Analysis of an activated *ABI5* allele using a new selection method for transgenic *Arabidopsis* seeds

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Abstract The *Arabidopsis* abscisic acid (ABA) insensitive (ABI)5 transcription factor participates in the ABA-dependent induction of late embryogenesis abundant (LEA) genes in the final stages of seed development. We tested whether the VP16 transcriptional activation domain is sufficient to provide ABI5 with the ability to activate the *AtEm* LEA genes in vegetative tissues. We took advantage of a new transgenic seed selection assay based on green fluorescent protein (GFP) fluorescence and found that VP16-ABI5 triggered growth retardation and ABA-independent induction of *AtEm1* in seedlings. These results indicate that ABI5 activation potential is a limiting step and might be a target for ABA signaling.

Key words: Abscisic acid insensitive 5; Abscisic acid; Transgenic seed selection; Late embryogenesis abundant gene regulation

1. Introduction

Towards the end of their development, seeds lose most of their water. In order to survive this process, they acquire desiccation tolerance. Late embryogenesis abundant (LEA) genes are expressed during the desiccation phase and are thought to help protect the embryo [1]. Abscisic acid (ABA) plays an important role in this process since mutants impaired either in ABA biosynthesis or sensitivity have reduced desiccation tolerance and/or LEA gene expression. In particular, the expressions of the model LEA genes *AtEm1* and *AtEm6* are decreased in the *Arabidopsis thaliana ABA insensitive 3* (*abi3*) and *abi5* mutants [2,3]. *ABI5* encodes a basic leucine zipper transcription factor [4,5] and binds in vitro to *cis* elements (called ABA responsive element or ABRE) present in *AtEm* promoters [6,7]. ABI5 is thus assumed to directly activate *AtEm* expression in plants. This activation is dependent on endogenous or externally applied ABA [3,8]. However, how it is achieved at the molecular level remains to be understood. ABI5 has a cryptic activation domain but does not, on its own, activate transcription in yeast [9]. Also, *ABI5* ectopic expression in vegetative tissues is not sufficient to induce *AtEm* [10]. ABI3 has been suggested to act as an ABI5 coactivator but this point remains controversial [8,9]. Here we test whether the strong activation domain of the VP16 protein (VP16-AD) [11] is sufficient to provide ABI5 with the ability to induce *AtEm* gene expression. For these experiments, we constructed a vector set combining the GATEWAY™ technology and a new selection method for transgenic seeds based on the expression of the green fluorescent protein (GFP). Our results show that ABI5 activation potential is indeed limiting and might be a target of the ABA signaling cascade.

2. Materials and methods

2.1. Materials

We used the *abi5-1* mutant [2] in the Wassilewskija (Ws) accession and *AtEm1::GUS* (*Em1143*) and *AtEm6::GUS* (*Em6-21c*) lines in the C24 accession [12].

2.2. Methods

2.2.1. Generation of transgenic plants. The FP100 vector was constructed by combining the *At2S3* promoter and *GFP* sequences [13] with a 35S terminator in pZP200 [14]. A double enhanced 35S promoter–NOS terminator cassette [3] was inserted in pFP100 to generate pFP101. A triple hemagglutinin (HA) tag, the VP16-AD [15] and the GATEWAY™ cassette RfA were assembled to generate vectors we named Alligator. ABI5 cDNA was inserted in Alligator vectors by recombination following Invitrogen recommendations. Transformation was performed by floral dip using the ASE *Agrobacterium* strain [16]. Seeds were selected using a Leica MZFLIII stereomicroscope equipped with GFP3 (470 nm/525 ± 50 nm) and B (470 nm/515 nm) filters.

