coli* led to enrichment of a 9-bp CT-rich motif that we termed BOXS2 (Figure 4A). In sampling the floral transition genes in Figure 3F, sequences resembling

BOXS2 could be found upstream of *SOC1*, *LFY*, *AP1*, *FUL* as well as *OXS2* coding regions. Since *SOC1* acts upstream of *LFY*, *AP1*, *FUL* and has a large CT-rich region with possibly additional *cis* elements, we focused on the *SOC1* promoter (Figure 4B). In an electromobility shift assay (EMSA), probe *x* (−446 to +22) spanning upstream and downstream of the transcript start (Hepworth *et al*, 2002) bound to the His-

tagged OXS2 protein, but the complex failed to enter the polyacrylamide gel (Figure 4B). Probe *y* (−141 to +6) encompassing the CT-rich region (−57 to +5) showed weak binding, whereas probe *z* (−351 to −289) that includes the −321 BOXS2 bound the His-tagged OXS2. In a 1.5% agarose gel, OXS2 retarded the migration of probe *z* (Figure 4B). Furthermore, this complex was displaced by

unlabelled probe, but not by non-specific dIdC DNA. Migration of labelled DNA further down the gel (indicated by *) was less specific, and might represent a DNA-bound

low molecular weight form of the protein or degradation products. This shows that there is at least one segment of the *SOC1* upstream sequence recognized by OXS2 *in vitro*.

Figure 4 OXS2 activation of floral integration genes associated with BOXS2 elements. (A) Selection and amplification binding (SAAB) assay using OXS2 recombinant protein. Sequence logo: letter sizes proportional to relative occurrence of the nucleotide in the SAAB population. $P^{(e)}$: estimated occurrence of the motif in *A. thaliana* promoter regions (C, G = 0.15; T, A = 0.35). (B) *SOC1* promoter and EMSA (electromobility shift assay) with *E. coli*-produced OXS2 protein using probe x, y, or z. Left panels, 4% acrylamide gel; right panel, 1.5% agarose vertical gel. FP, free probe; band x, y, or z; b, bound probe. *Low-molecular weight complexes. Cold probe is unlabelled z competitor; dIdC, non-specific competitor. (C) Immunoblot using anti-GFP antibody on cytoplasmic (C) or nuclear (N) fraction from 10-day-old seedlings transgenic for RB39, RB40, or histone H4-GFP (H4). (D) Chromatin immunoprecipitation (ChIP) on *SOC1* promoter (-446 to +23) from plants transgenic for GFP or OXS2-GFP (RB39); i, input; b, beads; αG, anti-GFP serum used for IP; *ACT2*, *ACTIN2* primers used as control; Enrichment established as α G/i *SOC1* / α G/i *ACT2* after qPCR. qPCR results presented as *SOC1* enrichment in OXS2-GFP plants versus GFP plants normalized to the *ACT2* DNA enrichment set to 1. (E) Transient expression assays. Firefly luciferase gene fused to promoter from *SOC1* (P_{SOC1}) (Hepworth *et al*, 2002), *LFY* (P_{LFY}), *AP1* (P_{AP1}), *FUL* (P_{FUL}), *TFL1* (P_{TFL1}) or OXS2 (P_{OXS2}) assayed for expression with (+) or without (-) co-transformed 35S-OXS2. Grey box: CT-rich region; black boxes: putative BOXS2 motifs with sequence and position shown relative to transcript start (arrowheads). Error bar = s.e. of triplicate experiments.

