Original Article

A new link between stress response and nucleolar function during pollen development in *Arabidopsis* mediated by AtREN1 protein

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ABSTRACT

Heat shock transcription factors (Hsfs) are involved in multiple aspects of stress response and plant growth. However, their role during male gametophyte development is largely unknown, although the generative phase is the most sensitive and critical period in the plant life cycle. Based on a wide screen of T-DNA mutant lines, we identified the atren1 mutation (restricted to nucleolus1) in early male gametophytic gene A1g77570, which has the closest homology to HSFA5 gene, the member of a heat shock transcription factor (HSF) gene family. The mutation causes multiple defects in male gametophyte development in both structure and function. Because the mutation disrupts an early acting (AtREN1) gene, these pollen phenotype abnormalities appear from bicellular pollen stage to pollen maturation. Moreover, the consequent prolagmic phase is compromised as well as documented by pollen germination defects and limited transmission via male gametophyte. In addition, atren1− plants are defective in heat stress (HS) response and produce notably higher proportion of aberrant pollen grains. AtREN1 protein is targeted specifically to the nucleolus that, together with the increased size of the nucleolus in atren1 pollen, suggests that it is likely to be involved in ribosomal RNA biogenesis or other nucleolar functions.

Key-word: heat shock response; heat shock transcription factor; male gametophyte development; nucleolus; pollen.

INTRODUCTION

Male gametophyte development in higher plants is a complex process requiring the coordinated participation of various cell types of flower tissues. Developmental events of the pollen grain formation are based on precise spatial and temporal gene expression activity co-regulated by transcription factors. At the structural level, haploid microspore released from a tetrad undergoes a massive reconstruction of the cell wall and volume increase accompanied by a progressive cycle of vacuole biogenesis. This process leads to a remarkable polarization of the microspore that is necessary for the first highly asymmetrical haploid mitosis producing two cells with different fates – a large vegetative cell enclosing a small generative cell. The generative cell then migrates in and undergoes the second pollen mitosis to produce two sperm cells that, together with the vegetative nucleus (VN), assemble into the male germ unit (MGU). At the functional level, pollen maturation is accompanied by an intensive carbohydrate and lipid metabolism and synthesis of RNAs and proteins to support the demanding progamic phase (Pacini 1996; Twell 2002; Gibalová et al. 2009). The accumulation of carbohydrates, proline, glycine-betaine and other osmoprotectants as well as heat shock proteins (HSPs), especially small HSPs (sHSPs), is necessary to keep the elementary cellular components vital until the pollen grain rehydrates again on the stigma surface (Schwacke et al. 1999).

Pollen desiccation is an internal stress-like process and represents a critical event in the pollen formation. While sporophytic stress response generates quantity of protectants, male gametophyte is more reluctant in terms of HSPs and reactive oxygen species (ROS) scavenger production on the whole. Hence, male gametophyte development is the most sensitive stage in the plant life cycle being easily affected by stressful environment (Barnabás, Jäger & Fehér 2008). Because of ongoing climate changes and because of most world crop production being based on successful sexual reproduction, great attention has been paid to the effect of high temperature on male gametophyte development in crop species (Sakata et al. 2000; Koonjul et al. 2005; Firon et al. 2006; Jain et al. 2007a, b; Jagadish et al. 2010), with special focus on HSP expression (Hopf, Plesofsky-Vig & Brambl 1992; Mascarenhas & Crone 1996; Young, Wilen & Bonham-Smith 2004; Volkov, Panchuk & Schöffl 2005). However, data explaining the effect of a heat shock on male gametophyte development in *Arabidopsis* are rather limited (Kim, Hong & Lee 2001; Zinn, Tunc-Ozdemir & Harper 2010; Tunc-Ozdemir et al. 2013).

HSPs, the crucial molecular chaperons, are functionally linked to heat shock transcription factors (HSFs) which stand as terminal regulators in stress responsive pathway and cross-talk to each other (Baniwal et al. 2004; Port et al. 2004; Shamovsky & Nudler 2008; Hu, Hu & Han 2009). The family of plant HSFs is remarkable for a large number of family members comparing to non-plant species (Scharf et al. 2012).
which pre-destinates them to unique plant functions operating at complex regulatory pathways. The structural analysis, functional prediction of each domain and classification of HSFs (A, B, C class) were described in detail elsewhere (Nover et al. 1996, 2001; von Koskull-Doring, Scharf & Nover 2007; Scharf et al. 2012). A huge investigation aimed at the identification of the regulatory functions of individual HSFs. There is a good evidence indicating that HSFA1 works as a master regulator either with dominant heat stress (HS) response (Mishra et al. 2002; Giorno et al. 2010; Bechtold et al. 2013) or low but constant expression subsequently activating HSFA2 if needed, which then acts as a ‘working horse’ enhancing thermostolerance (Nishizawa et al. 2006; Schramm et al. 2006; Charng et al. 2007; Liu et al. 2013). HsfA3, the major component in drought and HS signalling induced by DREB2A transcription factor, represents another example of stress-responsive HSF (Bharti et al. 2006). However, there are members of HSF family playing active role under non-stress conditions as well. This is the case of HsfA9, which is involved in embryo development and seed maturation in sunflower and Arabidopsis (Almoguera et al. 2002; Díaz-Martín et al. 2005; Kotak et al. 2007).

We attempt here to uncover the potential role of the closest homolog of HSFA5, AtREN1, in the nucleolus during the male gametophyte development. HsfA5 plays an unusual role among the A-class HSFs and acts as a specific repressor of the HS gene expression activator HsfA4 in tomato and Arabidopsis (Baniwal et al. 2007). However, to our best knowledge, there is no direct information available about the role of HsfA5 or other members of HSF family in male germ line.

