

The B2 domain of VIVIPAROUS1 is bi-functional and regulates nuclear localization and transactivation

Heather H. Marella · Ralph S. Quatrano

Received: 10 July 2006 / Accepted: 27 August 2006 / Published online: 15 September 2006
© Springer-Verlag 2006

Abstract The transcriptional regulator VIVIPAROUS1 (VP1) is composed of four functional domains that control different aspects of gene expression during seed development. The B2 domain is required for its role as a transcriptional activator, functioning at the site of transcription and/or for its transport into the nucleus. Previous work showed that the B2 domain was required for transactivation of the *Em* promoter. We demonstrate that VP1::GFP localizes to the nucleus of barley (*Hordeum vulgare*) aleurone cells, but when B2 is deleted, nuclear accumulation is lost. However, the B2 domain itself is not sufficient for nuclear localization of GFP::GUS. Using point mutagenesis on the putative NLS within B2, we show that the VP1::GFP still accumulates in the nucleus. Utilizing a comparative approach, through the alignment of B2 domains from various VP1/ABI3 proteins, including the ABI3 orthologs from *Physcomitrella patens*, revealed the involvement of other conserved amino acids. Mutating VP1 at the conserved threonine on the N-terminal side of the putative NLS and at a conserved arginine-glutamine-arginine sequence on the C-terminal side prevented nuclear localization of VP1. A single amino acid change, from alanine to threonine, within this NLS found in the *Arabidopsis abi3-7* mutant prevents transcription of *AtEm1* and *AtEm6* in vivo. We show that this same mutation in VP1 prevents transactivation of the *Em-GUS* reporter in barley aleu-

rone but does not interfere with nuclear localization. Our data demonstrate that the B2 domain of VP1 is bi-functional in nature regulating both nuclear localization and transactivation.

Keywords Abscisic acid · Barley · *Em* · Nuclear localization · *Physcomitrella* · VP1

Abbreviations

ABA	Abscisic acid
ABI3	Abscisic acid insensitive 3
ABI5	Abscisic acid insensitive 5
GFP	Green fluorescent protein
GUS	β -Glucuronidase
LEA	Late embryogenesis abundant
LUC	Luciferase
NLS	Nuclear localization signal
VP1	Viviparous 1

Introduction

The phytohormone abscisic acid (ABA) plays a vital role in plant development. It controls a variety of processes ranging from seed maturation and germination to regulation of stomatal opening. Classical genetic analysis of vivipary in *Zea mays* resulted in the isolation of VIVIPAROUS1 (VP1), a transcriptional regulator involved in maturation and developmental arrest of seeds, as well as anthocyanin biosynthesis (Robertson 1955; McCarty et al. 1991). Concurrently, generation of *ABSCISIC ACID INSENSITIVE* mutants of *Arabidopsis thaliana* led to the isolation of *ABSCISIC ACID INSENSITIVE3* (ABI3) mutants that germinate in the presence of inhibitory levels of ABA

H. H. Marella · R. S. Quatrano (✉)
Department of Biology, Washington University,
1 Brookings Drive, Campus Box 1137,
St Louis, MO 63130, USA
e-mail: rsq@wustl.edu

(Koorneef et al. 1984). Comparison of the amino acid sequence of ABI3 to that of VP1 revealed sequence similarity between the two proteins and in combination with the mutant seed phenotypes, led to the conclusion that they are orthologs (Giraudat et al. 1992). Eventually it was demonstrated that the major phenotypes of the severe *Arabidopsis abi3-6* mutant could be complemented in part by maize VP1 (Suzuki et al. 2001).

VP1/ABI3 are responsible for the transcriptional regulation of genes during seed development, maturation and germination, including the *LATE EMBRYOGENESIS ABUNDANT (LEA)* class (McCarty et al. 1991; Giraudat et al. 1992; Nambara et al. 1995; Parcy et al. 1997; Nambara et al. 2000). For example, the *Em* gene, a *LEA* family member (Marcotte et al. 1989), requires both ABA and VP1/ABI3 for expression (McCarty et al. 1991; Vasil et al. 1995; Bies-Etheve et al. 1999). VP1/ABI3 also appears to have a role outside of the seed regulating developmental transitions such as flowering, plastid differentiation, and meristem activity (reviewed in Rohde et al. 2000). VP1/ABI3 is involved in the crosstalk between ABA and auxin in lateral root development and germination (Suzuki et al. 2001; Brady et al. 2003). *VPI/ABI3*-like genes are present in a wide variety of seed plants (Hattori et al. 1994; Bobb et al. 1995; Chandler and Bartels 1997; Shiota et al. 1998; Lazaroova et al. 2002; Rohde et al. 2002; Footitt et al. 2003). In addition, we have also isolated three VP1/ABI3 family members from the moss, *Physcomitrella patens*, indicative of a vegetative function for this gene family (Marella et al. 2006). These results clearly indicate that VP1/ABI3 is not only involved in the ABA-regulation of seed development and germination, but also has broader functions in vegetative growth.

Previous work has demonstrated the existence of the ABA response pathway in *P. patens*, using the ABA-responsive wheat *Em* promoter linked to β -glucuronidase (GUS) as a reporter. The wheat *Em* promoter can be activated in protonemal tissue by exogenous ABA in a transient assay as well as in stable expression lines of *P. patens* (Knight et al. 1995). These results suggest that seed plants and *P. patens* share a similar ABA network and can be utilized for comparative functional studies of the ABA signaling network.

