Female gametophyte-specific gene induction system: Development and application to analyses of female gametogenesis in *Arabidopsis thaliana*

Dukhyun Hwang

Contents

Abbreviations

Cter	C-terminus
ER	endoplasmic reticulum
ES2	EMBRYO SAC2
FG	female gametophyte
GFP	green fluorescent protein
H2B	histone H2B
HS	heat shock
HSP	heat shock protein
J protein	DnaJ/Hsp40-family co-chaperone
KASH	Klarsicht/ANC/SYNE homology
LINC	linker of nucleoskeleton and cytoskeleton
NosT	NOPALINE SYNTHASE terminator
COXIV	cytochrome c oxidase subunit IV
SUN	Sad1/UNC-84
SUNDN	SUN dominant negative
T-DNA	transfer DNA
WIPs	WPP domain-interacting proteins
WPP	tryptophan-proline-proline

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Abstract

Flowering plants grow through somatic cell division, and leave their offspring through reproduction, which is an important lifetime. Plant reproduction involves gametophyte formation, fertilization, and embryogenesis. Female gametophyte (FG) is crucial for reproduction in flowering plants. Mutational analyses using *Arabidopsis thaliana* have identified genes and proteins involved in FG development. Techniques that induce gene expression by different external factors have been used as powerful tools for the analyses of various gene functions in plants. In this study, I developed a new method to analyze gene functions during female gametophyte development using heat-inducible Cre/*loxP* recombination system and FG-specific *EMBRYO SAC 2* (*ES2*) promoter. Gene expression from the *ES2* promoter can be detected from the four-nucleated FG stage. Efficient gene induction can be achieved by mild heat treatment at 35°C for 5 min. I applied this method to study gene functions in the fusion of polar nuclei during FG development. Sad1/UNC84 (SUN) proteins are conserved inner nuclear membrane proteins, which interact with the Klarsicht/ANC-1/Syne homology (KASH) proteins spanning the outer nuclear membrane. *A. thaliana* has five SUN protein genes. I used this system to express a dominant-negative mutant of SUN proteins (SUNDN) in developing FGs. Expression of SUNDN resulted in inhibition of the polar nuclear

fusion, while expression of a SUNDN mutant, which does not interact with KASH proteins, did not cause the polar nuclear fusion defect. These results suggest roles of SUN-KASH interactions in the fusion of polar nuclei. The heat-inducible gene expression system developed in this study provides a new strategy for analyzing gene functions in FG development and FG functions.

Introduction

Angiosperms have a distinct fertilization process in which two female gametes, the egg cell and the central cells, are both fertilized (Figure 1; Hamamura et al., 2011). Female gametophyte (FG) is crucial to reproductive process in angiosperms (Christensen et al., 1997). FG development requires several fundamental cellular process, such as vacuole formation, mitosis, nuclear migration, cell wall formation, and nuclear fusion (Christensen et al., 2002).

There are several patterns of FG developmental processes in angiosperms. Most flowering plants including *Arabidopsis thaliana* have *Polygonum*-type FGs, which is consisted of one egg cell, one central cell, two synergid cells and three antipodal cells (Figure 2; Yadegari and Drews 2004). The development of a FG within a developing ovule consists of two phases, megasporogenesis and megagametogenesis. During megasporogenesis, four haploid megaspores are produced after meiosis. During megagametogenesis, three of the four megaspores degenerate, while one surviving megaspore develops into the mature FG. The megaspore containing a single haploid nucleus (stage FG1) undergoes three rounds of mitoses. After the second mitosis, a four-nucleate cell, with two nuclei at each pole (stage FG4), is produced. Cellularization starts after the third mitosis, resulting in the formation of a seven-celled and eight-nucleate FG (stage FG5). The central cell contains two polar nuclei that migrate toward the center of the FG and fuse to form the secondary nucleus before fertilization (stage FG6) in *A. thaliana* and other species (Christensen et al., 1998; Yadegari and Drews, 2004; Drews and Koltnow, 2011).

