Heat shock factor HSFB2a involved in gametophyte development of Arabidopsis thaliana and its expression is controlled by a heat-inducible long non-coding antisense RNA

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Abstract Heat stress transcription factors (HSFs) are central regulators of the heat stress response. Plant HSFs of subgroup B lack a conserved sequence motif present in the transcriptional activation domain of class A-HSFs. Arabidopsis members were found to be involved in non-heat shock functions. In the present analysis we investigated the expression, regulation and function of HSFB2a. HSFB2a expression was counteracted by a natural long non-coding antisense RNA, asHSFB2a. In leaves, the antisense RNA gene is only expressed after heat stress and dependent on the activity of HSFA1a/HSFA1b. HSFB2a and asHSFB2a RNAs were also present in the absence of heat stress in the female gametophyte. Transgenic overexpression of HSFB2a resulted in a complete knock down of the asHSFB2a expression. Conversely, asHSFB2a overexpression leads to the absence of HSFB2a RNA. The knockdown of HSFB2a by asHSFB2a correlated with an improved, knockdown of asHSFB2a by HSFB2a overexpression with an impaired biomass production early in vegetative development. In both cases the development of female gametophytes was impaired. A T-DNA knock-out line did not segregate homozygous mutant plants, only heterozygous hsfB2a-tt1/+ were viable. Approximately 50 % of the female gametophytes were arrested in early development, before mitosis 3, resulting in 45 % of sterile ovules. Our analysis indicates that the “Yin–Yang” regulation of gene expression at the HSFB2a locus influences vegetative and gametophytic development in Arabidopsis.

Keywords HSFB2a · Heat shock factor · Gametophyte development · Antisense RNA · Arabidopsis thaliana

Introduction Heat stress transcription factors (HSFs) were originally identified as the central regulators of the heat stress response. In recent years HSFs have been found to be involved in the control of a multitude of stress responses and also in developmental processes. Unlike in other eukaryotes, there is a high diversity of HSFs in plants with 21 genes in Arabidopsis (Nover et al. 2001), 25 in rice (Guo et al. 2008), 30 in maize (Lin et al. 2011; Scharf et al. 2012) and 52 in soybean (Scharf et al. 2012) respectively. This may reflect a functional redundancy in controlling stress responses, evident for the A1-group of HSFs in Arabidopsis (Liu et al. 2011; Yoshida et al. 2011) and specific functions in non-heat stress processes as demonstrated for maize (Gagliardi et al. 1995) rice (Chauhan et al. 2011) and Arabidopsis (Kotak et al. 2007). Common features of all HSFs are the highly conserved DNA binding domain (DBD) that mediates binding to the heat stress elements (HSEs), repetitions of the “nGAAmTTCn” motif in promoters of target genes (Schöffl et al. 1998) and two hydrophobic regions A and B, that in case of plant class B HSFs are separated by a short linker (Nover et al. 2001). Plant HSFs are grouped in three classes A, B and C. Whereas almost all class A HSFs hold one or two acidic
AHA motifs conferring competence as transcriptional activators (Döring et al. 2000), all class B HSFs lack this domain and instead have a B3 repressing domain that was also found in 24 other transcription factors of *Arabidopsis* (Czarnecka-Verner et al. 2004; Ikeda and Ohme-Takagi 2009). The importance of HSF in developmental regulation in *Arabidopsis* has been demonstrated for HSFB4, which acts mainly in the root stem cell niche to control cell identity and cell fate (Begum et al. 2012; Pernas et al. 2010; ten Hove et al. 2010). On the other hand, *Arabidopsis* HSFB2b, together with HSFB1, was shown to be involved in the regulation of the defensin gene *PDF1.2* and pathogen resistance (Kumar et al. 2009).

These HSFs are also negative regulators of HSFA2, HSFA7a, and act in an autoregulatory manner (Ikeda et al. 2011). Only these class B HSFs have also been found to elicit mild cell death effects in *N. benthamiana* leaves (Zhu et al. 2012). For HSFB2a a mild repressing activity was also shown at the HSFA2 promoter (Ikeda et al. 2011), which is expressed in antipodal cells of mature female gametophytes (Kägi et al. 2010).