All of the *VPI/ABI3*-like genes cloned reveal highly conserved protein domains, designated A1, B1, B2, and B3, starting from the N-terminal (Giraudat et al. 1992; Suzuki et al. 1997). The A1 domain is a functional acidic activation domain found at the N-terminus of VP1 (McCarty et al. 1991). The B1 domain is involved in the physical interaction with the bZIP transcription factor, ABSCISIC ACID INSENSITIVE5 (ABI5) (Nakamura et al. 2001). The B3 domain has been shown to bind

DNA in vitro (Suzuki et al. 1997; Nag et al. 2005) and is found in other plant-specific transcription factors (Riechmann et al. 2000). The B2 domain consists of 16 amino acids, half of which are basic residues, and contains a putative nuclear localization signal (NLS), ARKKR (Giraudat et al. 1992). The B2 domain has been shown to be responsible for the ABA-dependent activation of ABA-regulated genes, like *Em*, through the ABA-response element (ABRE) (Hill et al. 1996; Bies-Etheve et al. 1999; Ezcurra et al. 2000), and facilitates the interaction with bZIP transcription factors (Hill et al. 1996) such as ABI5. The *Arabidopsis abi3-7* mutation converts a conserved alanine to a threonine in the putative NLS of the B2 domain and the mutants have reduced or no expression of *AtEm1* and *AtEm6*, respectively (Bies-Etheve et al. 1999). Deletion of the B2 domain from VP1 results in the loss of transactivation of the *Em* promoter but this could be due to the loss of the putative NLS (Hill et al. 1996). It is still unclear by which mechanisms this domain contributes to the overall function of VP1/ABI3.

To address the issue of localization, we used green fluorescent protein (GFP) fusion proteins to demonstrate that VP1 localizes to the nucleus of barley and *P. patens* cells and that the NLS resides in the amino-terminal half of VP1. An alignment of B2 domains of VP1/ABI3 proteins from widely diverged plant species revealed other conserved amino acids surrounding the putative NLS. Mutating the conserved threonine and an arginine-glutamine-arginine motif, in conjunction with the putative NLS, disrupted the nuclear localization of VP1. We also show that two different amino acid mutations in the NLS region prevent transactivation but do not disrupt nuclear localization. Hence, specific amino acids within the B2 domain of VP1 regulate both nuclear localization and gene expression.

Materials and methods

Plant material

Physcomitrella patens subspecies *patens* (Gransden) was used as the wild type strain. The strain was maintained as previously described (Bezanilla et al. 2003). Briefly, protonemal tissue was ground with a PowerGen 125 homogenizer (Fisher Science, Hampton, NH, USA) and inoculated onto cellophane overlaid on 0.7% agar plates of PpNH₄ medium (1.8 mM KH₂PO₄, pH 7.0, 3.4 mM Ca(NO₃)₂, 1 mM MgSO₄, 45 mM FeSO₄, 0.22 mM CuSO₄, 0.19 μ M ZnSO₄, 9.9 μ M H₃BO₃, 2 μ M MnCl₂, 0.23 μ M CoCl₂, 0.17 μ M KI, 0.1 μ M Na₂MoO₄, 2.7 μ M ammonium tartrate). The

tissue was cultured at 25°C under the cycle of 16 h light (90 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and 8 h dark.

Barley seeds (*Hordeum vulgare* cv *Himalaya*) from 1998 harvests at Washington State University in Pullman were used in all experiments.

DNA constructs

The effector construct of *Ubi-VP1* was made as follows: the coding sequence of VP1 was amplified by PCR from the cDNA, and subcloned into pGEM-Teasy (Promega, Madison, WI, USA). Accuracy of the amplified fragment was checked by sequencing and then placed downstream of the maize *Ubi1* promoter by a *SpeI* digestion (Christensen and Quail 1996), followed by the terminator of nopalinsynthetase gene, inserted by *NotI* digestion. The GFP fusion was generated by PCR to add GFP to the C-terminal end as a translational fusion, removing the stop codon of VP1 and the ATG of GFP, in the respective *Ubi-VP1-nos* vector. The *Ubi-VP1-N::GFP-nos* and *Ubi-VP1-C::GFP-nos* constructs were generated by PCR to add *SpeI* sites to the ends in order to swap out full-length VP1 from *Ubi-VP1::GFP-nos*. *Ubi-VP1-N::GFP-nos* consists of amino acids 1–501 of VP1, while *Ubi-VP1-C::GFP-nos* consists of a methionine followed by amino acids 502–691 of VP1. The *Ubi-VP1-ΔB2-nos* construct was generated by using PCR to add compatible restriction site ends (*SpeI*) on *VP1-ΔB2* (described in Hill et al. 1996) and cloning as described above for *Ubi-VP1::GFP-nos*. The resulting *Ubi-VP1-N::GFP-nos*, *Ubi-VP1-C::GFP-nos*, and *Ubi-VP1-ΔB2::GFP-nos* constructs were checked by sequencing. Point mutations to VP1 were generated with the Quik Change XL kit (Stratagene, La Jolla, CA, USA). The resulting point mutants were checked for accuracy by sequencing. *Em-GUS* (pBM113KP, Marcotte et al. 1989), *Ubi-LUC* (pAHC18, Bruce et al. 1989), and *Ubi-GFP* (Zentella et al. 2002), were described previously.

Transient assays

In principle, DNA delivery to barley aleurone cells was performed as described previously (Shen et al. 1993). Five micrograms of the GFP fusion constructs were used to prepare tungsten particles for two shots. Bombarded seeds were incubated for 18–24 h at room temperature before imaging. In the *Em-GUS* transactivation assays, we used 2 μg of each reporter construct (*Em-GUS* and *Ubi-LUC*) and effector construct to prepare tungsten particles for two shots. Seeds were incubated for 20 h at room temperature before protein extraction.

Transient transformation of *P. patens* by particle bombardment was carried out as described (Bezanilla et al. 2003). For nuclear localization of the GFP fusion constructs, 2 μg of DNA was used to prepare gold particles for four shots. The bombarded moss tissue was incubated for 45–48 h before imaging.

Four barley half-seeds for each replicate were ground in extraction buffer (Lanahan et al. 1992) and cell debris was spun down. GUS and luciferase (LUC) activities were measured as described (Lanahan et al. 1992). GUS activity was normalized by the LUC activity and represented as relative GUS activity (GUS/LUC) \pm SE. All the experiments consisted of four replicates.

Microscopy

Confocal microscopy was performed using barley aleurone layers or protonemal tissue of *P. patens* placed in water on a glass slide and covered with a cover slip. A Leica Confocal System TCS SP2 (Mannheim, Germany) was used for imaging GFP signal with an argon laser.