Genes required for megagametogenesis have been identified in large-scale screenings for FG mutants (Christensen et al., 1998; Pagnussat et al., 2005; Brukhin et al., 2011). Genes

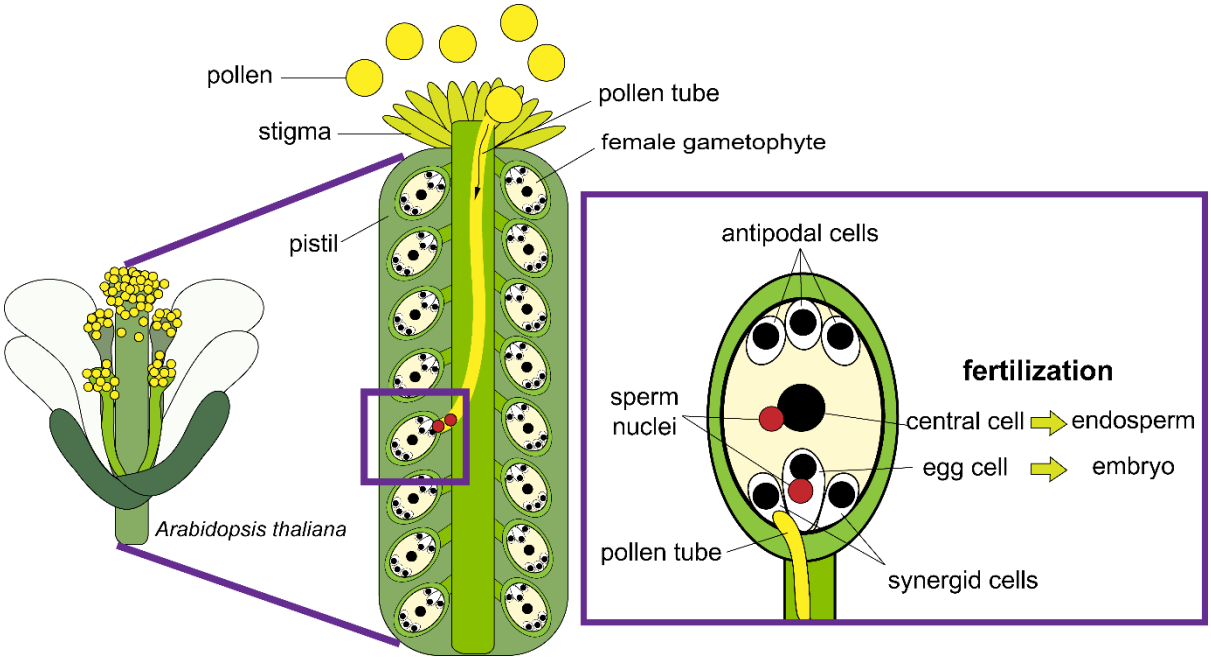

Figure 1. Fertilization of Arabidopsis

Fertilization of flowering plants starts from adhesion of pollen grains on the pistil. Pollen grains adsorb water from the pistil and germinate. Pollen tubes grow in the pistil and are guided to the female gametophytes. Upon entry of the pollen tube into the ovule, two sperm cells are released into the female gametophyte. One sperm fertilizes the egg cell producing embryo and the second fertilizes the central cell producing endosperm.