In this study we provide evidence that HSFB2a is required for the development of the female germ line and that its expression is controlled by a natural heat-inducible non-coding antisense RNA and vice versa.

**Results**

*HSFB2a* is required for plant fertility

In order to determine the function of HSFB2a we analysed four different T-DNA insertion lines. GT12254, GT10826, and SALK_137766 contain insertions in the 5′-Nontranslated region, the intron, and the 3′ terminal coding region, respectively (Fig. 1a), and did not exhibit any defects. By contrast, for SALK_012418, which contains a T-DNA insertion in the middle of the first exon, we were not able to recover homozygous plants (n = 458). We designated the mutant allele *hsfb2a*-tt1.

To better understand the mutant defect, we inspected the siliques of heterozygous *hsfb2a*-tt1 plants. Notably, 44.9 % of all ovules (n = 3,461) were sterile as compared to 2.2 % in wild type (n = 3,740, Fig. 2a, Figure S1a), suggesting that the mutant was gametophytic lethal. In order to test whether the *hsfb2a*-tt1 allele is passed on by both, male and female gametophytes, we conducted reciprocal crosses to Col-0 wild-type plants and found transmission rates of 21 and 22 % for female and male gametophytes, respectively (Table 1). Male transmission was substantially increased when the amount of pollen applied to the stigma was limited (Table 1), suggesting a reduced competitiveness of mutant pollen compared to wild-type.
To characterize the female gametophytic defect associated with the reduced \textit{hsfB2a\texttt{-tt1}} transmission, we performed cleared whole mounts of mutant and wild-type ovules 2 days after emasculation of the oldest closed flower bud, a stage where wild type gametophytes have undergone maturation. We found that 55.4\% of the gametophytes arrest before completion of all three mitotic divisions (Figure S1b). In 20.3\% of the arrested gametophytes four nuclei at the micropylar region of the gametophyte were visible (Figure S1b, Figure S2b). These results suggest that HSFB2a is required for the development of the female germline.

To determine whether the defect in \textit{hsfB2a\texttt{-tt1}} was, indeed, due to reduced HSFB2a activity, we in a first step characterized the expression of the gene at control temperature (20 °C) and after heat shock (38 °C, 1 h). At 20° the expression in wild type and mutant was 2.7 and 1.1\% with respect to the internal standard \textit{ACTIN2}. After heat induction, expression reached a fourfold higher level in both lines (9.8 and 4.5\%) (Figure S3). These results confirm that \textit{HSFB2a} is a heat-inducible gene and show that its activity is reduced by more than 50\% in \textit{hsfB2a\texttt{-tt1}} mutants.

We next asked, whether \textit{HSFB2a} could functionally complement the gametophytic lethal phenotype of

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**Table 1** Genotypes and transmission rates of crossings

| Genotypes F0   | Genotype F1                     | Transmission rate (%) | n   |
|----------------|---------------------------------|-----------------------|-----|
|                | \textit{wt} (%)                | \textit{hsfB2a\texttt{-tt1}/+} (%) |     |
| \textit{hsfB2a\texttt{-tt1}/+ × hsfB2a\texttt{-tt1}/+} | 77.7                           | 22.3                  | 44.6 | 1,446 |
| \textit{hsfB2a\texttt{-tt1}/+ × wt}                 | 89.6                           | 10.4                  | 20.8 | 154  |
| \textit{wt x hsfB2a\texttt{-tt1}/+}                | 89.2                           | 10.8                  | 21.6 | 329  |
| \textit{wt x hsfB2a\texttt{-tt1}/+\textsuperscript{a}} | 65.4                           | 34.6                  | 69.2 | 107  |

\textsuperscript{a} Reduced pollen
hsfB2a-tt1 and introgressed a genomic HSFB2a fragment, designated HSFB2a-tg into hsfB2a-tt1. We found only 4 (lines 75, 96, 165, 184) out of 189 BASTA resistant transformed plants in the T1 generation.

Microscopic inspection of female gametophytes of the T2 generation revealed an increase in the percentage of mature female gametophytes of 14.5 and 14.7 % in two out of four independent transgenic lines (lines 75 and 165, Figure S6). This small but significant improvement indicates that the female gametophytic defect relates to reduced HSFB2a activity, but also suggests that the expression levels conferred by HSFB2a-tg were not sufficient to reach those of the native HSFB2a allele.