Image analysis was performed with ImageJ software (Abramoff et al. 2004). The plot profile tool was used to quantify the GFP signal intensity in the cytoplasm and the nucleus of each transformed cell. The ratio of cytoplasmic to nuclear signal was used as the measure of localization of the GFP fusion proteins.

Results

VP1 localizes to the nucleus

We investigated the sub-cellular localization of VP1 by a GFP fusion construct (*VP1::GFP*) introduced into barley aleurone and *P. patens* protonemal cells via particle bombardment. The transient expression was analyzed by confocal microscopy. In both barley aleurone cells and *P. patens* bombarded with *VP1::GFP*, the GFP signal localizes at the nucleus (Fig. 1a, b), while the GFP control displays a diffuse cytoplasmic and nuclear localization (Marella et al. 2006).

In order to identify the region(s) responsible for the nuclear localization, we split VP1 into two parts, VP1-N and VP1-C, and fused each to GFP (Fig. 2a). We quantified the level of GFP localization between the cytoplasm and nucleus for VP1, GFP, VP1-N, and VP1-C. The N-terminal half of VP1, which contains the activation, B1, and B2 domains, localizes to the nucleus of barley aleurone cells (GFP signal ratio of 0.47), similar to that of VP1 (0.36) (Fig. 2b, d). The C-terminal half of VP1, which contains the B3 domain, fused to GFP shows a diffuse signal throughout the aleurone

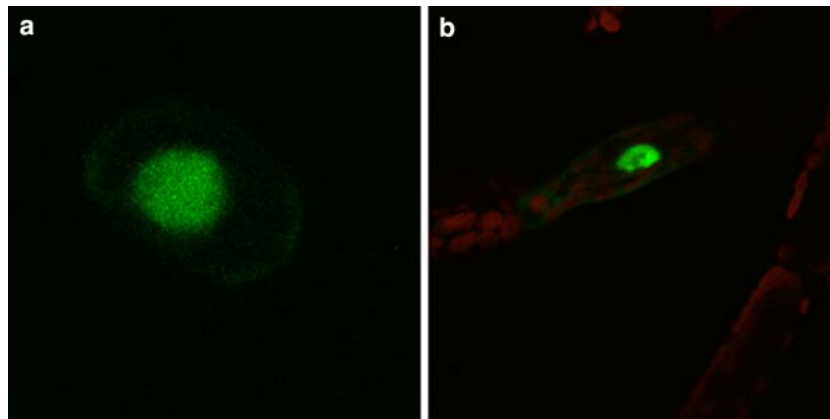


Fig. 1 VP1 localizes to the nucleus of barley aleurone and *Physcomitrella patens* cells. A construct for the over-expression of VP1::GFP was delivered into **a** barley aleurone cells and **b** *P. patens* protonemal tissue by particle bombardment. Images of trans-

formed cells were taken by laser-scanning confocal microscopy. The GFP signal and auto-fluorescence of chloroplasts are shown by *green* and *red* color, respectively

cell as does the GFP control (Fig. 2c, d). These data indicate that the NLS(s) resides in the N-terminal half of the VP1 protein.

Role of the B2 domain in nuclear localization

It has been noted that the B2 domain of the VP1/ABI3 protein family contains a NLS motif (ARKKR), which is similar to known NLSs from other organisms (Giraudat et al. 1992). Since the B2 domain was present in the part of VP1 that localized to the nucleus, we tested a B2 deletion ($\Delta 386\text{--}406$) mutant of VP1 (VP1- Δ B2::GFP) (Fig. 3a) and tested for nuclear localization by particle bombardment (Fig. 3b, c). The GFP signal ratio between the cytoplasm and nucleus of barley aleurone cells was slightly lower (1.0) than the GFP control (1.3) (Fig. 3d). This result indicates that the B2 domain is necessary for the nuclear localization of VP1.

We generated a series of point mutations in the putative NLS of the B2 domain, ARKKR (Fig. 4a). Mutating all five residues in ARKKR to TGEEG (construct VP1-A) (Fig. 4b) did not dramatically alter the nuclear localization of the VP1-A mutant (0.5) (Fig. 4c). In order to identify other critical residues in B2, we aligned the B2 domains of VP1 and ABI3 the *P. patens* ABI3 (Fig. 4a) since VP1 (Fig. 1a) as well as PpABI3A localize to the nucleus in aleurone cells (Marella et al. 2006). A conserved threonine, on the N-terminal side of the ARKKR motif, and a RQR motif, on the C-terminal side, were found and subjected to point mutagenesis (Fig. 4b). Altering the threonine to serine (construct VP1-B) did not affect nuclear localization of VP1 nor did changing the RQR to SDG (construct VP1-C) (Fig. 4c). However, these mutations in combination with the mutated putative NLS (con-

structs VP1-A + B and VP1-A + C), resulted in a loss of VP1 to specifically localize to the nucleus with signal ratios similar to that of the GFP control (Fig. 4c).

Since we determined the B2 domain was necessary for the nuclear localization of VP1, we asked if it is also sufficient as a NLS. The amino acids deleted from VP1 in the VP1- Δ B2::GFP construct were placed at the N-terminus of a GFP::GUS fusion protein (Fig. 5a). The resulting B2::GFP::GUS fusion failed to specifically localize to the nucleus when transiently expressed in barley aleurone cells and *P. patens* cells (Fig. 5b, c). The GFP signal ratio of the B2::GFP::GUS was exactly the same level seen in the GFP control (Fig. 5d). These data indicate that the B2 domain of VP1 is necessary but not sufficient for nuclear localization.

The B2 domain is also involved in transactivation

Previous work has shown that the B2 domain is critical to VP1/ABI3's ability to transactivate ABA-regulated genes (Hill et al. 1996; Bies-Etheve et al. 1999; Ezcurra et al. 2000). However, these results are complicated by the role of the B2 domain in nuclear localization, since a loss of nuclear localization could also alter the transactivation of genes by VP1/ABI3. We determined if the amino acids required for transactivation were within the NLS. The *abi3-7* mutant contains the A458T point mutation in the B2 domain of *Arabidopsis* ABI3 and results in the absence of *AtEm6* and proper *AtEm1* expression (Bies-Etheve et al. 1999). Recapitulated in VP1 as the A391T mutation, this point mutation localizes to the nucleus but fails to transactivate *Em-GUS* expression in barley aleurone cells (Fig. 6). We then tested two other sets of mutations, which retained nuclear localization, for defects in *Em-GUS* expression.