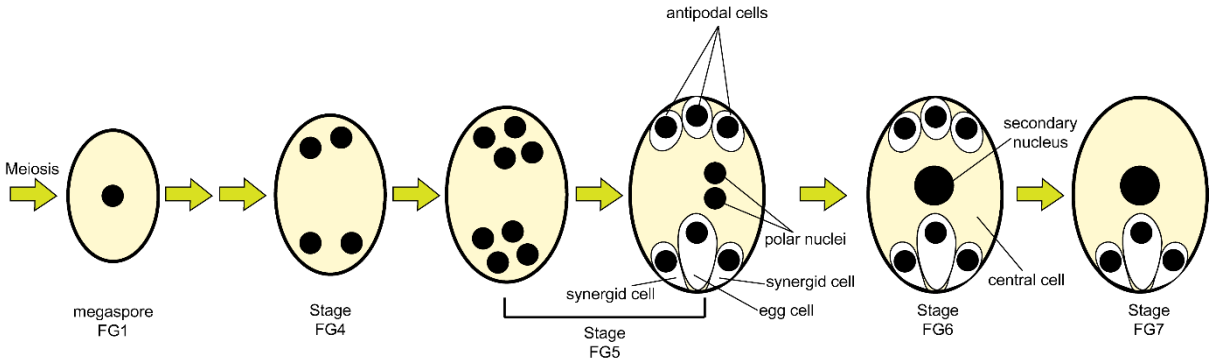

Figure 2. FG development in Arabidopsis

The megaspore contains a single nucleus (FG1), which undergoes three rounds of mitosis. After the second mitosis, a four nucleate cell is produced (FG4). After the third mitosis, cellularization is started and resulted in the formation of a seven-celled and eight-nucleate FG (FG5). The central cell contains two polar nuclei that fuse to the secondary nucleus (FG6). The antipodal cells degenerate, producing the stage FG7.

required for FG functions also have been identified in screenings of genes expressed specifically in FGs (Kasahara et al., 2005; Yu et al., 2005; Johnston et al., 2007; Steffen et al., 2007; Wuest et al., 2010; Wang et al., 2010; Takeuchi and Higashiyama, 2012; Sprunck et al., 2012). Phenotypic analyses of mutants identified in these screenings or those obtained using reverse genetics approaches have been used to reveal the role of the identified genes in FG development and FG functions. Techniques that induce gene expression by different external factors have been used as powerful tools for the analyses of various gene functions in plants (Moore et al., 2006).

In order to analyze the function of genes, Cre-*loxP* mediated recombination has been used to analyze the specific gene functions in cell populations from an extensive range of tissues (McLellan et al., 2017). By using this system, gene expression by targeted insertion and deletion of a DNA fragment from the chromosome can be regulated temporally and spatially and also precisely. (Wang et al., 2005). The heat-inducible gene induction system has been used for mosaic analysis (Sieburth et al., 1998), conditional gene knockout (Nagahara et al., 2015), and transgene induction in somatic cells (Ogawa et al., 2015).

Promoters that allow gene expression in developing and mature FGs have been identified. The *EMBRYO SAC2* (*ES2*) promoter gene encodes a protein related to self-incompatibility proteins and its promoter activity was detected in the embryo sac from the FG3 stage to FG7 (Steffen et al., 2007; Yu et al., 2005). An inducible gene expression system that allow gene expression in developing FGs can be constructed using heat-inducible Cre-*loxP* mediated recombination and the *ES2* promoter.

Linker of nucleoskeleton and cytoskeleton (LINC) complexes are protein complexes

in the nuclear envelope, which are crucial for various cellular processes including duplication, chromosome decondensation, anchorage of spindle pole body and centrosomes to the nuclear envelope (NE), meiotic chromosome bouquet formation, and telomere anchorage (Graumann et al., 2010). The LINC complex is consisted of the Sad1/UNC-84 (SUN) protein in the inner nuclear envelope and the Klarsicht/ANC/SYNE homology (KASH) proteins in the outer nuclear envelope (Figure 3). SUN proteins contain a conserved SUN domain in the luminal side, which interacts KASH proteins (Graumann et al., 2010).

Plant SUN proteins are divided two types, Cter-SUN proteins conserved in land plants and mid-SUN proteins conserved throughout the plant kingdom (Zhou et al., 2015a; Groves et al., 2018). Cter-SUN proteins contain a nucleoplasmic domain, a transmembrane domain, a coiled-coil domain, and the C-terminus SUN domain (Groves et al., 2018). Cter-SUN proteins in plants are similar in size to SUN proteins of yeast and mammals. Arabidopsis SUN 1 (AtSUN1) and AtSUN2 are classified as Cter-SUN proteins (Zhou et al., 2015a). Arabidopsis has three mid-SUN proteins (AtSUN3, AtSUN4, and AtSUN5) that contain SUN domains located between two transmembrane domains (Groves et al., 2018). T-DNA mutant lines for Arabidopsis *SUN* protein genes are available. However, there are difficulties in construction of multiple *sun* knock-out plants because SUN proteins are essential for plant viability (Graumann et al., 2014). A dominant-negative mutant of SUN protein (SUNDN) was developed, and SUN protein functions in fertility, NE formation, nucleoskeletal anchorage and plant meiosis have been shown using SUNDN (Zhou et al., 2015a; Zhou et al., 2015b; Varas et al., 2015). Mutant analysis of WPP domain-interacting proteins (WIPs), a class of Arabidopsis KASH proteins, showed involvement of these proteins in nuclear shape and size and pollen fertility, suggesting functions of LINC complexes in these processes (Zhou et al., 2015b; Poulet et al., 2017).