Figure 3 OXS2 localization affects flowering time. (A) Subcellular localization in onion epidermal cells of OXS2 or derivative proteins tagged with GFP or dsRed expressed from 35S promoter; numbers indicate amino-acid positions; ankyrin repeats (ANK), ZF domain (ZF), and polyglutamine stretches (Q) as Figure 1B; L708P is point mutation in OXS2 NES; inverted triangles show NES from OXS2, O2L1, or HIV-1 Rev (rev); NLS is from SV40 protein. Bar = 20 μm. (B, C) Confocal microscopy of (B) protoplasts from plants transgenic for OXS2-GFP (RB39) treated 3 h without or with ABA (100 μM) or leptomycin B (LMB, 50 ng/ml). Bar = 1 μm; or (C) adaxial side of *Arabidopsis* leaves from RB39 (full-length OXS2), RB43 (N-terminus deletion), RB44 (NES-rev in place of one ankyrin repeat), RB40 (C-terminus deletion); c: cytosol, chl: chloroplast, gc: guard cell, n: nucleus. (D) Localization determined by transient expression in onion cells and confirmed in transgenic *Arabidopsis* (except for RB206 (L708P aa substitution) as none of the tested transgenic plants ($n > 12$) yielded sufficient GFP signal for imaging, although transgene expression was confirmed by RT-PCR); + or - indicate the relative presence or absence, respectively, of OXS2 or derivative proteins tagged with GFP in (c) cytoplasm, (n) nucleus, or (n^s) nucleus of cells subjected to stress; ND, not determined. Flowering time recorded for primary leaves at bolting on two separate lines expressing similar levels of detectable OXS2-GFP; grown in greenhouse corresponding to mild stress conditions; s.e. from 12 plants; *Ler*, wild type. (E) OXS2 transactivation of Gal4 promoter-luciferase gene fusion. Each activator construct encodes a fusion to the Gal4 DNA binding domain (G4DBD). G4AD, Gal4 activation domain; ΔZF, deletion of ZF domain (aa 251–446, as in RB41); ΔC1 and ΔC2, 186 aa (as in RB40) and 112 aa (as in RB50) C-terminal deletions of the putative activation domain, respectively; ΔQ: deletion of the polyglutamine rich domain (aa 508–634). (F) RT-PCR analysis of flowering genes in plants transgenic for RB43, RB206, RB39 and control RB40 that exhibit wild-type flowering time; 7-day-old seedlings grown on agar plates 21 °C, continuous light. *Expression of *SOC1* and *LFY* on 4-day-old seedlings; *EF1α*, internal control. Unlike other OXS2 variants, inert RB40 is expressed at higher level, hence higher OXS2 mRNA due also to the RB40 transgene. (G) Relative expression of *SOC1* and *LFY* determined by quantitative RT-PCR. Measurements of the mean ± s.d. of triplicates extrapolated on standard curves of each tested gene.

Chromatin immunoprecipitation (ChIP) was used to examine if OXS2 binds the *SOC1* promoter *in vivo*. Nuclear fractions were purified from 10-day-old seedlings producing the full-length OXS2–GFP (RB39), a C-terminal truncated form (RB40), or a histone H4–GFP control. As expected for the RB40 form that is mainly localized to the nucleus, anti-GFP antibodies easily detected its presence in the nuclear-enriched fraction (Figure 4C). The same conditions used also detected the RB39 form, although at lower levels than in the cytoplasmic fraction. Real-time quantitative PCR on the immunoprecipitated chromatin showed a ninefold enrichment of an *SOC1* promoter fragment (–446 to +22) in RB39 plants relative to GFP control plants. Gel analysis also shows a similar eightfold enrichment with *SOC1/ACT2* band intensity at $0.5 \times$ in GFP plants and $4 \times$ in RB39 plants (Figure 4D). This is consistent with the EMSA data that OXS2 binds the *SOC1* promoter.

Since protein binding to DNA does not necessarily indicate gene activation, we tested for OXS2 transactivation using a luciferase reporter gene fused to a *SOC1* upstream fragment that includes the –321 BOXS2 (–446 to +552, ATG start is +555 due to 2 bp addition). In a transient expression assay, a fivefold greater expression was observed when an OXS2-activator construct (35S-OXS2) was included (Figure 4E). Similarly, the inclusion of 35S-OXS2 enhanced expression of promoter fragments from *LFY* (–583 to +1), *API* (–551 to +214), and *FUL* (–1007 to +100), each containing a putative BOXS2. For some genes, it is possible that the OXS2 effect is indirect, through the activation of endogenous *SOC1* known to activate *LFY* or endogenous *LFY* known to activate *API* (Parcy *et al*, 1998). OXS2, however, is not a general activator of gene expression, as it did not activate a *TFL1* promoter fragment (–992 to +37) that lacks a BOXS2-like sequence.

As noted earlier, the higher accumulation of OXS2 mRNA in L708P plants raised the possibility of OXS2 autoactivation. However, putative BOXS2 motifs upstream of the OXS2 ATG start, all lie downstream of the transcript start with two of them within the first intron. Nonetheless, the luciferase reporter fused to this fragment (–1147 to +853) was stimulated sixfold by the co-introduction of 35S-OXS2, consistent with a possibility of OXS2 autoactivation through this leader-intron segment.