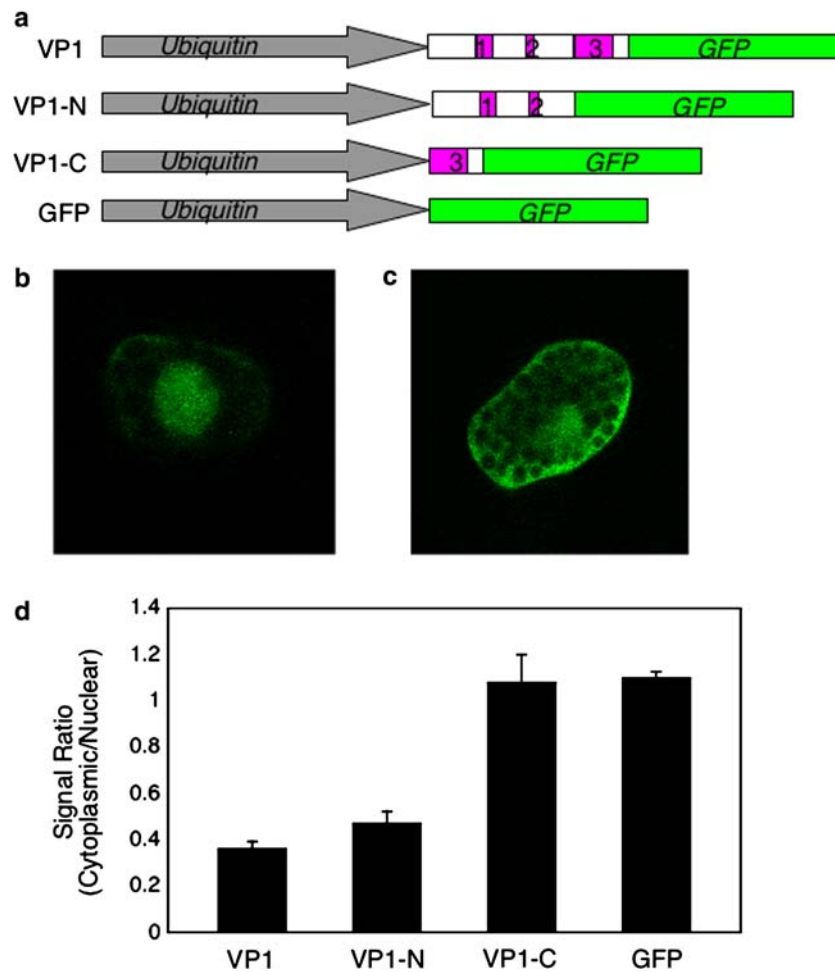


Fig. 2 The N-terminal half of VP1 localizes to the nucleus. **a** Schematics of the constructs used for the over-expression of VP1, the N- and C-terminal halves of VP1, and GFP. The basic domains of VP1 are indicated by the pink boxes. The maize *Ubiquitin* promoter was used to drive expression in both barley aleurone and *P. patens* cells. **b** Confocal image of VP1-N::GFP localization in a barley aleurone cell. **c** Confocal image of VP1-C::GFP localization in a barley aleurone cell. **d** Five micrograms of the constructs

from panel **a** were bombarded into barley aleurone cells and the half seeds were incubated for 20 h. The aleurone layers were isolated and transformed cells were imaged by confocal microscopy so that the center of the nucleus was in focus. ImageJ software was used to quantify the levels of GFP in the nucleus and cytoplasm of the cells. The signal ratio between the cytoplasmic and nuclear GFP was calculated as a measure of localization. VP1 ($n = 10$), VP1-N ($n = 13$), VP1-C ($n = 15$), GFP ($n = 13$)

The VP1-K393,394E mutant also has reduced transactivation of *Em-GUS*, although the level of reduction is not quite as strong as the VP1-A391T mutant (Fig. 6). The VP1-R392,395G mutant does not display a defect in *Em-GUS* transactivation (Fig. 6). These data indicate that residues embedded within the NLS of VP1 also play a role in transactivation of ABA-regulated genes.

Discussion

We demonstrate that the B2 domain of VP1 is bi-functional in nature, regulating both nuclear localization and transactivation. Using a GFP fusion protein, we show that VP1 localizes to the nucleus of barley aleurone and

P. patens cells, indicating a conservation of nuclear receptors. Similar results are observed for AtABI3 and PpABI3A (Marella et al. 2006) showing a conservation of NLSs of these orthologs as well. VP1 has several regions of basic amino acids throughout the protein that may act as a NLS. We initially determined that an NLS resides in the amino-terminal half of the protein, which contains the basic amino acid domains B1 and B2. Since the ARKKR motif in the B2 domain appears most similar to a monopartite NLS, we deleted the entire B2 domain (VP1-ΔB2::GFP) and found it is required for nuclear localization. Since VP1, AtABI3, and PpABI3A are localized to the nucleus of cells from both barley aleurone and *P. patens* protonemal filaments, we compared the B2 domains of *P. patens* and seed plants.