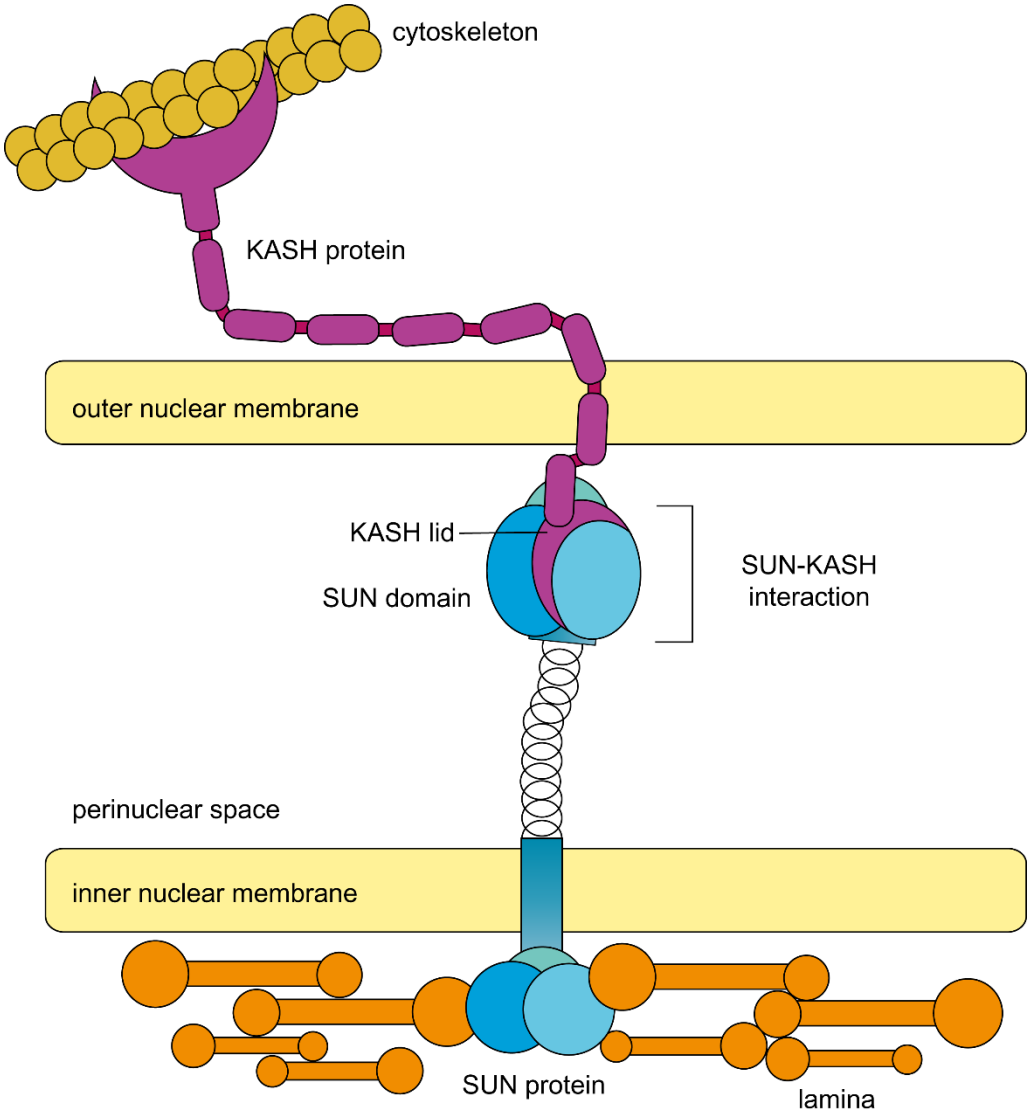

Figure 3. SUN and KASH proteins

A trimer of SUN proteins located in the inner nuclear membrane interacts with a KASH protein of the outer nuclear membrane in the perinuclear space. The SUN-KASH protein interaction links cytoskeleton and nuclear lamina.