The nucleolus is a prominent, highly dynamic structure with diverse molecular functions (Hernandez-Verdun 2006; Kim 2009). The relationship between its typical zonal organization and the most significant role in the fabrication of ribosomal subunits was described and extensively reviewed (Raška et al. 2004; Raška, Shaw & Cmarko 2006; Sáez-Vásquez & Medina 2008). However, ribosomal RNA (rRNA) and ribosomal biogenesis are not the only key functions of the nucleolus. It is appreciated as a multifunctional sub-nuclear body controlling cell-cycle progression and proliferation, processing other small RNAs and producing multiple ribonucleoprotein particles, and controlling spliced mRNAs (Boivert et al. 2007; Brown & Shaw 2008; Sirri et al. 2008; Shaw & Brown 2012). Alongside the above listed canonical functions, the nucleolus is also a notable stress-sensing structure playing the central role in the coordination of cellular stress response (Mayer, Bierhoff & Grummt 2005; Lorković 2009; Boulon et al. 2010). Heat shock itself can cause transcription inactivation of both RNA polymerase I and RNA polymerase II complexes (Zhai & Comai 2000) as a strategy to keep cellular homeostasis (Sengupta, Peterson & Sabatini 2010). However, the knowledge about nucleolar functions during pollen development is largely missing, and up to date, only few scattered analyses have considered this topic. Firstly, the mutation of AtRH36, an RNA helicase which is involved in rRNA biogenesis, affects female gametogenesis and to a lesser extent also male gametophyte development (Huang et al. 2010). Secondly, the cohesion protein SYN3 acting in nucleolus plays an important role in pollen development (Jiang et al. 2007), and thirdly, SLOW WALKER1 encoding WD-40 protein, which is involved in 18S rRNA biogenesis, is essential for gametogenesis (Shi et al. 2005).

Hence, the aim of our work was to functionally characterize the atren1 mutation as a first record of the sensitivity to the heat shock and impact on nucleolar dynamics at the structural and functional levels with respect to male gametophyte development.

**MATERIALS AND METHODS**

**Plant material**

We used T-DNA insertion line SALK_141481 from The European Arabidopsis Stock Centre with the insert in the only exon of the At1g77570 gene (Fig. 1a). Plants were grown in soil in a cultivation room at standard conditions of 22 °C, 16 h/8 h day/night regime with illumination of 150 μmol m⁻² s⁻¹.

**Isolation of pollen developmental stages**

Four pollen developmental stages including microspores [uninucleate microspores (UNM)], bicellular pollen (BCP), tricellular pollen (TCP) and mature pollen grains (MPG) were separated in order to isolate RNA for further experiments by modified protocol of Honys & Twell (2004). Inflorescences of fully blooming atren1/− mutant plants (thousands) were cut and collected into 1 L flask on ice. Later, 100 mL of cold 0.3 M mannitol was added and shaken (twice with the same volume of fresh solution) for 5 min to wash out mature pollen from open flowers. The total

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**Figure 1.** (a) At1g77570 gene model containing the only exon (black box) including SALK_141481 T-DNA insertion site (triangle) and positions of respective primers (arrows); untranslated regions (grey boxes); LB and RB (left and right borders of T-DNA); the position of START and STOP codons and T-DNA insertion are in brackets. (b) Expression analysis of both end regions of AtREN1 transcript in wild-type (WT) and atren1 pollen; RT-PCR of AtREN1 mRNA 5′-end (upstream of T-DNA insertion, primers REN_F1/R1) and 3′-end regions (downstream of T-DNA insertion, primers REN_F2/R2) as well as eIF5A control transcript (primers eIF5A_F/R).
suspension of mature pollen was filtered through 100 and 50 μm nylon membrane and centrifuged at 450 g, 5 min, 4 °C. By repeating the centrifugation under the same conditions, the initial volume of 200 μL was reduced to 1 mL, and after the final centrifugation step, the dried pollen pellet was frozen at −20 °C. Remaining washed inflorescences were gently mashed in small portions of 0.3 M mannitol in mortar on ice to break the immature anthers and to release the developing spores. The suspension was filtered and centrifuged under the same conditions. Similarly, the initial volume was decreased to 800 μL. Meanwhile, Percoll step gradient I (85%/45%/20%) was prepared in four centrifuge tubes with 2 mL of each layer. A total of 200 μL of immature pollen suspension was applied on the top of the gradient and centrifuged in swing rotor at 450 g, 5 min, 4 °C. Three separated layers (from the top: one UNM, two mixtures of UNM and BCP, and three TCPs) were collected in microcentrifuge tubes on ice. Suspensions of pure UNM and TCP were pelleted, dried and stored at −20 °C. The middle layer containing UNM and BCP was again applied on Percoll step gradient II (25%/35%/45%) and centrifuged in swing rotor at 450 g, 5 min, 4 °C. The upper layer containing UNM and middle and lower layers containing BCP were pelleted, dried and stored at −20 °C.

Phenotype analysis

At least three flower buds from a plant containing immature pollen stages (UNM, BCP and TCP) were collected into DAPI/GUS buffer (400 ng mL−1; Park, Howden & Twell 1998; GUS solution: 50 mM Na2HPO4, 50 mM NaH2PO4, 10 mM EDTA; Gallagher 1992). Pollen from young anthers was mechanically released and stained on glass slides. Mature pollen (MPG) was collected from at least three fully opened flowers and directly released and stained in DAPI/GUS buffer and observed in visible (VIS) and ultraviolet (UV) epifluorescence microscopy (Nikon Eclipse TE2000-E; Nikon Corp., Shinjuku, Tokyo, Japan).