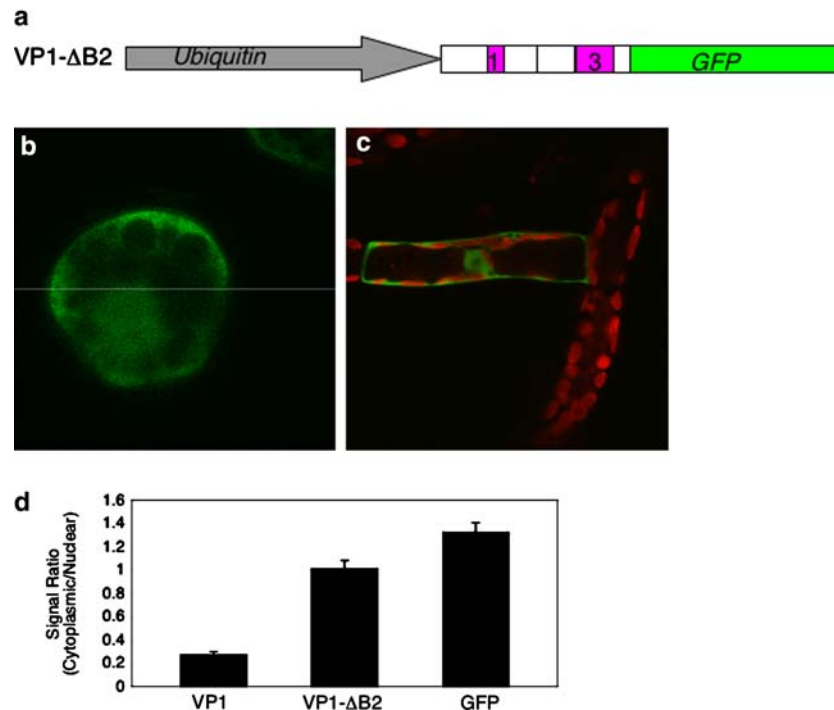


Fig. 3 The deletion of the B2 domain of VP1 results in a loss of nuclear localization. **a** A schematic of the VP1-ΔB2::GFP construct used for localization in barley aleurone and *P. patens* cells. **b** Confocal image of VP1-ΔB2::GFP localization in a barley aleurone cell. **c** Confocal image of VP1-ΔB2::GFP localization in *P. patens* protonema. **d** Five micrograms of the VP1-ΔB2::GFP construct and VP1 and GFP controls were bombarded into barley

aleurone cells and the half seeds were incubated for 20 h. The aleurone layers were isolated and transformed cells were imaged by confocal microscopy so that the center of the nucleus was in focus. ImageJ software was used to quantify the levels of GFP in the nucleus and cytoplasm of the cells. The signal ratio between the cytoplasmic and nuclear GFP was calculated as a measure of localization. VP1 ($n = 26$), VP1-ΔB2 ($n = 36$), GFP ($n = 37$)

Alignment of the B2 domains of VP1/ABI3 proteins from *P. patens*, maize, and *Arabidopsis* revealed other conserved amino acids. Mutating the conserved threonine (T388) and an arginine-glutamine-arginine motif, along with the amino acids in the putative NLS (ARKKR), prevented the nuclear localization of VP1.

The identification of T388 in VP1 as an important residue for nuclear localization raises the possibility that phosphorylation plays a role in the localization of VP1. Phosphorylation is a common mechanism for regulation of nuclear localization (cf. Poon and Jans 2005). In fact, a phosphorylated threonine residue found close to the NLS (like T388 of VP1) has been implicated in the nuclear localization of a vertebrate transcription factor (Sheng et al. 2006). Mutation of the conserved arginine-glutamine-arginine motif in addition to the ARKKR also resulted in a loss of nuclear localization. This RQR motif is also found in the B2-like domain of FUSCA3 (FUS3), another B3 domain containing transcriptional activator of seed development in *Arabidopsis* (Luerssen et al. 1998). It will be interesting to see if these amino acids also function in the nuclear localization of FUS3, as they do for VP1.

The B2 domain itself is not sufficient to cause the specific nuclear localization of a GFP::GUS fusion protein, however. Instead, we observe a diffuse GFP signal throughout the nucleus and cytoplasm. In order for a NLS to interact with importin it needs to be on an exposed portion of the folded protein. In the case of OPAQUE2, the fusion of the NLS-B region N-terminal to GUS was clearly more efficient at nuclear localization than the C-terminal fusion (Varagona et al. 1992). It is possible that the NLS is not properly exposed in the B2::GFP::GUS fusion. It may also be possible that another NLS exists in VP1. The VP1-ΔB2::GFP is found in both the nucleus and cytoplasm but yet its localization is consistently and significantly slightly more nuclear than the GFP control. A protein of this size (approximately 100 kDa) would not be expected to diffuse into the nucleus like the GFP control (cf. Weis 2003). The fact that we still observe a nuclear GFP signal, albeit weak, may be an indication that another NLS in VP1 is functioning to cause an intermediate accumulation in the nucleus. If so, both NLSs are required for full localization, as is the case with OPAQUE2 (Varagona and Raikhel 1994).

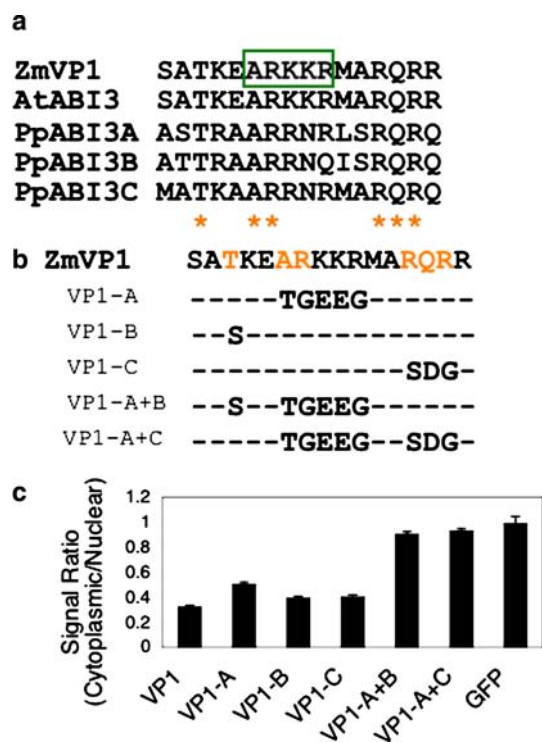


Fig. 4 A comparison of B2 domains across species identifies residues important for nuclear localization. **a** The alignment of the B2 domains from maize VP1, *Arabidopsis* ABI3, and *P. patens* ABI3A, ABI3B, and ABI3C. Perfectly conserved amino acids are indicated by orange asterisks, while the putative NLS is outlined by a green box. **b** A series of point mutations were generated altering the amino acids of the B2 domain of VP1. The amino acids in orange are those perfectly conserved in the VP1/ABI3 family from panel **a**. **c** Five micrograms of the mutant constructs in panel **b** and VP1 and GFP controls were bombarded into barley aleurone cells and the half seeds were incubated for 18 h. The aleurone layers were isolated and transformed cells were imaged by confocal microscopy so that the center of the nucleus was in focus. ImageJ software was used to quantify the levels of GFP in the nucleus and cytoplasm of the cells. The signal ratio between the cytoplasmic and nuclear GFP was calculated as a measure of localization. VP1 ($n = 23$), VP1-A ($n = 24$), VP1-B ($n = 22$), VP1-C ($n = 22$), VP1-A + B ($n = 20$), VP1-A + C ($n = 20$), GFP ($n = 19$)