Functional redundancy for inducing floral transition

The overexpression studies suggested that OXS2, in particular the nuclear OXS2–NES^{L708P}, has the capacity to promote early flowering. Paradoxically, however, the loss of OXS2 function fails to abolish the stress-induced reproductive response, as *oxs2* mutants do not flower later than the wild type when stressed (Figure 1C). Therefore, we considered the possibility of functional redundancy to account for this aspect of the OXS2 pathway, with likely candidates being the O2L proteins (Supplementary Figure S1). A role in stress-induced flowering would likely require gene expression and/or nuclear localization during stress. Transient expression studies with GFP fusion proteins indicated that O2L1 is similar to OXS2, cytoplasmic in the absence of stress but nuclear during stress (4°C, 3 h; Figure 2E). A putative NES found in O2L1 could also substitute for the OXS2 NES (Figure 3A: RB210). In contrast, O2L2, O2L3, and O2L4 did not show stress-induced translocation. O2L2 appears in the cytoplasm, while O2L3 and O2L4 are in the nucleus.

To examine a potentially redundant role for floral induction, we generated all combinations of double, triple, quadruple, and quintuple mutants (Figure 5A). When grown in the greenhouse without a stress treatment, 11 of 32 combinations showed a late flowering phenotype, averaging >15 leaves compared with the wild-type range of 12–14. Of these 11 combinations, the common denominator was the *o2l1-1* allele, and even in the single *o2l1-1* mutant, a mild late flowering effect was observed. Given that this late flowering effect was seen without a stress treatment, this could indicate that O2L1 is necessary for the normal flowering process. Recalling that loss of OXS2 function in the absence of stress leads to early flowering, it was surprising to see that 8 of the 11 combinations have in common both *oxs2-1* and *o2l1-1*, as though the two mutations cooperatively promote late flowering rather than exerting opposite effects. None of the triple mutants exhibited flowering time later than the double *oxs2-1*, *o2l1-1* combination, although even later flowering was found in genotypes that included *o2l4-1*, with either *o2l2-1* or *o2l3-1*. The quintuple mutant (*o2l⁵*) also was late flowering but showed growth defects in roots and aerial structures, and the stunted growth phenotype may indicate that a minimum of one family member is necessary for a critical, though not essential, aspect of development.

Stress-induced flowering and *SOC1* expression abolished in quadruple mutant

If our hypothesis that OXS2 paralogues compensate for the loss of OXS2 function in stress-induced flowering is correct, then stress-responsive flowering ought to be absent in *o2l⁵*. However, since *o2l⁵* shows pleiotropic defects, and a stress treatment further impairs its growth, we examined a quadruple mutant. As shown in Figure 5A, the latest flowering time was recorded for the quadruple mutant *oxs2-1 o2l1-1 o2l3-1 o2l4-1*, named *o2l⁴*. In control conditions with or without a drought stress, the wild type hastened flowering when stressed, but *o2l⁴* failed to respond (Figure 5B). Given that the *SOC1* promoter is a direct target of OXS2, we examined whether *SOC1* expression might be impaired in *o2l⁴*. *SOC1* expression is regulated by environmental cues, by CO induction under LD. To test the effect of stress on *SOC1* expression in a CO-independent manner, plants were grown under SD (Figure 5C). Three hours of *t*-BOOH treatment yielded a 40-fold increase of *SOC1* expression in the wild type but only a 3- or 11-fold increase in *o2l⁴* or *o2l⁵*, respectively. This shows that the OXS2 protein family is needed for stress-induced *SOC1* expression, and this could be the basis of stress-induced flowering mediated by the OXS2 family of proteins. O2L2, however, appears to have a limited role in that function as shown in the mutant analysis and as expected from its mainly root-specific expression profile (Genevestigator: Zimmermann *et al*, 2004).