DNA isolation and genetic analysis

A leaf sample was frozen in liquid nitrogen, mashed with glass beads (MagNA Lyser; Roche Applied Science, Penzberg, Germany) and mixed with 250 μL CTAB extraction buffer (1.4 M NaCl, 20 mM EDTA, 100 mM Tris pH 8.0, 3% CTAB) at room temperature (RT) for 20 min. Then, 250 μL of chloroform:isoamylalcohol mixture (CH:IAA, 24:1) was added and mixed. Samples were centrifuged (8000 g, 10 min, RT), and 200 μL of the upper aqueous phase was transferred into a tube containing 140 μL of isopropanol. After mixing, tubes were left for 5 min for DNA denaturation and centrifuged once again (8000 g, 2 min, RT). DNA pellets were washed with 1 mL of 70% ethanol and, after a final centrifugation (8000 g, 2 min, RT), carefully dried, resuspended in 50 μL H2O and stored at −20 °C. Progeny of self-crossed heterozygous plants was genotyped for the segregation analysis. Segregated plants of three genotypes were tested for the co-segregation of the phenotype defects with the mutation. Outcrosses between Col-0 wild type (WT) and heterozygotes in both directions were done to check the transmission analysis. For that, flowers were emasculated in flower buds, isolated and pollinated in the following day when stigmas were fully developed. After the seeds developed, the segregation ratio of the progeny was analysed.

**RNA isolation and expression analysis**

Two sets of samples were used: (1) pollen developmental stages, root, stem, leaf and inflorescence from Col-0 WT to verify the At1g77570 gene expression profile; and (2) all pollen developmental stages from atren1/− mutant plants to verify the knockout or knockdown of the AtIREN1 mRNA in mutant pollen. Total RNA was isolated by RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) from the respective samples according to the manufacturer’s instruction. The quality and quantity of total RNA were determined on agarose gel and spectrophotometrically (Nanodrop; Thermo Fisher Scientific, Waltham, MA, USA). After the DNase treatment (RQ1 RNase-Free DNase; Promega, Madison, WI, USA), RT-PCR was performed using ImProm-II Reverse Transcription System (Promega) with oligo(dT); or random primers and RNA from Col-0 or atren1 pollen; cDNA from Col-0 WT was then used to verify the gene expression profile using gene-specific primers REN_F:ATCTTGACGCCCTTTGAAT and REN_R:AGACTTGAAATCTACTTTGGA GA, and cDNA from atren1/− mutant plants was used to verify the knockout mutation using cDNA_REN_F1: CTTCTTAGTCTAAATCTTCG, cDNA_REN_R1:TTGTA ACCATCCCTAAAC, cDNA_REN_F2:GAGATAAAC TGGAGCTTATG and cDNA_REN_R2:ATTTGCGAGTG TTGGAAGAG. Because the AtIREN1 gene (At1g77570) has no intron, to exclude the contamination by gDNA, the following set of primers was used for the HSF5A gene (At4g13980) over the intron providing the PCR products of 1402 bp (−intron) or 1730 bp (+intron), eIF5A (eukaryotic translation initiation factor 5A, At1g13950) was used as a reference housekeeping gene; eIF5A_F:ACGCTTCACA TCGTATCAAA, eIF5A_R:TAACATGCGGGCACATCA CAA.

**Promoter analysis**

A total of 1 kb upstream region from +1 ATG was amplified and introduced via D-TOPO donor vector (Invitrogen, Carlsbad, CA, USA) into the Gateway-compatible pKGWS7 destination vector (VIB, Ghent, Belgium; Karimi, Inzé & Depicker 2002) for β-glucuronidase (GUS) assay. Expression clones were transformed into Agrobacterium tumefaciens strain GV3101 and transformed into Arabidopsis by floral dip method (Clough & Bent 1998). Seeds were sown onto LB Agar containing 50 μg mL−1 Kanamycin. Resistant plants were later transferred into soil and grown. Top inflorescences were cut and immersed into the GUS-staining buffer (0.1 M phosphate buffer, pH 7.0; 10 mM EDTA, pH 8.0; 0.1% triton X-100 supplemented with 1 mM X-glC and 4 mM ferricyanide). After the 2 d incubation at 37 °C, samples were decolorized in ethanol:aceton (3:1) and...
observed in bright field and epifluorescence under the Nikon Eclipse TE2000-E microscope.

Complementation

The WT allele from Col-0 DNA including 1 kb upstream region from +1 ATG and following coding region + 3′UTR was introduced via D-TOPO donor vector (Invitrogen) into the Gateway-compatible pKGW destination vector (VIB; Karimi et al. 2002). Expression clones were transformed into A. tumefaciens strain GV3101 and transfected into atren1/+ plants by floral dip method. Seeds were sown onto LB agar containing 50 µg mL⁻¹ Kanamycin. Selected plants were later transferred into soil and grown under standard conditions. Mature pollen was collected from open flowers and checked for the phenotype in VIS and UV microscopy.

Transient expression

The whole coding sequence of AtREN1 was subcloned into the pGWB5 and pGWB6 vectors (VIB) in order to create C- and N-terminal GFP fusion proteins. The expression clones were transformed into A. tumefaciens strain GV3101 and transfected by syringer into the epidermal layer of tobacco (Nicotiana benthamiana) leaves. After 1 d dark period, leaf segments were cut and mounted in 10% glycerol on a glass slide for confocal microscopy.

AgNOR staining

Freshly collected whole Arabidopsis flowers were fixed in Carnoy’s solution. Subsequently, the material was hydrated in decreasing concentrations of ethanol (70-50-25-10%), washed in 5% acetic acid and finally twice in distilled water, 5 min in each solution in a 15 mL tube. After maceration, flowers were impregnated by 50% AgNO₃ in water, 55 °C, 20 min; then incubated in 1:1 mixture of 2% gelatin in 1% formic acid and 50% AgNO₃ for 20 min in dark. The plant material was then washed with distilled water for 10 min and then treated with 5% sodium thiosulfate for 10 min (Zienkiewicz & Bednarska 2009; modified). Individual anthers of sequential developmental series were prepared on slide for confocal microscopy.