2.2.2. Expression analyses. For Western blot analyses, seeds were mechanically ground in liquid nitrogen and resuspended in 200 μl Laemmli buffer (1 M β-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 80 mM Tris–HCl (pH 6.8), 10% glycerol, 0.004% bromophenol blue). 10 μg of proteins were resolved on 10% acrylamide gels by SDS–polyacrylamide gel electrophoresis (PAGE) and blotted onto Hybond C nylon membranes (Amersham Biosciences). The primary antibody (rat anti-HA 3F10, Amersham Biosciences) diluted 1/10 000 in Tris-buffered saline (TBS), 0.3% Tween, 0.5% skimmed milk and the secondary antibody (anti-rat IgG peroxidase conjugate, A5795, Sigma-Aldrich) diluted 1/20 000 in TBS, 0.3% Tween, 0.5% skimmed milk, were incubated with the membranes for 90 min at room temperature. The enhanced chemiluminescence chemiluminescence (ECL+) kit (Amersham Biosciences) was used for revelation. Northern blot

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Abbreviations: VP16-AD, VP16 activation domain; ABA, abscisic acid; ABI, abscisic acid insensitive; LEA, late embryogenesis abundant; DAP, day after pollination; GFP, green fluorescent protein; ABRE, abscisic acid responsive element

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and GUS staining were performed on seedlings grown on germination medium as described in [3,7,13] except that RNA was extracted using the RNeasy kit (Qiagen).

3. Results

3.1. Designing new vectors for Arabidopsis transformation

We have designed a new series of plant binary vectors – named Alligator – that allows for the selection of transgenic *Arabidopsis* seeds via the GFP expression driven by the *At2S3* seed-specific promoter (Figs. 1 and 2A,B). The Alligator 1 and 2 vectors contain a GATEWAY™ cassette in order to easily generate fusions between any cDNA and a triple HA tag or the HA tag plus the VP16-AD. This domain [11] can confer constitutive activation potential to plant transcription factors (see [15] for an example).

3.2. T1 complementation of the abi5-1 mutants

We first tested whether tagged versions of ABI5 (obtained by inserting *ABI5* cDNA in Alligator 1 and 2 vectors) were able to complement the *abi5-1* mutant. We transformed the *abi5-1* mutant with the two constructs, selected the primary transformed seeds based on their fluorescence (as shown in Fig. 2A) and sowed them directly on 3 μM ABA. As opposed to wild-type seeds (0% germination, $N = 24$), most of the *abi5-1* mutant seeds germinated (82%, $N = 58$). However, only 14% ($N = 45$) of the *35S::HA-ABI5 abi5-1* and 5% ($N = 18$) of the *35S::HA-VP16-ABI5 abi5-1* seeds germinated indicating that both types of fusion proteins were able to compensate for the lack of the endogenous ABI5 protein. We performed this experiment with T1 seeds of different brightness and did not observe any link between fluorescence intensity and ABA sensitivity (data not shown) indicating that fluorescence intensity is not necessarily correlated with transgene expression level. Our selection method enabled easy complementation tests of multiple independent transgenic lines, without the need for plant propagation and homozygous line selection. Such a complementation test was not previously possible using standard selection assays because the transgenic ABA sensitive seeds could not be distinguished from non-germinating untransformed seeds.

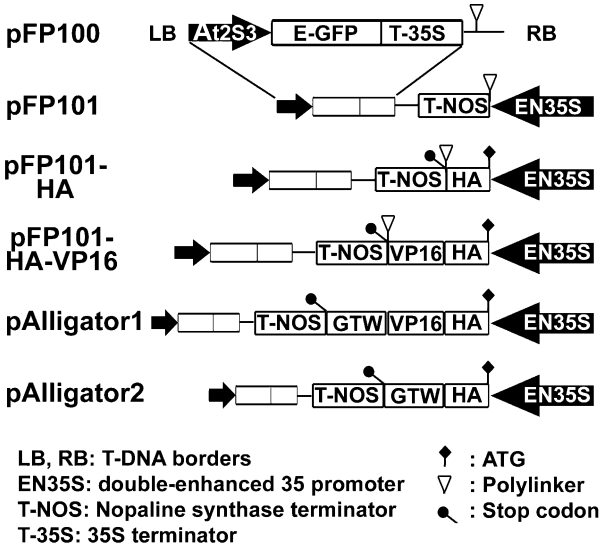

Fig. 1. Maps of vectors allowing transgenic selection based on GFP fluorescence. All vectors contain the coding sequence of an endoplasmic reticulum targeted GFP (E-GFP) under control of the *At2S3* promoter. The GATEWAY™ recombination cassette (GTW) allows insertion of cDNAs in frame with a triple HA tag (HA) and the VP16-AD.