Induction of *SOC1* in the absence of stress

As stress-induced expression of *SOC1* depends on the OXS2 gene family, we considered the possibility that they may also regulate non-stress-induced expression of *SOC1*. If this were the case, it would account for the late flowering observed in *o2l⁴*, *o2l⁵*, and other mutant combinations in the absence of stress (Figure 5A). To reduce the impact of photoperiod regulation, *SOC1* expression was examined in plants grown in continuous light (21°C, Figure 5D). A peak of *SOC1*

Figure 5 OXS2 and O2L genes regulate stress-induced flowering. (A) Flowering time of greenhouse grown plants measured as total leaf number; mean $\pm$ s.d. ($n = 12$ –16); filled square, mutant allele; open square, wild-type allele. (B–E) Plants grown in environmental chambers. (B) Stress-induced flowering in wild type and the most delayed mutant combination *o2l4* (*oxs2-1 o2l1-1 o2l3-1 o2l4-1*); drought stress applied 7 days after germination by withholding water until bolting. (C–E) *SOC1* expression assessed by RT-qPCR. (C) Wild-type seedlings, the quadruple mutant *o2l4* and the quintuple mutant *o2l5* grown 10 days in short days—to prevent photoperiodic induction of *SOC1*—and treated with or without *t*-BOOH for 3 h. (D) Wild type and *o2l5* from days 4 to 11 under continuous light. (E) *SOC1* induction in wild type and *o2l5* by shifting to long-day growth following 10 short days.

expression at day 7 after germination corresponded to the timing of floral transition in the wild type. In *o2l5*, this peak was absent and the overall level of *SOC1* expression was greatly reduced. This suggests that even in the absence of stress, OXS2 and O2L proteins contribute to *SOC1* expression.

To test *SOC1* induction by the photoperiod, plants grown in SD for 10 days were then subjected to three consecutive LD treatments to induce the CO-dependent expression of *SOC1* (Figure 5E). A peak of *SOC1* induction was observed during the third LD with both the wild-type and the *o2l5* mutant. However, *SOC1* expression was severely reduced in the *o2l5* mutant. This suggests that OXS2 and O2L proteins mediate *SOC1* induction in response to upstream input signals,

including the photoperiod regulator CO. Given that Hepworth *et al* (2002) had described the *SOC1* promoter as being positively regulated at an unspecified *cis* element in a CO-dependent process, it seems possible that OXS2 (and perhaps O2L) recognition to BOXS2 could represent that positively regulated component described.

Conclusions

The stress-induced expression of OXS2 in *Arabidopsis* roots and apices hints at a role in stress biology in these organs. In a root elongation assay, *oxs2* null mutants are clearly more sensitive than the wild type to oxidative stress. Roots are

directly exposed to numerous stresses that could cause oxidative damage, and it is likely that OXS2 ameliorates this stress challenge through the activation of tolerance mechanisms, to which we have not yet investigated this aspect of OXS2 biology. Moreover, we can only speculate that OXS2 recognizes homologous tolerance pathways in the fission yeast, since OXS2 expression in this host leads to greater stress tolerance.

Our attempt to overexpressing OXS2 failed to yield plants with higher stress tolerance. However, the higher accumulation of OXS2 in the nucleus did correlate with early flowering. Hence, we surmised that OXS2 expression at the apex might not be related to tolerance mechanisms for survival of the individual, but to escape mechanisms for survival of the species. Indeed, OXS2 was found to activate several floral integrator genes, and in particular, to bind and activate the *SOC1* promoter at a region that includes a BOXS2 element. Recent studies indicate that the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors are targets of regulatory microRNAs (miR156 and miR172) that control phase transitions (Wang *et al*, 2009; Wu *et al*, 2009). SPL9, in particular, has been associated with *SOC1* expression (Yamagouchi *et al*, 2009) and would act as a transcriptional facilitator (Fornara and Coupland, 2009). Hence, OXS2/O2L proteins may not be the only transcriptional activators of *SOC1*. It would be of future interest to investigate the genetic interactions between miR172-SPL9 and OXS2/O2L regarding the expression of *SOC1*.

In the early flowering line OXS2-NES^{L708P}, we observed higher transcript levels of *FLC* and *TFL1* that encode repressors of the flowering pathway. *FLC* is known to bind to a CArG element on the *SOC1* promoter (Hepworth *et al*, 2002) to repress its transcription (Searle *et al*, 2006). We can, therefore, infer that the OXS2-mediated activation of *SOC1* prevails over *FLC* repression. Although OXS2 does not directly activate *FLC* and *TFL1*, it is possible that their upregulation may serve a needed function. Should the stress intensity subside, the presence of a monitoring role for *FLC* and *TFL1* may aid a reversal of the decision towards floral repression.