Additionally, we tested two more lines with a T-DNA insertion in HSFB2a for an altered female gametophytic phenotype. These lines, GT10826 and GT12254, neither heterozygous nor homozygous mutant plants, showed a phenotype differing from wildtype. However, these lines are in a different genetic background (Ler) compared to hsfB2a-tt1 (Col0) and the T-DNA insertions are located in the 5′-UTR (GT12254) and the intron (GT10826), respectively. In addition, we generated HsfB2a-mutants by generating overexpression lines for sense and antisense RNA (see below).

Expression of HSFB2a

In order to examine HSFB2a expression during early development we analysed promoter:GUS reporter expression in transgenic Arabidopsis plants. Using an 855 base pair (bp) DNA fragment upstream of the transcription start site of HSFB2a fused to the β-glucuronidase gene, we detected signals in the root tip and nodes of 8, respectively 15 day old seedlings (Fig. 3a, b). In the reproductive tissues we found staining in anthers of flowers at stage 13, according to Smyth et al. (1990), in walking stick embryos, and in the junction between receptacle and silique (Fig. 3c, e, f, g). At this stage, however, there was no staining detected in ovules. GUS expression in leaves was only detectable at the beginning of senescence in the vascular tissue (Fig. 3d).

**HSFB2a is expressed in the female gametophyte**

Due to the gametophytic defect of hsfB2A-tt1/+ mutants we tested HSFB2a expression by in situ hybridization experiments. Sections of carpels were hybridized with a probe covering part of the 3′-UTR (Fig. 1a). We detected strong and specific hybridization signals in female gametophytes.
with the antisense probe, while no signal was obtained with the sense control probe (Fig. 2b, c).

By contrast, by using a probe covering the last 71 bp of the HSFB2a ORF and the first 40 bp of the 3′-UTR (Fig. 1a), hybridizations signals were not only obtained with the antisense probe, but an unprecedented strong signal was also detected in the sense probe control (Figure S4). These data suggest that in this part of the coding region, both strands of HSFB2a are transcribed. This interpretation is supported by the presence of an annotated cDNA clone, RAFL21-83-H09, with an 1,138 bp overlap with the genomic sequence of HSFB2a. This cDNA contains no ORF starting with an ATG and hence most probably is a non-coding RNA.

Expression from the antisense strand of HSFB2a

The natural antisense RNA expression (asHSFB2a) at the HSFB2a locus, identified by in situ hybridization in the female gametophyte, was further analysed in leaves of wild-type plants. At different temperatures RNA levels were quantified by real-time qRT-PCR (Fig. 1c). The antisense RNA (asHSFB2a) was almost absent (0.01 % of ACTIN standard) at control temperature but was more than 1100-fold induced (11.5 % of ACTIN RNA standard) after one hour heat stress at 38 °C. Thus heat-induction of asHSFB2a RNA is more than 180-fold higher than that of HSFB2a mRNA, suggesting that not only HSFB2a but also asHSFB2a is a heat shock gene. We screened the HSFB2a and asHSFB2a upstream region for the presence of HSEs, which are characteristic to heat-inducible genes. In fact, in both putative promoter regions we found one perfect HSE with the consensus sequence nGAAnTTCn located upstream of the transcriptional start sites of HSFB2a (22 bp) and asHSFB2a (71 bp) (Fig. 1a). The 1 kb upstream region of asHSFB2a contained, in addition, more than twice as many imperfect HSEs (with one bp change in consensus HSE) than the respective upstream region of HSFB2a.

The heat-induction of HSFB2a depends on the presence of class A heat shock factors HSFA1a and -A1b (Busch et al. 2005). This prompted us to examine also the asHSFB2a RNA levels in hsF1a/hsF1b double knock-out plants (Busch et al. 2005). We found on average a 50-fold reduction of asHSFB2a RNA after heat shock as compared to wild type (Figure S7), indicating that also the antisense HSFB2a transcript is under regulatory control by class A heat shock factors.