Heat treatment

The progeny of atren1/+ from seedling stage to fully developed inflorescences was grown (6 weeks) in cultivation chamber Sanyo MLR-351H (Sanyo, Osaka, Japan) at 16 h/8 h day light regime, 22 °C and 70% humidity. After that, the temperature was set up to 30 °C for 2 d, other parameters were kept the same. At least three open flowers were collected before and after the 2 d HS from the same plants to observe the pollen phenotype in VIS and UV epifluorescence. The heat-treated flowers were collected the second day at 30 °C to assure that all collected flowers had completed pollen development from meiosis under the HS conditions.

Analysis of pollen tube growth

Col-0 WT flowers were emasculated and pollinated the following day by fresh pollen from Col-0 WT (control) and homozygous atren1/+ plants. Five hours after the pollination, the pistils were cut and fixed in ethanol:acetic acid (3:1) mixture for 1 h at RT. After overnight softening in 8 M NaOH, the pistils were washed in distilled water and stained for callose by aniline blue solution (0.1% aniline blue in 0.1 M K₂HPO₄-KOH buffer, pH 11) for 3 h as described by Ishiguro et al. (2001) to compare pollen tube growth with WT control in planta. For in vitro assay of pollen tube growth, 20 flowers were collected into 750 µL of 0.3 M mannitol, shaken and centrifuged. Mature pollen from the pellet was dispensed on an agarose pad (1.5% low-melting agarose in 0.01% H₂BO₃, 5 Mm CaCl₂·2H₂O, 1 mM MgSO₄·7H₂O, 5 mM KCl, and 10% sucrose, pH 7.5; Boavida & McCormick 2007) on a glass slide. The pollen germinated in a dark humid chamber at 22 °C and grew overnight, and then observed in VIS microscopy.

RESULTS

Phenotype defects of pollen developmental stages

The gene At1g77570 was selected for a detailed analysis as a candidate of early-expressed pollen putative transcription factors. Originally, it was annotated as a member of the heat shock factor family. However, according to the TAIR annotation, At1g77570 was described to contain only an HSF-like domain while other databases (DATF, PntTDFB) directly included the gene into the HSF family. Therefore, it can be considered as a side member of the HSF family of transcription factors annotated as HSFA5-like. The appropriate SALK_141481 T-DNA insertion line harbouring the insertion in the only exon of At1g77570 gene was evaluated for possible phenotype defects caused by the atren1 mutation (Fig. 1a). In order to evaluate the level of AtREN1 mRNA knockdown caused by the T-DNA insertion, RNA was isolated from four pollen developmental stages (UNM, BCP, TCP and MPG) of atren1−/− homozygous mutant plants and used for semi-quantitative RT-PCR with two sets of primers flanking the T-DNA insertion site. The AtREN1 transcript was absent in all samples verifying its complete knockdown (Fig. 1b – see Methods).

The At1g77570 gene was previously selected for its early and significantly pollen-enriched expression according to the microarray data (Fig. 2a) (Honys & Twell 2004). Its expression profile was verified by semi-quantitative RT-PCR of mRNA isolated from four pollen developmental stages (UNM, BCP, TCP, MPG), inflorescence, leaf, stem and root of Col-0 plant. The AtREN1 transcript was detected only in the
early-stage spores (UNM and BCP) and in the inflorescence containing both these stages (Fig. 2b). AtREN1 promoter activity was further verified in pAtREN1::GUS transgenic plants. In selected T1 plants, the GUS signal was restricted only to early pollen stages including UNM and BCP. No sporophytic tissues (root, stem, and leaf) were stained with the sole exception for tapetum in young anthers (Fig. 2c–f).

All phenotypic defects associated with the atren1 mutation were observed only in male gametophyte (Fig. 3), no other visible abnormalities in female gametophyte or in sporophytic tissues including tapetum were found. The mutant phenotype was first observed in mature pollen of homozygous plants showing up to 30% MGU disruption (20% dispersed plus 5–10% eccentric MGU) (Fig. 3h) in terms of previously published classification (Renáč, Dupláková & Honys 2012). However, such expressivity was not found in all homozygous plants, instead, some homozygotes showed milder phenotype defects indicating lower mutation penetrance and expressivity (Fig. 4a). Nevertheless, the percentage of MGU disruption from heterozygous plants reached about one half of the level found in homozygotes, as expected for a gametophytic mutation. The phenotype co-segregated with the genotype, which was proven also in a larger population of about 200 plants (data not shown).

Because AtREN1 was an early expressed gene, we included the early developmental stages of immature pollen into our analysis in order to find out at what stage the MGU disruption started. Firstly, it was necessary to characterize the phenotype of Col-0 WT developing spores because such overview was not available so far. In this respect, the presented survey of the Arabidopsis WT pollen appearance during its development represents the first published record of this kind. Five healthy Col-0 and atren1−/− plants grown at two different places (cultivation room and growth chamber) under standard conditions were used for the analysis. There was no visible difference between plants cultivated at both places. All samples showed very little phenotype variation in all developmental stages. Isolated microspores from both Col-0 and mutant plants showed a uniform image (Fig. 4b, UNM). In the BCP stage, however, we distinguished two variants in nuclei arrangement. Both the vegetative nucleus (VN) and the generative nucleus (GN) were in 93% BCP tightly bound near the centre of the grain forming MGU. The remaining portion of pollen grains represented a population with more dispersed organization of the two nuclei. We have measured the distance between VN and GN as a fold of diameter of GN. Such distribution varied in this population of BCP from 1.5 to 2 GN diameters. This type represented 7% BCP in WT while in mutant pollen this percentage raised up to 30% (Fig. 4b, BCP). In the TCP, we clearly distinguished even three types of MGU arrangement. Firstly, it was the common image of closely bound MGU in the centre. Such type represented 41% in Col-0 and 35% in the mutant. Secondly, the MGU figure was not so tightly bound but rather relaxed, representing 44% in WT and 28% in mutant pollen. Thirdly, the MGU arrangement was even looser with various eccentric or separated nuclei figures counting for 15% in Col-0 and as many as 38% in atren1−/− mutant. Only this pollen phenotype was considered as strongly MGU disrupted in our analyses.