Multiple NLSs are commonly found in nuclear localized proteins and it may be that multiple NLSs help large proteins enter the nucleus more efficiently (Dingwall et al. 1982; Dworetzky and Feldherr 1988). Since VP1 has 691 amino acids, more than 200 amino acids larger than OPAQUE2, it is feasible that another NLS exists and acts in coordination with the NLS found in the B2 domain. A likely candidate for the location of another NLS is the B1 domain, especially since it is rich in lysines and arginines that could compose either a monopartite or bipartite basic-type NLS. This hypothesis is further supported by our result showing that the amino-terminal half of VP1 (which contains B1 and

B2) could localize GFP to the nucleus as efficiently as full length VP1. For this study, we conclude that B2 is absolutely required and that some part of B1, most likely, may aid in the localization.

Since the B2 domain has also been implicated in the transactivation of ABA-regulated genes, we tested our NLS point mutants for their ability to transactivate the wheat *Em* promoter in barley aleurone to determine if the B2 domain has multiple functions. It is not uncommon for plant transcriptional regulators to have a NLS in domains with other functions, especially DNA binding domains (Liu et al. 1999; Cokol et al. 2000), so we sought to resolve the two functions of the B2 domain of VP1. First, we showed that the VP1-ΔB2 failed to transactivate *Em-GUS* (Fig. 6; Hill et al. 1996), but this failure could be attributed to its defect in nuclear localization. Utilizing point mutations in the B2 domain, rather than the full deletion of the domain from VP1, allowed us to examine the role of the amino acids in transactivation apart from their role in nuclear localization. Amongst the point mutations in the B2 domain that still properly localize to the nucleus, two mutations, A391T and K393,394E, significantly reduce the transactivation of *Em-GUS*. The A391T mutation, which alters the highly conserved alanine located in the NLS, also has a distinct role in *Em-GUS* transactivation; the effect of this single amino acid change is nearly as strong as the deletion of the B2 domain from VP1. A possible explanation is that the A391T mutation introduces a phosphorylated threonine that inhibits the proper functioning of VP1. This hypothesis is supported by the phosphorylation prediction software, Net Phos 2.0 Server (Blom et al. 1999). The K393, 394E mutations also reduce *Em-GUS* transactivation. These mutations switch basic amino acids for acidic amino acids, which could disrupt the chemistry of the observed non-specific interaction between the B2 domain and DNA (Hill et al. 1996). In contrast, the R392,395G mutations within the NLS do not alter the transactivation of *Em-GUS*, indicating that not all the residues involved in nuclear localization are also essential for *Em-GUS* expression. Perhaps the net change in charge of the R392,395G mutations is not as dramatic as the K393,394E mutations in weakening the interaction between DNA and the B2 domain. Further biochemical analysis of VP1 and the point mutations may reveal more about the specific role of the B2 in transactivation.

In summary, we have been able to uncouple several overlapping functions of the B2 domain using point mutations specifically conserved between bryophytes (moss) and angiosperms (barley). This type of comparative approach resulted in the experimental

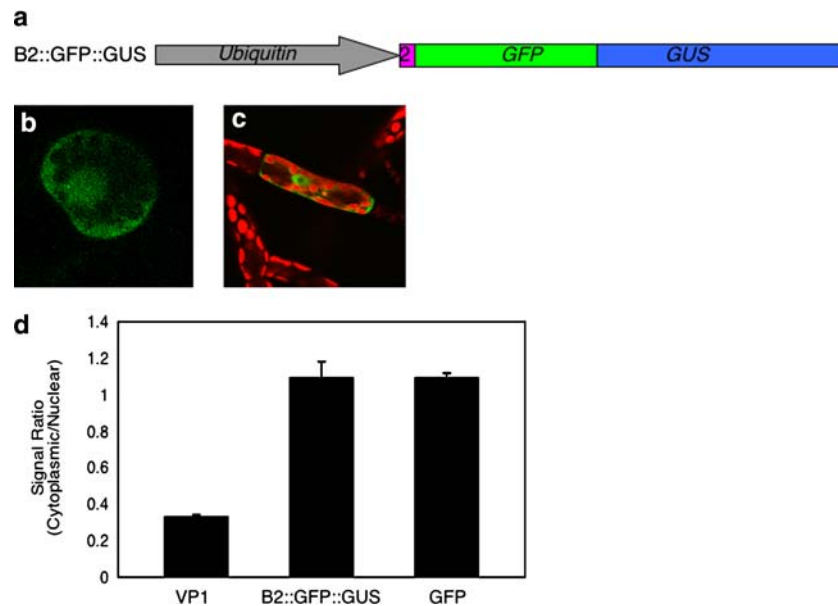


Fig. 5 The B2 domain is not sufficient for nuclear localization. **a** A schematic of the B2::GFP::GUS construct. **b** Confocal image of B2::GFP::GUS localization in a barley aleurone cell. **c** Confocal image of B2::GFP::GUS localization in *P. patens* protonema. **d** Five micrograms of the B2::GFP::GUS construct and VP1 and GFP controls were bombarded into barley aleurone cells and the half seeds were incubated for 22 h. The aleurone layers were iso-

lated and transformed cells were imaged by confocal microscopy so that the center of the nucleus was in focus. ImageJ software was used to quantify the levels of GFP in the nucleus and cytoplasm of the cells. The signal ratio between the cytoplasmic and nuclear GFP was calculated as a measure of localization. VP1 ($n = 15$), B2::GFP::GUS ($n = 6$), GFP ($n = 13$)