Manipulation of sense and antisense RNA expression

The strictly heat-inducible natural non-coding antisense RNA may specify a regulatory mechanism targeting the expression of specific genes. In order to test whether HSFB2a expression was affected by the antisense transcript asHSFB2a and vice versa we fused the CaMV 35S promoter to the full-length cDNA of asHSFB2a and HSFB2a. Transgenic Arabidopsis plants overexpressing either one of these constructs were examined by qRT-PCR for both, HSFB2a sense and asHSFB2a antisense expression at different temperatures. Compared with wild type, 35S:HSFB2a plants showed a high constitutive level of the HSFB2a transcript, however, in these plants the asHSFB2a RNA was undetectable at all temperatures. Conversely, 35S:asHSFB2a plants, expressing high constitutive levels of asHSFB2a RNA, did not express detectable HSFB2a mRNA levels at any temperature (Fig. 1b, c). These results indicate that HSFB2a and asHSFB2a are potent inhibitors of the respective other transcript. Together with our observation that asHSFB2a is massively induced upon heat stress, these results suggest that the noncoding asHSFB2a RNA buffers the HSFB2a-dependent heat shock response.

HSFB2a links heat response and seedling size

The intriguing “Yin–Yang relationship” of RNA levels exhibited after transgenic manipulations of HSFB2a and asHSFB2a offers the opportunity to examine the effects of HSFB2a downregulation in the sporophyte by overexpressing asHSFB2a. For proof of concept, we in a first step examined the effect of asHSFB2a-mediated HSFB2a downregulation during ovule development. We found that 45 % of all ovules in 35S:asHSFB2a lines exhibited an arrest in embryo sac development at various stages (Fig. 4a), reminiscent of the phenotype observed in hsfB2A-tt1/+ plants. Given that 35S activity is not detectable in the female gametophyte (Roszak and Köhler 2011), these data suggest that the amount of asHSFB2a in the sporophytic ovular tissue is decisive for the development of the female germline. We next examined the effect of asHSFB2a-mediated HSFB2a downregulation in sporophytic tissue. Notably, in four independent 35S:asHSFB2a antisense lines, we observed 10 days after germination an increase in biomass by 58 % as compared to wildtype. Conversely, plants overexpressing HSFB2a (35S:HSFB2a) exhibited a 15 % reduced fresh weight compared to wild type (Figure S5). These results indicate that HSFB2a has a negative impact on early plant growth and reveal an unprecedented link between the plant heat shock response and the regulation of seedling size.

Discussion

Regulation of HSFB2a and asHSFB2a expression

Using in situ hybridization and qRT-PCR HSFB2a mRNA was detected in female gametophytes and leaves
Our expression is controlled by HSFA1a/HSFA1b (Busch et al. 2005). Our observations have shown that heat-induced HSEs in the respective 1 kb promoter upstream regions. It correlates with the differences in the numbers of imperfect mRNA. Differences in strength of heat-induction as HSFB2a is much stronger than of the heat-induction of the respective gene. In leaves HSFB2a is transcribed in opposite orientation starting shortly beyond the 5′-end of the HSFB2a coding region and terminating in the 3′-end of the HSFB2a coding region and terminating. This gene, asHSFB2a, is very strong and strictly heat stress dependent. As expected from previous studies (Busch et al. 2005), but surprisingly, our analyses revealed also a strong negative effect on the level of asHSFB2a RNA. Under these conditions the sense RNA acts as a “silencing antisense RNA” on asHSFB2a expression. The molecular mechanism of this “Yin–Yang” control of sense and antisense RNA expression is unknown. Long non-coding RNAs may cause transcriptional silencing through promoting the formation of chromatin-modifying complexes (Beisel et al. 2007; Schuettengruber et al. 2007; Shilatifard 2008; Wang and Chang 2011). In Arabidopsis non-coding sense transcripts termed COOLAIR, originating from the first intron of FLC, have recently been shown to be sufficient to maintain repression of FLC during vernalization (Helliwell et al. 2011). The COOLAIR antisense transcripts (Swiezewski et al. 2009) originating from a promoter adjacent to the FLC 3′ untranslated region seems to have only a redundant function in silencing the FLC locus.