Figure 2. (a) The levels of AtREN1 (At1g77570) expression during pollen development according to microarray data visualized by a GFP database (http://agfp.ueb.cas.cz; Dupláková et al. 2007). The expression is specifically active within early stages [uninucleate microspores (UNM) and bicellular pollen (BCP)] while absent in late stages [tricellular pollen (TCP) and mature pollen grains (MPG)]. (b) AtREN1 expression profile verified by semi-quantitative RT-PCR. The expression was detected in microspores (UNM, BCP) and in inflorescences (INF) containing these stages. No expression signal was detected in other tissues, both gametophytic (TCP and MPG) and sporophytic [leaf (LEF), stem (STM) and root (ROT)]. GUS staining of pAtREN1::GUS-transformed plants confirmed the AtREN1 promoter activity only in young anthers (c–d) containing UNM (e) and BCP (f). Other tissues including premeiotic stages, TCP and MPG remained unstained.

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Because of the complex phenotypic nature of TCP, 2405 grains of Col-0 were observed in total from different plants (with comparable results) to avoid accidental fluctuation of the phenotype (Fig. 4b, TCP). Finally, the MPG stage, already described above, contained 5% MGU disruption in Col-0 plants and up to 30% in mutants (Fig. 4b, MPG).

Considering the cellular structure and nuclear arrangement in tapetal cells, we observed this tissue only in young anthers with UNM and BCP. We noticed the developmental difference between the two stages, particularly rounded cells and large nuclei in cells at the UNM stage, while irregular cells and nuclear shape in tapetum with the first signs of degeneration at the BCP stage. Nevertheless, such development was similar both in Col-0 and atren1−/− anthers with no significant difference in subcellular/nuclear organization in tapetum.

Genetic analysis showed the distortion of segregation ratio

Because we observed the lower proportion of homozygous plants in the population, we performed the segregation analysis of the progeny of self-crossed atren1 heterozygous plants. We found the segregation ratio of 1:1.7:0.8, which was distorted in comparison to the Mendelian 1:2:1 ratio (Table 1). The question remained whether this distortion was caused only by the reduced transmission through the male or whether the transmission through the female was also compromised. Therefore, we outcrossed heterozygous plants to Col-0 in both directions and observed the reduced transmission through both the male (1:0.6) and the female (1:0.8) (Table 1).

Analysis of pollen germination and pollen tube growth

Considering the reduction of male gamete transmission, we speculated whether the reduction was based on pollen germination ability and/or on the rate of pollen tube growth. To test this possibility, we monitored pollen tube growth in vivo. For that reason, pistils of Col-0 plants were pollinated either by WT pollen from Col-0 plants or by pollen from atren1−/− plants. After 5 h, the pistils were collected and stained for callose. The length of pollen tubes was comparable between WT and mutant in several replicates of each pollination experiment (Fig. 5a). In both cases, we observed that pollen tubes in both samples reached the lowest ovules in the ovary after 5 h of growth. To estimate the in vitro pollen germination efficiency, WT and mutant pollen was applied on agarose pads and cultivated for 12 h in the humid chamber. After the cultivation, the proportion of germinated pollen was checked by light microscopy. The in vitro germination efficiency of pollen grains differed significantly as it dropped from 80% in WT pollen down to 20% in atren1−/− mutants (Fig. 5b).

AtREN1 complementation rescued the WT phenotype

To further verify the functional link between the insertion in the At1g77570 gene and the phenotype observed, we cloned the whole genomic sequence including the upstream region of −1000 bp and 3′UTR into the pKGW vector for the complementation analysis. Out of 42 transformants in T1 generation in atren1−/− background, we observed the WT phenotype in 26 plants (<5% pollen abnormalities) and the mild mutant phenotype in 14 plants (5–10% pollen abnormalities) indicating the functional rescue of the WT phenotype (data not shown). Only two plants showed even stronger MGU-disrupted phenotype (36 and 51%) than the atren1−/− background itself. On the basis of these results, we confirmed that the pollen phenotype defects of SALK_141481 line result from the mutation of At1g77570 gene.

Transient expression revealed the AtREN1 nucleolar localization

The localization of AtREN1 fusion proteins with GFP (both N- and C-terminal fusions) in transiently transformed...
tobacco leaf epidermis was restricted to the nucleus, with significantly stronger signal in a large sub-nuclear region presumably corresponding to the nucleolus (Fig. 6a). In addition, the AtREN1-GFP protein localized also into other nuclear bodies. On the contrary, free GFP control was localized diffusely in the cytoplasm and the nucleoplasm but not in the nucleolus (Fig. 6a).

To further specify the AtREN1-GFP localization in the sub-nuclear region, we used standard markers fibrillarin, SRp34 and coilin (Fig. 6c–e). Fibrillarin is present specifically in the nucleolus, coilin is restricted to the Cajal bodies and the splicing-associated protein SRp34 is distributed throughout the nucleoplasm and sometimes concentrated into splicing speckles but excluded from the nucleolus. The co-localization with fibrillarin unambiguously targeted AtREN1 protein into the nucleolus (Fig. 6c). AtREN1-GFP also appeared to co-localize with a subset of Cajal bodies (Fig. 6e). These Cajal bodies were often localized close to the nucleolus or touching its edge, in some cases they were even harboured in the nucleolus.