Collectively, the data suggest a model of dual OXS2 function (Figure 6). Under normal growth conditions, OXS2 would be excluded from the nucleus by XPO1, optimizing vegetative growth. Stress activation of OXS2 triggers its translocation to the nucleus, which likely activates a protection program to alleviate the encountered stress. Reduction of stress leads back to XPO1-mediated nuclear export of OXS2. Should stress increases, OXS2 autoactivation may provide commensurate amplification of the response through *de novo* OXS2 protein synthesis. Sustained or severe adverse conditions may trigger reproduction through activation of the floral integrators *SOC1* and *LFY*. Should the stress intensity subside, however, it is likely that the stress escape response would be aborted. This might be accomplished through a reversal of the stress modification of OXS2, triggering nuclear export of the protein, and/or by a fast degradation of the active nuclear form. The cytoplasmic form of OXS2 may help build resources under favourable conditions for delayed but more abundant progeny. The mechanism by which cytoplasmic OXS2 is associated with floral repression is yet unknown, but might involve cytoplasmic recognition and sequestration of floral inducers. Since OXS2 orthologues are found in dicots

Figure 6 Model of OXS2 regulation of stress tolerance or stress escape. OXS2^C: cytoplasmic OXS2; OXS2^N: nuclear OXS2; bottom arrow: vegetative to reproductive transition. T-bars and arrows indicate repression and induction, respectively. In the absence of stress (green lines), OXS2^C is due to XPO1-mediated nuclear exclusion and is needed to delay flowering. During low stress intensity (orange lines), nuclear accumulation of OXS2 activates stress tolerance and the alleviation of stress replenishes OXS2^C for delayed flowering. Under high stress intensity (red lines), higher accumulation of OXS2^N results from autoactivation and translocation from the cytoplasm for a stress escape response, and in conjunction with family members O2L1, 3, and 4, activate the reproductive phase via the floral integrator genes.

and monocots (Supplementary Figure S1B), this fine-tuning mechanism for reproduction may be as old as the phylum of Angiosperms.

Materials and methods

Accessions

Accession numbers are TAIR (<http://www.arabidopsis.org/>) OXS2: At2g41900, O2L1: At5g12850, O2L2: At5g58620, O2L3: At3g55980, O2L4: At2g40140, SOC1 (AGL20): At2g45660, LFY: At5g61850, AP1: At1g69120, FUL: At5g60910, TFL1: At5g03840, FLC: At5g10140.

Biological materials

S. pombe strain SJ23 (*h*⁺, *ura4*, *leu1.32*) and procedures for yeast experiments have been described (Blanvillain *et al*, 2009). *Arabidopsis thaliana* seeds of *oxs2* alleles and *o2l* alleles (*oxs2-1*: SALK_037470, *oxs2-2*: SALK_030754, *oxs2-4*: SALK_122614, *oxs2-6*: SALK_126148, *o2l1-1*: SALK_020612, *o2l2-1*: SALK_097914, *o2l3-1*: SALK_141550, *o2l4-1*: SALK_024800) from T-DNA mutagenesis populations, and Col-0: SALK_6000, *lfy-1*: cs6228, *tfl1-14*: cs6238 were obtained from the Arabidopsis Biological Resource Center and confirmed for the T-DNA insertion through PCR analysis (primers: o2R with oLBB1 or o2F). Plant expression constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 for floral dip infiltration. Details for the constructs in Figures 1–4 and the primer sequences are available online in Supplementary data.

Gene expression and protein subcellular localization

RNA extraction and retrotranscription was conducted using RNeasy plant minikit and RT superscript III (Stratagene). Standard procedures were used for northern blots. Transient expression in onion cells (bulb sliced to ~16 cm²) was conducted using the Biolistic PDS 1000/He Particle Delivery System (Bio-Rad) (1100 psi, 10 cm travelling distance) with DNA, 1 M CaCl₂, 16 mM spermidine precipitated onto 1 μm gold particles. After 16–40 h in the dark at 24°C, the epidermis was peeled and observed by epifluorescence microscopy with FITC filters or under a Zeiss confocal microscope. Protein localization of stably transformed *Arabidopsis* was examined on the adaxial side of *Arabidopsis* leaves or on protoplasts

prepared by standard procedures, with a 2-h recovery period allowed for the stress induced by the protoplast preparation to subside.