Interestingly, CaMV 35S-driven HSFB2a overexpression imposes also a strong negative effect on the level of asHSFB2a RNA. Under these conditions the sense RNA acts as a “silencing antisense RNA” on asHSFB2a expression. The molecular mechanism of this “Yin–Yang” control of sense and antisense RNA expression is unknown. Long non-coding RNAs may cause transcriptional silencing through promoting the formation of chromatin-modifying complexes (Beisel et al. 2007; Schuettengruber et al. 2007; Shilatifard 2008; Wang and Chang 2011). In Arabidopsis non-coding sense transcripts termed COOLAIR, originating from the first intron of FLC, have recently been shown to be sufficient to maintain repression of FLC during vernalization (Helliwell et al. 2011). The COOLAIR antisense transcripts (Swiezewski et al. 2009) originating from a promoter adjacent to the FLC 3′ untranslated region seems to have only a redundant function in silencing the FLC locus.

Is asHSFB2a-dependent regulation of HSFB2a also implemented in the female gametophyte? By in situ hybridization asHSFB2a RNA was detected in ovules. Manipulation of HSFB2a and respectively asHSFB2a expression via 35S-promoter-driven transgenes resulted in significant changes in the proportions of defective gametophytes (Fig. 4). These defective phenotypes are overall more pronounced in asHSFB2a transgenic lines, which is in support of the detrimental effect of the hsb2a-tt1 mutation. There is some variation in the proportions of defective ovules, but at present it is unknown how strong constitutive 35S-promoter-driven gene expression is implemented in cells of the gametophyte. It is not clear whether the 35S-promoter is fully active at all stages during female gametophyte development in Arabidopsis (Jiang et al. 2010; Liu et al. 2008; Roszak and Köhler 2011). Our observation that 35S-driven expression of asHSFB2a interferes with female gametophyte development might relate to the fact that the unicellular embryo sac inherits its cytoplasm from sporophytic, i.e. asHSFB2a containing tissue. Alternatively, HSFB2a is required sporophytically to ensure proper female gametophyte development.
development. Our data suggest that changes in HSFB2a expression levels are crucial for proper female gametophyte development. Our finding that the female gametophyte and seed defects of hsfb2a-tt1/+ could only be rescued to a minor extend by a genomic HSFB2a fragment might hint to a lack of critical cis-regulatory elements in the sequence provided.

Growth phenotype of HSFB2a and asHSFB2a overexpression lines

The 35S-driven overexpression of HSFB2a and asHSFB2a in transgenic lines had clear effects on the gametophyte and on the plant growth phenotype. The asHSFB2a-oX lines showed an improved, the HSFB2a-oX lines an impaired biomass production, but only in the early phase of plant growth. This suggests that during development HSFB2a activity temporarily represses vegetative growth and following heat stress the antisense regulation by asHSFB2a counteracts this effect to restore growth and further development.

HSFB2a promotes nuclear proliferation in the female gametophyte

Characteristic to gametophytic mutants is the non-Mendelian inheritance of mutant alleles, which is also referred to as segregation distortion (Drews et al. 1998; Drews and Yadegari 2002; Moore et al. 1997; Page and Grossniklaus 2002). The hsfb2a-tt1/+ plants, heterozygous for the heat shock factor HSFB2a, exhibit 50% sterile ovules and a substantially reduced male and female transmission, indicating that the absence of the gene is detrimental to the development of the male and female germ lines. The finding that male transmission is enhanced by reducing the amount of pollen applied to the pistil suggests that the defect is at least partially due to retarded pollen development or tube growth.

The analysis of hsfb2a-tt1 female gametophytes revealed an impaired development during megagametogenesis. Most of the phenotypically abnormal ovules exhibited four nuclei of similar size and the crosses of hsfb2a-tt1/+ with the egg cell marker line DD45::NLS_GUS provided no evidence for the specification of an egg cell. Notably, a small proportion of hsfb2a-tt1 female gametophytes gave rise to functional seeds when pollination was delayed, reminiscent to results gained for swa1–swa3 mutants (Li et al. 2009; Liu et al. 2010; Shi et al. 2005). Swa1–3 mutants are defective in different rRNA processing factors and exhibit retarded division cycles in the female gametophyte. Whether HSFB2a can be linked to the biogenesis of ribosomal RNA is currently unclear.