Nucleolar size changed during pollen development

After AgNOR staining, the nucleolus was clearly detected as a brown spot in light microscopy and it appeared even more contrastively in UV light as a dark spot against DAPI-stained nucleoplasm. The nucleolar size was calculated as a proportion of the Ag-stained area from the total nuclear area observed under UV fluorescence. We observed that in both WT and mutant pollen, the relative nucleolar size was noticeably larger in early stages (UNM, BCP) when compared with later ones (TCP, MPG) reaching its maximum in BCP (Fig. 6f–h). In mature pollen, the nucleolus was almost undetectable. Considering the mutant pollen nucleolus, we observed that its relative size was constantly larger than that of Col-0 pollen. The sole exception was the MPG where the size of both nucleoli was comparable.

Heat treatment affected the pollen development

Although several HSF family members are known to be involved also in the standard development, stress response is considered to be the principal function of HSF proteins. Moreover, the nucleolus is well known for its stress-sensing functions as well (Raška et al. 2004; Mayer et al. 2005). Therefore, we tested whether the application of HS affects the atren1 pollen development. Plants grown in the cultivation chamber at standard conditions were tested for pollen phenotype before the application of HS. The progeny of segregating heterozygotes was used together with Col-0 as a control. The phenotype of heterozygote progeny co-segregated with the genotype of individual plants as expected. Before HS, the abnormal pollen phenotype ratio was 14.3, 7.1 and 5.4% in homozygous, heterozygous and separated WT plants, respectively, in comparison to 4% abnormal pollen in Col-0 plants. The proportion of MGU disruption in heterozygotes approximately corresponded to one half of that in homozygotes. However, after 2 d HS (30 °C), the pollen phenotype of plants bearing the atren1 mutation changed. Most pollen phenotype disruptions were observed within homozygous plants, reaching 40–50% (3–3.5 times more than under non-stress conditions) of total pollen in several individuals. On average, the percentage of MGU disruptions reached 31.6% in homozygous plants, 21.6% in heterozygotes, 10.9% in segregated WT plants and 9.5% in Col-0. The phenotype of segregated WT plants remained more or less stable after HS and was comparable to the response of Col-0 control plants (Fig. 7). On the whole, the portfolio of pollen phenotype abnormalities (in terms of phenotype classes) in homozygotes after the heat shock was comparable to the phenotype under standard cultivation conditions, which means that the pollen with eccentric and dispersed MGU dominated among pollen abnormalities. The fitness of all observed plants during 2 d at 30 °C remained stable without any visible sign of suffering except for faster growth and branching of inflorescences.

Table 1 Transmission of the atren1 allele

<table>
<thead>
<tr>
<th>Cross-type</th>
<th>Total</th>
<th>+/+</th>
<th>+/-</th>
<th>−/−</th>
<th>Observed ratio (+/+ : ± : −/−)</th>
<th>Mendelian ratio</th>
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<td>Self-cross</td>
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<td>atren1/+ × atren1/+</td>
<td>280</td>
<td>80</td>
<td>135</td>
<td>65</td>
<td>1 : 1.7 : 0.8</td>
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<td>Cross-type</td>
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<td>Observed ratio (+/+ : ±)</td>
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<td>Outcross</td>
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<td>Col-0 × atren1/+</td>
<td>211</td>
<td>132</td>
<td>79</td>
<td></td>
<td>1 : 0.6</td>
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<tr>
<td>atren1/+ × Col-0</td>
<td>216</td>
<td>120</td>
<td>96</td>
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<td>1 : 0.8</td>
<td>1 : 1</td>
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in a humid chamber.

and growth (12 h) of Col-0 and atren1 pollen tubes on agarose pad in a humid chamber. atren1 pollen germination rate (20%) was four times reduced in comparison to the Col-0 pollen (80%).

DISCUSSION

The At1g77570 gene encoded a protein that contained an HSF-like domain and was annotated as HSFA5-like. Guo et al. (2008) performed the phylogenetic analysis of the HSF family and found out that the protein encoded by At1g77570 contained only DBD (DNA-binding domain) and a part of HR-A/B (heptad hydrophobic repeat-A/B) domain with less conservation probably as a result of an incomplete gene duplication during its evolution. Because of this fact, AtREN1 protein encoded by the At1g77570 gene cannot serve as a direct transcriptional activator as it lacks the aromatic, hydrophobic and acidic amino acids (AHA) (activation domain), and it is questionable whether it is able to cooperate with other HSFs via its HR-A/B region.

As stated above, HSFA5, the closest homolog of AtREN1, plays a unique role among HSFs as a repressor of HsfA4 which controls the HS response possibly triggered by HSFA4 (Yamanouchi et al. 2002; Baniwal et al. 2007; Shim et al. 2009). Surprisingly, the transcription of HSFA5 gene is specifically up-regulated in soybean and Arabidopsis pollen (von Koskull-Doring et al. 2007; Haerizadeh et al. 2009) indicating its function in pollen development especially at late developmental stages. HSFA4c (At5g45710) was also described as pollen specific but its expression started earlier, from bicellular stage on, and it increased during pollen maturation. If true, HsfA4c cannot be repressed at the BCP stage by HSFA5 and this begs the question whether the closely related AtREN1 protein could play such a role at least before the expression of HSFA5. In general, the oligomerization domain is necessary for the intermolecular interaction. However, the blocking of the HSEs (cis-elements) in regulated downstream genes may be sufficient to exert the repressing function. Alternatively, and more likely, AtREN1 protein can act in a different regulatory pathway. The goal of this work was to describe and characterize the atren1 pollen mutation caused by T-DNA insertion in At1g77570 gene.