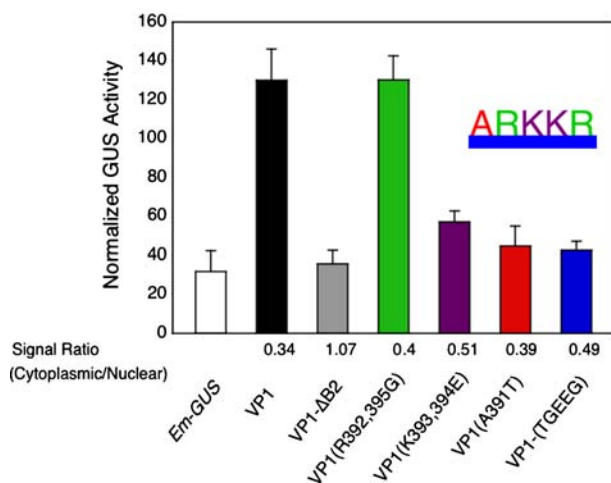


Fig. 6 Amino acids in the B2 domain are required for transactivation of *Em-GUS*. *Em-GUS* and *Ubi-LUC* were co-bombarded into barley aleurone cells with or without the *Ubi-VP1* effector constructs, using 2 μ g of each construct. Bars indicate the relative GUS activities \pm SE after 20 h of incubation ($n = 4$). The bar color of the B2 domain mutant constructs matches the amino acids altered in the putative NLS sequence in the upper right of the graph. The nuclear localization of each construct is indicated by the signal ratio (cytoplasmic/nuclear) under the graph

identification of the amino acids of the B2 domain involved in the nuclear localization of VP1. We also show that the phenotypes of the *abi3-7* mutant are not

the result of altering the NLS, but that rather they are strictly tied to transactivation. Currently, structure/function analysis of the VP1/ABI3 family is limited by the lack of an overall three-dimensional structure, however recent progress has been made with determining the structure of B3 domains from other *Arabidopsis* proteins (Yamasaki et al. 2004; Waltner et al. 2005). Until similar structural information is available for the B2 domain, it will be necessary to use point mutations, such as those generated in this study, to further investigate the roles of this pivotal domain.

Acknowledgments We thank Dr. Tuan-hua David Ho and Dr. Jose Casaretto for providing the DNA constructs of *Ubi-GFP* and *Ubi-LUC*, and also for their technical assistance with the particle bombardment of barley aleurone cells. We thank Aihong Pan for her assistance. This research was supported by Washington University to R.S.Q.

References

- Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image processing with ImageJ. *Biophoton Int* 11:36–42
- Bezanilla M, Pan A, Quatrano RS (2003) RNA interference in the moss *Physcomitrella patens*. *Plant Physiol* 133:470–474
- Bies-Etheve N, da Silva Conceicao A, Giraudat J, Koornneef M, Leon-Kloosterziel K, Valon C, Delseny M (1999) Importance of the B2 domain of the *Arabidopsis* ABI3 protein for *Em* and 2S albumin gene regulation. *Plant Mol Biol* 40:1045–1054