Experimental procedures

Plant materials and growth conditions

Arabidopsis thaliana accession Columbia (Col-0) was used as the wild type. Seeds of SALK_012418 were obtained from the Nottingham Arabidopsis Stock Centre, seeds of GT10826 and GT12254 were provided by Robert Martienssen (Cold Spring Harbor Laboratory).

Seeds were sown in soil, kept at 4 °C for 2 days in the dark and grown in a chamber under white fluorescent light (80 µmol m⁻² s⁻¹) with a 16 h/8 h light/dark cycle at 20 °C and 60% relative humidity. For selection of mutant plants, seeds were surface-sterilized and sown on plates containing Murashige & Skoog medium including vitamins with 0.8 % (w/v) phyto agar (both DUCHefa, Haarlem, Netherlands) supplemented with 2 % (w/v) sucrose and 50 µg/ml Kanamycin. Following cold treatment at 4 °C for 2 days in the dark, plates were incubated under standard growth conditions. After 10 days, resistant plants were transferred to soil. Transgenic lines carrying a phosphinotricin resistance gene were grown on soil and for selection sprayed with 0.1 % (v/v) BASTA (AgrEvo, Germany) 8–10 days after germination.

For mRNA analysis two leaves from each of 20 five-week-old plants were incubated for 1 h at the respective temperature in SIB-puffer (1 mM potassium phosphate, pH 6.0, 1 % sucrose) in the dark in a shaking water bath. After treatment leaves were immediately frozen in liquid nitrogen and stored at −80 °C.

DNA constructs and the generation of transgenic plants

All PCR reactions for cloning were conducted with PHUSION polymerase (New England Biolabs) and correctness of the clones was confirmed by sequencing. For complementation of hsfb2a-tt1/+ plants a 3,418 bp genomic fragment spanning the HSFB2a sequence with 1,180 bp upstream of the transcription start site and 684 bp downstream of the 3′-UTR was cloned by restriction of P1 clone MITG10 (Liu et al. 1995), obtained from the Arabidopsis Biological Resource Center, (http://abrc.osu.edu/) with SpeI and MscI and insertion of the fragment into SpeI/Smal of the binary vector pCB308 (Xiang et al. 1999).

For HSFB2a overexpression in plants a 1,555 bp fragment was amplified by PCR from clone MITG10 with the primers B2aOXFKpnI and B2aOXRSacI and joined via SacI to a 813 bp CaMV promoter sequence that was amplified from pBI121 (Chen et al. 2003) with the primers CamVSacF2 and CamVKpnR2. The resulting construct was inserted into SacI of pCB308. For asHSFB2a overexpression in plants a 1,458 bp fragment with the complete sequence of the annotated cDNA clone RAFL21-83-H09
was amplified by PCR from clone MITG10 with the primers ASFKpnl and ASRSacI and joined via SacI to the CaMV promoter sequence as above. The resulting construct was inserted in SacI of pCB308. For β-glucuronidase (GUS) expression under the control of the HSFB2a promoter a 949 bp fragment comprising 855 bp upstream of the transcription start site and 94 bp of the 3′-UTR of HSFB2a was amplified with XbaI recognition site containing primers B2aPromF and B2aPromR. There were no relevant promoter elements detectable in the extra 300 bp region of the 1,180 bp promoter (used for complementation, see above) compared to the 855 bp promoter. After digestion of the PCR product with XbaI the fragment was inserted in the XbaI site in pCB308. The respective constructs were introduced into Agrobacterium tumefaciens strain GV3101 by electroporation and transgenic plants were created by the vacuum infiltration method (Bechtold et al. 1993).

Southern blot hybridization

To rule out a second insertion elsewhere in the genome, we digested genomic DNA of the heterozygous mutant, segregating plants with no T-DNA insertion in HSFB2a and Col0 wild type with HindIII and subjected it to southern blot hybridization probed with a radioactively labeled 203 bp fragment amplified with the primers TDNALBF/TDNALBR, specific for the left border sequence of the T-DNA. Two specific bands were detected only in the digest of the heterozygous mutant, not in wild type segregants.

Isolation of mRNA and quantitative real-time RT-PCR

RNA was isolated from 100 mg frozen leaf powder with the CHEMAGIC mRNA Direct Kit (Chemagen, Germany). Reverse transcription was performed with one fifth of the eluted mRNA using the ISCRPT cDNA Synthesis Kit (Biorad, Hercules, CA).