The introduction of AtREN1 WT allele into the atren1/+ background rescued the WT phenotype in most plants (40/42) and clearly demonstrated the functional link between mutation and phenotype. However, two plants in T1 generation produced even more MGU-disrupted pollen than in atren1 background itself. This observation may suggest an accidental disruption of another gene active in pollen development by the transformation.

The mutation of AtREN1 gene caused a significant disruption (up to 30%) of male gametophyte development. In addition, the type of observed phenotype defects (separated and eccentric MGU) may have no specific link to the T-DNA insertion in At1g77570 but may rather reflect the overall cellular disruption because it was commonly found in numerous T-DNA lines (Renák, Dušláková & Honys 2012). Considering phenotype defects throughout pollen development, no effect of AtREN1 gene disruption was observed in UNM although the gene expression was confirmed at this stage (Figs 2 & 3). Additionally, the latter case was also significant for tapetal expression in young anthers as the only sporophytic tissue detected. This finding would be in agreement with the phenomenon of 'haplo-diploid transcription' (Honys, Renák & Twell 2006) because microsporogenesis and tapetal functions are physiologically bound and a number of genes are transcribed in tandem in both tissues. However, to explain the absence of phenotype defects caused by the AtREN1 gene disruption in UNM, we assume that the gene may be either expressed just after the global settlement of microspore arrangement, and thus it was too late to affect it, or the protein may be translated/become active at the UNM/BCP transition, and hence may be able to influence cellular processes rather in BCP. Therefore, different types of BCP and later of TCP are considered to represent an outcome of the MGU dislocation after pollen mitosis I (PMI). The discovery that the atren1 mutation affected BCP to the same extent as observed in MPG (Fig. 4b) suggested that the mutation actually specifically affected this developmental stage and that the effects of this functional disruption persisted throughout the male gametophyte development including the pollen fitness. The loose MGU organization in the BCP and the amplified manifestation of this defect in the TCP are likely to result from the fact that early TCP just after pollen mitosis II (PMII) is a dynamic structure with global cytoskeletal rearrangement related to PMII which makes it more sensitive to disturbances (Zonia, Tupý & Staiger 1999; Oh et al. 2010, 2012). Potentially, AtREN1 could be involved in such massive microtubule or microfilament rearrangement during PMI and PMII as described by Zonia, Tupý & Staiger (1999) for non-Arabidopsis species. Considering Arabidopsis, the data on cytoskeletal dynamics during pollen development are rather missing. The rapidly increasing volume of post-PMII pollen grain is also likely to add up to this phenomenon. To explain the MGU phenotype defects in atren1 pollen in the whole context, additional analyses will be needed. In any case, the loose MGU figure was...
re-consolidated again at the transition from TCP to MPG resulting in vanishing of previous settings. As mentioned earlier in promoter analysis, the only sporophytic expression was observed in tapetum of young anthers containing UNM and BCP. Because tapetum largely consists of binuclear cells, we focused on its cellular structure and nuclear arrangement as well. However, no visible difference was found between Col-0 and atren1/− tapetum cells. Perhaps, AtREN1, if translated, is involved in a different way than in male gametophyte or the effect of its absence in tapetum may be masked by the activity of another protein.

Another manifestation of atren1 mutation effects was observed at the level of the segregation ratio distortion in the population of heterozygous plants (Table 1). The shift from Mendelian ratio 1:2:1 to the observed 1:1.7:0.8 was caused mainly by the reduced male gamete transmission. This ratio...
corresponded to the abundance of MGU-disrupted mature pollen evolving from the BCP stage. It also suggested that such a proportion of pollen grains bearing the mutant allele compromised not only the pollen development but also the progamic phase. This idea was further supported by the fact that mutant pollen exhibited significantly reduced germination rate and pollen tube growth in vitro. On the contrary, once germinated, the subsequent pollen tube growth was not affected in vivo.

Based on the transient expression, the AtREN1 protein was localized in the nucleus and predominantly in the nucleolus (Fig. 6). Considering this, it still may act as a transcription factor, the first suggestion of its function being the involvement in the biogenesis of rRNA. Because AtREN1 is specifically expressed at early stages of pollen development, it may be required for the expression of pollen/stage-specific expression of rRNA. It has been published that a tissue-specific heterogeneity was observed in the population of 45S pre-rRNA in rat (Higashi et al. 1972) or even alternative pre-rRNA splicing in testis and other tissues of Ctenomys (Melen et al. 1999). Similarly, it has been shown that most ribosomes were made before PMII in the developing Arabidopsis pollen (Honys & Twell 2004). In connection with this, we isolated rRNA from atren1 and Col-0 pollen produced under normal or HS conditions. However, we found no differences in the rRNA pattern (data not shown). Alternatively, the AtREN1 protein may be involved in the regulation of active/inactive state of ribosomal DNA. This also represents a key regulatory event during the development to satisfy the actual demand – although there are hundreds of tandem repeat copies of rDNA, only 5–50% of them are actively transcribed (French et al. 2003; Grummt 2003). There are several transcription factors known to be involved in the rRNA biogenesis and necessary for male/female gametophyte development or embryogenesis. (Shi et al. 2005; Sugikawa et al. 2005; Portereiko et al. 2006; Griffith et al. 2007; Harscoët et al. 2010; Huang et al. 2010).