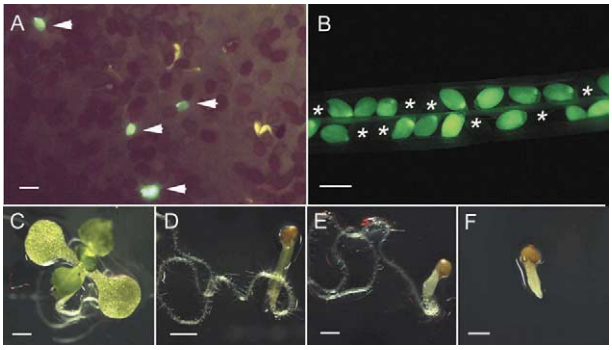

Fig. 2. Alligator vectors enable selection of transgenic seeds and isolation of *35S::HA-ABI5 abi5-1* arrested seedlings. A: Transgenic T1 seeds (indicated by arrows) are identified via their fluorescence among the progeny of a T0 plant. B: Segregation of the transgene can be directly monitored by looking for fluorescent T2 seeds. White stars indicate non-transgenic seeds. C–F: Phenotypes of *35S::HA-VP16-ABI5 abi5-1* T1 transgenic seedlings growing normally (C) or showing severe (D,E) or extreme (F) growth retardation. Bars = 5 mm.

3.3. 35S::HA-VP16-ABI5 transgene triggers a growth retardation

Non-germinating fluorescent seeds from a complementation experiment similar to the one described above were transferred onto ABA-free medium. We noticed that 26 (out of 82) *35S::HA-VP16-ABI5 abi5-1* seedlings displayed phenotypes never seen among *35S::HA-ABI5 abi5-1* seedlings. These phenotypes ranged from an almost complete growth arrest after germination to growth retardation confined to cotyledons (Fig. 2D–F). In a regular screen for transgenic seedlings, severely affected seedlings would have been missed and counted as herbicide or antibiotic sensitive.

Although the most severely retarded seedlings never reached the rosette stage, several others reached maturity and produced T2 seeds. The T2 seedlings showed a range of growth retardation phenotypes (severe as in Fig. 2D,E or milder (not show)) that were observed even in the absence of ABA treatment.

3.4. The HA-ABI5 and HA-VP16-ABI5 proteins are expressed at similar level

In order to determine if the growth retardation phenotype was truly due to the presence of the VP16-AD and not to different expression levels of the transgenes, we analyzed expression of both types of fusion proteins. Transgenic seeds from a hemizygous T1 plant were identified via their fluorescence (Fig. 2B) and used for Western blot analysis with antibodies directed against the HA tag. As shown in Fig. 3, we detected similar expression level in *35S::HA-ABI5 abi5-1* and *35S::HA-VP16-ABI5 abi5-1* seedlings. We concluded that the growth retardations are due to the VP16-AD and not to differences in transgene expression levels. In addition, we did not observe the ABA-triggered stabilization of ABI5 that was described in previous studies [8,17].