Quantitative PCR reactions were performed using SYBR® Green technology on the ICYCLER system (Biorad, Hercules, CA). HSFB2a cDNA was amplified with primers HSFB2AF2 and HSFB2AR2, asHSFB2a cDNA with the primers ASB2AF and ASB2AR, ACTIN2 cDNA with the primers ACTIN2F3 and ACTIN2R3. For each genotype, at least three independent biological replicates were analysed in triplicate PCR reactions. Relative expression of transcripts was quantified with respect to Actin2 (At3g18780) as internal standard.

Determination of 5′ and 3′-ends of mRNA

To define transcriptional start and termination sites of asHSFB2a a 5′- and 3′-RACE was performed by using the Invitrogen GeneRacer® Kit with SuperScript® III RT and TOPO TA Cloning® Kit for sequencing. Following the protocol of the manufacturer we used mRNA from heat shocked (1 h, 38 °C) wild-type leaves and the primers ASGSP5′RACE and ASGSP3′RACE as the respective 5′- and 3′-specific primers for amplification and sequencing. Nine and eight clones of the PCR products for the 5′-end and the 3′-end, respectively, were sequenced and the resulting nucleotide sequences compared to the TAIR annotation of the RIKEN cDNA clone RAIJ21-83-H09. The 5′ end of seven clones was identical or 2–3 nucleotides shorter than the TAIR sequence, while two clones showed a 52 and 51 nucleotide shorter sequence, respectively. The 3′-end of five PCR clones was the same or within a range of five nucleotides as the annotated sequence. Three PCR products were truncated in a range of 32–181 nucleotides.

In situ hybridisation

Flowers of stage 13 and 14 (Smyth et al. 1990) were harvested into FAA solution (3.7 % formaldehyde, 50 % ethanol, 5 % acetic acid) and embedded in paraplast. Sections of eight μm thickness were prepared with a microtome (Leica Biocut 2,035) and transferred on microscope slides (SUPERFROST ULTRA PLUS, Thermo Scientific). Probes were synthesized with the DIG RNA Labelling Kit (Roche) on a PCR fragment of HSFB2a generated with primers SP6-2B2aR2 and T7-B2aF2 (HSFB2a-specific) or with primers HSFB2aF2 and HSFB2aR2. Hybridization at 50 °C and detection were carried out as described by (Wollmann et al. 2010).

Crossings and genotyping of T-DNA lines

Wild type and insertion mutants were emasculated 2 days before anthesis and cross-pollinated 2 days later. Crosses with limited pollen were conducted by transferring mature pollen of hsfB2a-At1t+ planters to a wild type stigma with the help of a ciliary. SALK_012418 and the progeny of the respective crossings were genotyped for the hsfB2a-At1t allele using T-DNA specific primer LBa1 and either of the gene specific primers N512418R or N512418L, which amplify the wild type allele. PCR products were sequenced to confirm the insertion site of the T-DNA after nucleotide 260 of the TAIR annotated cDNA. On both ends of the insertion we found the left border sequence of the T-DNA. To rule out a second insertion elsewhere in the genome, we digested genomic DNA of the heterozygous mutant, segregating plants with no T-DNA insertion in HSFB2a and Col0 wild type with HindIII and subjected it to southern blot hybridization probed with a radioactively labeled 203 bp fragment amplified with the primers TDNALBF/TDNALBR, specific for the left border
sequence of the T-DNA. Two specific bands were detected only in the digest of the heterozygous mutant, not in wild type segregants.

Microscopy and staining of ovules

For microscopy of female gametophytes the oldest closed flower bud of an inflorescence was emasculated and harvested 2 days later. Whole-mount clearings of ovules were performed as described (Yadegari et al. 1994). Cleared whole-mounts were visualized using a Zeiss AXIOSCOP Microscope (Zeiss, Oberkochen, Germany).

Seed analysis in hsfB2A-ttl/+ heterozygotes and wild type

For seed counting siliques at position no. 6–10 of the primary inflorescence were placed on double-sided tape on a microscope slide and opened with a scalpel under a dissecting microscope.

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