Luciferase assay

Onion epidermal cells were transfected by microprojectile bombardment with 0.5 µg pRLC (*Renilla* luciferase), 1 µg of the luciferase reporter construct and 2 µg of the transactivating construct (Figure 3E), or by 0.6 µg pRLC, 3 µg of pPromoter-luc, and when needed, 5 µg of the activator construct (Figure 4E), kept dark 20 h, 21°C, ground in liquid nitrogen. Proteins were extracted in 1 ml of PBLuc buffer (200 mM NaPO₄, pH 7, 4 mM EDTA, 2 mM DTT, 5% glycerol, 10 mg/l BSA and 1 mM PMSF) and assayed (5–20 µl) using the Dual-Luciferase[®] Reporter Assay System (Promega). Mean ± s.d. determined from three independent experiments was normalized to negative control set at 1.

Protein expression

E. coli (DE3) pRARE2 strain (Novagen) transformed with OXS2 cloned in pET21d was grown at 30°C. Cells were induced with 0.2 mM isopropyl-β-D-thiogalactoside for 5 h, harvested by centrifugation, resuspended in 25 mM HEPES pH 7.9, 500 mM NaCl, 5 mM imidazol, 1 × protease inhibitor cocktail (Boehringer) and disrupted in a French press. In all, 0.5% triton was added to the lysate, centrifuged (25 000 g, 20 min at 4°C) and the supernatant used for purification of the His6-tagged protein using a Nickel resin (Novagen).

Selection of OXS2 DNA-binding sequence

A pool of degenerate 60-bp SAAB oligonucleotides (5'-gagaggatcagtcagcatg(n)₂₀ctcagcctcgagaattccaa-3') was converted to double-stranded DNA through treatment with DNA polymerase I (Klenow) and the 3' primer (5'-ttggaattctcgaggctgag-3'), and used to identify the OXS2 DNA-binding motif, using 3' and 5' (5'-gagaggatcagtcagcatg-3') primers to amplify the bound SAAB oligos. In all, 94 sequences were identified and analysed using MEME software version 3.0, online submission at <http://meme.sdsc.edu>.

Electromobility shift assay

DNA probes were labelled by PCR or Klenow fill-in using [α -³²P] dCTP and oligonucleotides (see Supplementary data). Binding reactions were prepared by mixing 500 ng of protein in 20 mM

HEPES pH 7.9, 20 mM NaCl, 10 µM ZnCl₂, 1 mM EDTA, 1 mM DTT, 100 ng poly (dIdC), 0.1% tertigol, 10% glycerol and 10 000 c.p.m. of the PCR-labelled probe in a final volume of 30 µl. After 1 h incubation on ice, complexes were resolved by gel electrophoresis (4°C, 2 h, 20 V/cm, polyacrylamide, 0.5 × TBE or 1.5% agarose, 0.5 × TAE) and detected by autoradiography of the dried gels.

Chromatin immunoprecipitation

ChIP was performed according to the Pikaard laboratory protocol, with details available in Supplementary data.

SOC1 profiling

Plants were grown *in vitro* for 11 days at 21°C, in SDs (8 h of light/24 h) or in continuous light. Plants were transferred to LDs for induction (16 h of light/24 h) or stressed with *t*-BOOH (300 µM final) added in the plate for 2 h. Samples (50–120 mg of fresh weight) were flash frozen after treatment. RT-qPCR is detailed in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank S Ruzin and D Schichnes for advice on confocal microscopy; H Smith for advice on SAAB assay; A Lima and Y Zenk for technical assistance; B Al-Sady, C Carles, G Chuck, J Fletcher, PH Quail, ZR Sung, and J Thomson for comments on early versions of the manuscript; D Ehrhardt for pE2S, and the Salk Institute and the Arabidopsis Biological Resource Center for seed stocks. Funding provided by US Department of Agriculture, US Department of Energy, and South China Botanical Garden.

Author contributions: SW contributed Figure 5C–E, PW contributed Supplementary Figure S2B, JHK isolated AT3 cDNA, and RB contributed the remaining figures. RB and DWO designed the experiments and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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