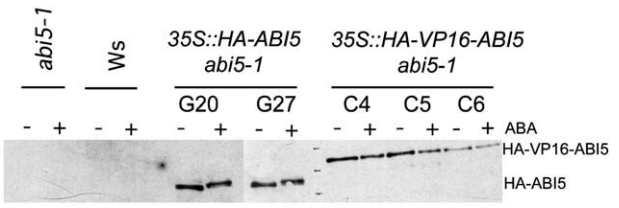

Fig. 3. Expression of HA-ABI5 and HA-VP16-ABI5 fusion proteins. Wild-type (Ws), *abi5-1* and transgenic T2 seeds of indicated genotypes were grown for 2 days with (+) or without (−) 30 μM ABA. Fusion proteins were detected in a Western blot experiment with an antibody directed against the HA tag. Expected sizes are 51 kDa for HA-ABI5 and 59 kDa for HA-VP16-ABI5. Observed sizes are 55 and 95 kDa, respectively.

3.5. *HA-VP16-ABI5 is sufficient to induce LEA gene expression*

Although ABI5 is known to bind the ABRE present in the *AtEm1* and *AtEm6* promoters, and to possess a cryptic activation domain, its constitutive expression is not sufficient to induce the expression of these genes in vegetative tissues [10]. We tested whether the presence of the VP16-AD allowed ABI5 to induce the *AtEm* genes in 10-day-old seedlings. We found that HA-VP16-ABI5, but not HA-ABI5, induced the expression of both *AtEm1* and *AtEm6* genes, even in the absence of ABA treatment (Fig. 4). Furthermore, only *AtEm6*, and not *AtEm1*, expression was enhanced by ABA treatment.

We also determined if the VP16-AD-induced gene expression was mediated through the *AtEm* promoters and analyzed the tissue specificity of expression. To do this, we crossed *AtEm1::GUS* and *AtEm6::GUS* carrying reporter lines [12] to hemizygous *HA-VP16-ABI5 abi5-1* plants. We sorted the fluorescent (*AtEm::GUS*/. *35S::HA-VP16-ABI5*/.) and non-fluorescent seeds (*AtEM::GUS*/../.) in the F1 progeny of the cross and performed GUS staining after 10 days of growth, thereby comparing two pools of seedlings that only differ by the presence of the *35S::HA-VP16-ABI5* transgene. We detected ectopic GUS activity in the severely affected seedlings arising from fluorescent seeds but never in seedlings from non-fluorescent seeds (Fig. 5A for *AtEm1* and data not shown for *AtEm6*). This staining was mostly present in the cotyledons and the crown.

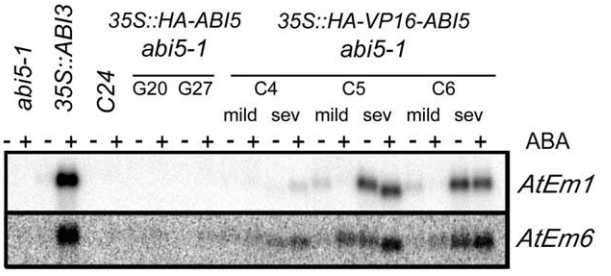

Fig. 4. *AtEm1* and *AtEm6* genes are constitutively expressed in 10-day-old *35S::HA-VP16-ABI5 abi5-1* but not in *35S::HA-ABI5 abi5-1* plants. 10-day-old seedlings were transferred for 4 h on a medium with (+) or without (−) 30 μM ABA. *35S::HA-VP16-ABI5 abi5-1* plants were harvested in two separate classes depending on their phenotype (mild or severe (sev.)). 5 μg of total RNA were loaded per lane except for the severe class (1.5 μg). A *35S::ABI3* line and the corresponding wild-type (C24) are shown as controls.