- Blom N, Gammeltoft S, Brunak S (1999) Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* 294:1351–1362
- Bobb AJ, Eiben HG, Bustos MM (1995) PvAlf, an embryo-specific acidic transcriptional activator enhances gene expression from phaseolin and phytohemagglutinin promoters. *Plant J* 8:331–343
- Brady SM, Sarkar SF, Bonetta D, McCourt P (2003) The *ABSCISIC ACID INSENSITIVE 3 (ABI3)* gene is modulated by farnesylation and is involved in auxin signaling and lateral root development in *Arabidopsis*. *Plant J* 34:67–75
- Bruce WB, Christensen AH, Klein T, Fromm M, Quail PH (1989) Photoregulation of a phytochrome gene promoter from oat transferred into rice by particle bombardment. *Proc Natl Acad Sci USA* 86:9692–9696
- Chandler JW, Bartels D (1997) Structure and function of the *vpl* gene homologue from the resurrection plant *Craterostigma plantagineum* Hochst. *Mol Gen Genet* 256:539–546
- Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res* 5:213–218
- Cokol M, Nair R, Rost B (2000) Finding nuclear localization signals. *EMBO Rep* 1:411–415
- Dingwall C, Sharnick SV, Laskey RA (1982) A polypeptide domain that specifies migration of nucleoplasm into the nucleus. *Cell* 30:449–458
- Dworetzky SI, Feldherr CM (1988) Translocation of RNA-coated gold particles through the nuclear pores of oocytes. *J Cell Biol* 106:575–584
- Ezcurra I, Wycliffe P, Nehlin L, Ellerstrom M, Rask L (2000) Transactivation of the *Brassica napus* napin promoter by ABI3 requires interaction of the conserved B2 and B3 domains of ABI3 with different cis-elements: B2 mediates activation through an ABRE, whereas B3 interacts with a RY/G-box. *Plant J* 24:57–66
- Footitt S, Ingouff M, Clapham D, von Arnold S (2003) Expression of the *viviparous 1 (Pavp1)* and *p34cdc2 protein kinase (cdc2Pa)* genes during somatic embryogenesis in Norway spruce (*Picea abies* [L.] Karst). *J Exp Bot* 54:1711–1719
- Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM (1992) Isolation of the *Arabidopsis ABI3* gene by positional cloning. *Plant Cell* 4:1251–1261
- Hattori T, Terada T, Hamasuna ST (1994) Sequence and functional analyses of the rice gene homologous to the maize *Vp1*. *Plant Mol Biol* 24:805–810
- Hill A, Nantel A, Rock CD, Quatrano RS (1996) A conserved domain of the *viviparous-1* gene product enhances the DNA binding activity of the bZIP protein EmBP-1 and other transcription factors. *J Biol Chem* 271:3366–3374
- Knight CD, Sehgal A, Atwal K, Wallace JC, Cove DJ, Coates D, Quatrano RS, Bahadur S, Stockley PG, Cumings AC (1995) Molecular responses to abscisic acid and stress are conserved between moss and cereals. *Plant Cell* 7:499–506
- Koornneef M, Reuling G, Karssen CM (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. *Physiol Plant* 61:377–383
- Lanahan MB, Ho TH, Rogers SW, Rogers JC (1992) A gibberellin response complex in cereal alpha-amylase gene promoters. *Plant Cell* 4:203–211
- Lazarova G, Zeng Y, Kermod AR (2002) Cloning and expression of an *ABSCISIC ACID-INSENSITIVE 3 (ABI3)* gene homologue of yellow-cedar (*Chamaecyparis nootkatensis*). *J Exp Bot* 53:1219–1221
- Liu L, White MJ, MacRae TH (1999) Transcription factors and their genes in higher plants functional domains, evolution and regulation. *Eur J Biochem* 262:247–257
- Luerssen H, Kirik V, Herrmann P, Misera S (1998) *FUSCA3* encodes a protein with a conserved VP1/ABI3-like B3 domain which is of functional importance for the regulation of seed maturation in *Arabidopsis thaliana*. *Plant J* 15:755–764
- Marcotte WR Jr, Russell SH, Quatrano RS (1989) Abscisic acid-responsive sequences from the *em* gene of wheat. *Plant Cell* 1:969–976
- Marella HH, Sakata Y, Quatrano RS (2006) Characterization and functional analysis of *ABSCISIC ACID INSENSITIVE3*-like genes from *Physcomitrella patens*. *Plant J* 46:1032–1044
- McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK (1991) The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. *Cell* 66:895–905
- Nag R, Maity MK, Dasgupta M (2005) Dual DNA binding property of *ABA insensitive 3* like factors targeted to promoters responsive to ABA and auxin. *Plant Mol Biol* 59:821–838
- Nakamura S, Lynch TJ, Finkelstein RR (2001) Physical interactions between ABA response loci of *Arabidopsis*. *Plant J* 26:627–635
- Nambara E, Keith K, McCourt P, Naito S (1995) A regulatory role for the *ABI3* gene in the establishment of embryo maturation in *Arabidopsis thaliana*. *Development* 121:629–636
- Nambara E, Hayama R, Tsuchiya Y, Nishimura M, Kawaide H, Kamiya Y, Naito S (2000) The role of *ABI3* and *FUS3* loci in *Arabidopsis thaliana* on phase transition from late embryo development to germination. *Dev Biol* 220:412–423
- Parcy F, Valon C, Kohara A, Misera S, Giraudat J (1997) The *ABSCISIC ACID-INSENSITIVE3*, *FUSCA3*, and *LEAFY COTYLEDON1* loci act in concert to control multiple aspects of *Arabidopsis* seed development. *Plant Cell* 9:1265–1277
- Poon IK, Jans DA (2005) Regulation of nuclear transport: central role in development and transformation? *Traffic* 6:173–186
- Riechmann JL, Heard J, Martin G, Reuber L, Jiang C, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu G (2000) *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. *Science* 290:2105–2110
- Robertson D (1955) The genetics of vivipary in maize. *Genetics* 40:745–760
- Rohde A, Kurup S, Holdsworth M (2000) *ABI3* emerges from the seed. *Trends Plant Sci* 5:418–419
- Rohde A, Prinsen E, De Rycke R, Engler G, Van Montagu M, Boerjan W (2002) PtABI3 impinges on the growth and differentiation of embryonic leaves during bud set in poplar. *Plant Cell* 14:1885–1901
- Shen Q, Uknes SJ, Ho TH (1993) Hormone response complex in a novel abscisic acid and cycloheximide-inducible barley gene. *J Biol Chem* 268:23652–23660
- Sheng T, Chi S, Zhang X, Xie J (2006) Regulation of Gli1 localization by the cAMP/protein kinase A signaling axis through a site near the nuclear localization signal. *J Biol Chem* 281:9–12
- Shiota H, Satoh R, Watabe K, Harada H, Kamada H (1998) C-ABI3, the carrot homologue of the *Arabidopsis* ABI3, is expressed during both zygotic and somatic embryogenesis and functions in the regulation of embryo-specific ABA-inducible genes. *Plant Cell Physiol* 39:1184–1193
- Suzuki M, Kao CY, McCarty DR (1997) The conserved B3 domain of VIVIPAROUS1 has a cooperative DNA binding activity. *Plant Cell* 9:799–807
- Suzuki M, Kao CY, Coccione S, McCarty DR (2001) Maize VP1 complements *Arabidopsis abi3* and confers a novel ABA/auxin interaction in roots. *Plant J* 28:409–418
- Varagona MJ, Raikhel NV (1994) The basic domain in the bZIP regulatory protein Opaque2 serves two independent

- functions: DNA binding and nuclear localization. *Plant J* 5:207–214
- Varagona MJ, Schmidt RJ, Raikhel NV (1992) Nuclear localization signal(s) required for nuclear targeting of the maize regulatory protein Opaque-2. *Plant Cell* 4:1213–1227
- Vasil V, Marcotte WR Jr, Rosenkrans L, Cocciolone SM, Vasil IK, Quatrano RS, McCarty DR (1995) Overlap of Viviparous1 (VP1) and abscisic acid response elements in the *Em* promoter: G-box elements are sufficient but not necessary for VP1 transactivation. *Plant Cell* 7:1511–1518
- Waltner JK, Peterson FC, Lytle BL, Volkman BF (2005) Structure of the B3 domain from *Arabidopsis thaliana* protein At1g16640. *Protein Sci* 14:2478–2483
- Weis K (2003) Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. *Cell* 112:441–451
- Yamasaki K, Kigawa T, Inoue M, Tateno M, Yamasaki T, Yabuki T, Aoki M, Seki E, Matsuda T, Tomo Y, Hayami N, Terada T, Shirouzu M, Osanai T, Tanaka A, Seki M, Shinozaki K, Yokoyama S (2004) Solution structure of the B3 DNA binding domain of the *Arabidopsis* cold-responsive transcription factor RAV1. *Plant Cell* 16:3448–3459
- Zentella R, Yamauchi D, Ho TH (2002) Molecular dissection of the gibberellin/abscisic acid signaling pathways by transiently expressed RNA interference in barley aleurone cells. *Plant Cell* 14:2289–2301