Besides its canonical role, there are many other functions assigned to the nucleolus including siRNA/miRNA transcription, nucleotide modification, mRNA export/splicing control, nonsense-mediated decay, transcriptional silencing and maturation of snRNAs/snoRNAs, the processes in which Cajal bodies also participate (Tani et al. 1995; Boisvert et al. 2007; Brown & Shaw 2008; Jellbauer & Jansen 2008; Pontes & Pikaard 2008; Kim 2009; Koroleva et al. 2009; Shaw & Brown 2012). The fact that the AtREN1 protein was dynamically localized also in Cajal bodies indicated its possible alternative function besides rRNA biogenesis. In some cells, the GFP signal was more intense in the nucleolar outer ring where this granular component is usually localized. There is an evidence that not only ribosomal subunits are assembled here but that it represents also the shared sub-nuclear domain for miRNA, SRP-RNA biogenesis, AFR (Karayan et al. 2001) and nucleostemin (Ritland Politz et al. 2006; Romanova et al. 2009). Considering AtREN1 as a side member of HSF family, it is important to note that nucleolus....
is well known for its stress-sensing functions as well (Raška et al. 2004; Mayer et al. 2005). Indeed, atren1−/− plants were more sensitive to HS, which can be fatal for pollen development. On the contrary, some members of the HSF family are known to be involved rather in normal development than in stress response (HSFA9). The most similar case was described for spermatogenesis regulated by HsfA1 and HsfA2. Disruption of both genes led to disorganized synaptonemal complex and caused male sterility in mice (Eddy 1998; Nakai, Suzuki & Tanabe 2000). Potentially, the AtREN1 protein can ensure both these regulatory functions because both HS response and normal development were affected.

Initially, we used modified AgNOR staining to visualize nuclear bodies and Cajal bodies in particular (Zienkiewicz & Bednarska 2009) since REN1 protein co-localized there as well. Unfortunately, we were not able to observe these structures in Arabidopsis pollen probably due to smaller size of pollen grains and their rougher surface than in Hyacinthus for which this protocol was originally developed. Instead, the nucleolus was clearly distinguishable especially under UV light (Fig. 6). Considering the relative nucleolar size, it was apparent that the nucleolus was larger at early stages (UNM, BCP) than at later stages (TCP, MPG). It may reflect massive biogenesis of ribosomal subunits (Honys & Twell 2004) accompanying intense metabolism and rapid growth in early developmental stages and its gradual decrease towards pollen maturation. Moreover, such change may be connected also to the global developmental programme alternation in terms of the transcription of different gene sets (Honys & Twell 2003, 2004). Focusing on mutant pollen, the nucleolus was constantly noticeably larger (UNM, BCP, TCP) than in WT pollen. Because we do not know the exact function of the AtREN1 protein, we can only speculate what caused this enlargement. May the absence of the AtREN1 protein be compensated by the overexpression of functionally at least partly redundant genes whose abundant protein products accumulate in the nucleolus? Or may the absence of the AtREN1 protein as a potential repressor cause the overexpression of downstream-regulated genes whose proteins accumulate in the nucleolus? In any case, the atren1 mutation disturbs normal nucleolar processes as manifested on structural and functional levels.

The involvement of the AtREN1 protein (side member of the HSF family) in stress response was tested by the application of HS (30 °C/2 d) on developing pollen grains (Fig. 7). We applied a 2 d period of heat treatment to make sure that mature pollen collected for phenotype analysis had undergone its whole development in elevated temperature. The pollen phenotype co-segregated with the genotype and, more importantly, there was an apparent trend in heterozygous and especially homozygous plants to produce higher proportion of aberrant pollen grains. It was no surprise that HS condition negatively influenced the pollen development, which agreed with the published observations (Kim et al. 2001; Barnabás et al. 2008; Zinn et al. 2010). The proportion of MGU-disrupted pollen before the application of stress (5.4, 7.1 and 14.3% in segregated WTs, heterozygotes and homozygotes, respectively) was calculated from limited number of plants involved in HS experiment. However, the proportion of mutant pollen in homozygous plants (14.3%) corresponded to the analysis of AtREN1 penetrance and expressivity (Fig. 4a) performed in larger population.

Aside from the disruption of pollen development, the progamic phase was also affected. The reduction of pollen germination and pollen tube growth in vitro was significant compared with the Col-0 pollen. It seemed unusual that the mutation in early acting protein influenced progamic phase at least at its beginning. To our surprise, the decrease in germination was from 80% in Col-0 to 20% in atren1 pollen. One would expect that this massive drop should be accompanied by the reduction in seed set production. However, no such phenomenon was observed. We believe it was partly conditional-based phenotype because artificial in vitro growth test on agarose provides different environment than on stigma. Perhaps some not necessarily dysfunctional background changes in atren1 pollen were magnified and worsened under in vitro conditions. On the contrary, in planta pollen tube growth did not show any visible difference between atren1 and WT pollen tube growth. It could be caused by the excess of pollen used for the pollination so that only less MGU-disrupted pollen grains (which represented the majority) germinated and grew leading to no visible difference. Overall, the natural self-pollination of heterozygous plants led to the production of a certain portion of mutant pollen with structural and/or functional disorder, however not to such level as observed in vitro but robust enough to cause the reduction in mutant allele transmission.

Our work presents a functional characterization of the SALK_141481 line harbouring the T-DNA insertion in At1g77570 gene encoding a side member of HSF family. We found out that the atren1 mutation affected male gametophyte development while the sporophyte showed no changes. Mature pollen of mutant homozygous plants showed a range of pollen phenotype defects starting from the BCP stage and influencing the progamic phase as well. The AtREN1 protein localized almost exclusively to the nucleolus and the atren1 mutation caused an enlargement of nucleolar volume, suggesting the AtREN1 role in rRNA biogenesis and/or other nucleolar processes. Moreover, the atren1 mutation increased the HS sensitivity during pollen development, thus being the first reported nucleolar protein involved both in normal pollen development and in the stress response in the male germ line.

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