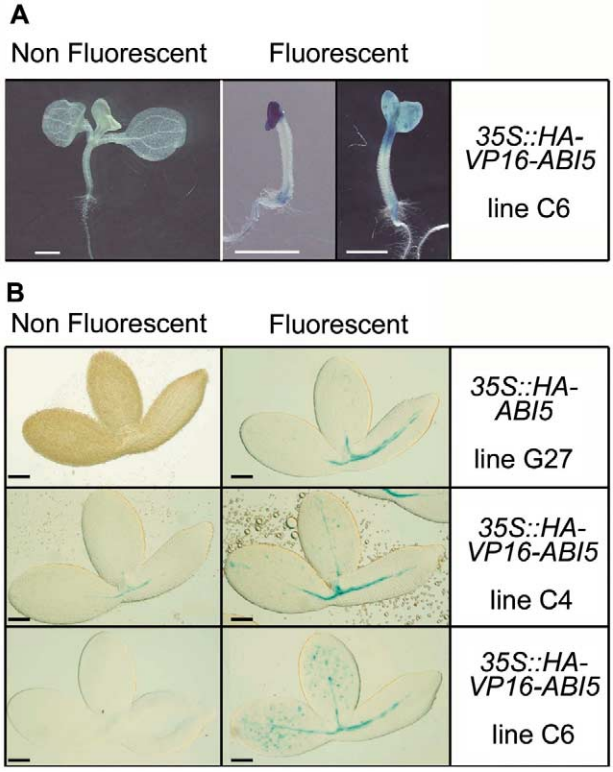

Fig. 5. Expression of *AtEm1::GUS* is upregulated by ABI5 fusion proteins. *AtEm1::GUS* plants were pollinated by flowers hemizygous for the transgene indicated on the right. Transgene inheritance was followed via embryo fluorescence. Histochemical GUS assays were performed on 10-day-old seedlings (A) or on 10 DAP embryos extruded from maturating siliques (B). Bars = 4 mm in A and 100 μm in B.

3.6. AtEm *expression is only slightly modified by* ABI5 *constitutive expression in embryos*

Both *ABI5* and the *AtEm* genes are expressed in the later stages of embryo development, when seeds start to desiccate [3–5,18]. To test if the late expression of *AtEm* genes is due to the late expression of *ABI5*, we analyzed whether HA-ABI5 or HA-VP16-ABI5 constitutive expression during early embryo development was sufficient to trigger early expression of *AtEm* genes. We again used the *AtEm::GUS* reporter lines crossed to hemizygous lines overexpressing *ABI5*. The use of the fluorescent selection marker offered a reliable internal control. Indeed, because of the dynamic *AtEm* expression pattern, it is crucial to analyze the different embryos at exactly the same age. Here, transgenic and non-transgenic seeds were harvested in the same developing silique and identified based on their fluorescence. In early stages (heart stage embryos), we never observed any staining. However, staining of 10-day-after-pollination (DAP) embryos revealed a slightly earlier *AtEm1::GUS* staining in fluorescent seeds (*35S::HA-ABI5* or *35S::HA-VP16-ABI5*, Fig. 5B) than in non-fluorescent ones, demonstrating that *ABI5* was one of the limiting factors for *AtEm1* expression. Similar results were obtained with *AtEm6::GUS* (not shown).

4. Discussion

4.1. *Specific advantages of the Alligator vector system*

In this study, we describe a new set of vectors that combine

transgenic selection based on GFP fluorescence with the GATEWAY™ technology and the HA tag. Some of the advantages of vectors using seed-expressed fluorescent markers were recently discussed [19]. As illustrated in this study, this list of advantages can now be increased. This system affords T1 complementation with multiple independent lines. It also allows for rapid differentiation between phenotypes of maternal and embryonic origin. Working with crosses or detecting outcrosses that occur in the greenhouse is greatly facilitated. Identification of homozygous lines is also quicker (Fig. 2B) and we noticed increased percentages of homozygous lines among the brightest seeds of hemizygous plants (data not shown). This system has been used successfully by other laboratories to express different proteins such as a metal transporter, an homeobox transcription factor or a phytochrome (S. Thomine, C. Fankhauser and M. Proveniers, personal communication). The only drawback is the occurrence of fluorescent T1 seeds that give rise to non-fluorescent T2 progeny. These false positives usually represent 5–10% of the fluorescent T1, although we did notice up to 60% false positives in one individual experiment. We suspect that these escapes are due to chimeric embryos where the transgene was not inserted in meristematic cells.

4.2. AtEm *regulation by ABI5 in seedlings*

Since *AtEm* gene expression is reduced in *abi5* mutants and the ABI5 protein binds to *AtEm* promoters, it is thought that ABI5 regulates the *AtEm* genes directly. Still, *ABI5* ectopic expression was shown not to be sufficient to activate *AtEm* expression in vegetative tissues [10]. Our results show that the VP16-AD is sufficient to allow ABI5 to induce both *AtEm1* and *AtEm6* in seedlings. This induction was observed concomitantly with seedling growth retardation but we do not know whether these two phenotypes are causally linked. The effect of the added activation domain suggests that the ABI5 activation potential is limiting. Consistent with this view, ABI5 is not able to activate transcription on its own in yeast [9]. ABI5 might thus work in planta through interaction with a coactivator such as ABI3 [9]. ABA was known to positively regulate *AtEm* expression in seeds, early seedling or vegetative tissues ectopically expressing *ABI3* [3,8]. We have shown here that *AtEm1* induction by *HA-VP16-ABI5* is ABA independent. This result is consistent with the hypothesis that ABA could act by increasing ABI5 activation potential (as suggested for an ABI5 homolog from rice [20]) and that the HA-VP16-ABI5 constitutively activated version cannot be further activated. For *AtEm6*, ABA addition does increase transcription suggesting that the mechanism might be slightly different. This result is in accordance with the increased ability of ABI5 to bind the *AtEm6* promoter following ABA treatment [8]. ABA has also been shown to stabilize ABI5 [17]. We did not observe this stabilization in very young seedlings (Fig. 3) or in 10-day-old seedlings with 4 and 22 h ABA treatment (not shown), perhaps because of the additional protein tag. We might have thus, accidentally, engineered ABI5 alleles that decouple ABA's effect on ABI5 stability from its effect on ABI5 activation potential or DNA binding.

4.3. AtEm *regulation in seeds*

ABI5 temporal expression pattern closely follows the pattern of its target genes *AtEm1* and *AtEm6* suggesting that *ABI5* could be the limiting factor for *AtEm* gene expression. However, we showed that overexpression of HA-ABI5 or HA-VP16-ABI5 activated *AtEm* expression only slightly earlier than in wild-type seeds. This result indicates that *ABI5* transcription is not the only limiting factor during seed development. The observation that the VP16-AD does not always confer *ABI5* with the ability to induce *AtEm* expression (as in young embryos or in the seedling root) suggests the existence of additional cofactors expressed during late seed development and required for *AtEm* induction. Alternatively, negative regulators (such as *EEL* [7]) might also prevent *AtEm* induction in certain tissues. The nature of this post-transcriptional regulation is unknown and could affect the accumulation or the activity of the ABI5 protein.

4.4. *ABI5 function in preventing germination*

How ABI5 and ABA prevent germination at the molecular level is not known. In particular, we do not know whether ABI5 acts as a transcriptional activator or repressor. Because *HA-VP16-ABI5* is able to complement the *abi5-1* mutation in germination assays (and even confer ABA hypersensitivity, data not shown), it is likely that ABI5 fulfills this function as an activator. Potential target genes for this activation activity include the cell cycle inhibitor *ICK1* [21].

4.5. *Prospects*

Our results illustrate how our new transgenic seed selection system allowed us to gain insight into the function of an important regulator of seed maturation. Many more aspects of ABI5 function, however, remain to be elucidated. Similarly as experiments showing that ABI5 constitutive expression can complement the weak *abi3-1* mutant [8], it will be interesting to determine if constitutive expression of ABI5 or VP16-ABI5 can complement an *abi3* null allele.

Finally, we are convinced that vectors derived from the series that we presented here will be of considerable help in high throughput genomics, especially when combined with commercially available seed sorters.

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