In budding yeast (*Saccharomyces cerevisiae*), the SUN protein Mps3/Nep98 was shown to be required for nuclear fusion (karyogamy) during mating (Nishikawa et al., 2003). The mechanisms of nuclear fusion in yeast (karyogamy) and Arabidopsis (polar nuclear fusion) are similar. Both processes proceed without nuclear envelope breakdown; nuclear fusion is achieved by sequential fusions of the outer and inner nuclear membranes. Studies report the involvement of BiP, a molecular chaperone HSP70 in the endoplasmic reticulum (ER), and its regulatory partners, J-protein family co-chaperones (J proteins), in these processes (Rose et al., 1989; Nishikawa and Endo, 1997; Brizzio et al., 1999; Maruyama et al., 2010; Maruyama et al., 2014). The SUN protein Mps3/Nep98 interacts with Jem1, a J protein required for karyogamy (Nishikawa and Endo, 1997; Nishikawa et al., 2003). Therefore, potential role of SUN proteins in polar nuclear fusion can be tested by expression of SUNDN in developing FGs.

In this study, I developed a new gene induction system in developing FGs, Using this system, expression of SUNDN in developing FGs was achieved. Expression of SUNDN caused defects in the polar nuclear fusion, indicating that the roles of SUN proteins in this process. The gene induction system can be used as a new tool for analyses of gene functions in plant reproduction research.

Materials and Methods

Plant materials and growth conditions

The heat shock promoter (HS)-Cre line of *A. thaliana* (Ogawa et al., 2015), provided by Dr. Taku Takahashi at the Okayama University, Okayama, Japan, was used in this study. Seeds were sterilized on the surface and sown on soil or Murashige and Skoog (MS) medium (Wako, Osaka, Japan) containing 0.7% agar and 1% sucrose. Plants were grown at 22°C under continuous light.

Plasmid construction and plant transformation

Primers used for plasmid construction were listed in Table 1. A 1.5 kb DNA fragment containing the *loxP-HISTONE H2B-NosT-loxP* cassette was amplified by PCR from pDME100 using loxP-H2BF-infusion and loxP-H2BR-infusion2 primers. pDME100 was generated by cloning the 1.5 kb *HISTONE H2B-NosT-loxP* fragment, which was amplified from *pRPS5A::H2B-GFP* (Adachi et al., 2011) using pENTR_H2B_F and loxP-NOSter_R primers, into the pENTR/D-TOPO vector (Invitrogen, Massachusetts, USA). The amplified fragment was inserted into the *Xba*I site of pGWB501 (Nakagawa et al., 2007) using the In-Fusion HD Cloning Kit (Clontech, Mountain View, USA) to generate pSW1. And 1.1 kb DNA fragment containing the *ES2* promoter, and amplified from the Arabidopsis genomic DNA using 1g26795-F and 1g26795-R primers, which was cloned into the *Hind*III site of pSW1 using In-Fusion HD Cloning Kit; therefore, generates pSW9. Also, 0.8 kb DNA fragment containing *pCOXIV-GFP* was amplified using pCOXIV-F and GFP-R primers and cloned into the pENTR/D-TOPO vector to generate pSW11.

The fusion SUNDN gene was constructed as follows. DNA fragment corresponding to amino acid residues 129–455 of SUN, containing the C-terminal ER retention signal was amplified from a cDNA clone RAFL19-33-G08 (provided by RIKEN Bio Resource Center) using Sun2LmF and Sun2LmR primers. Then it cloned into the pENTR/D-TOPO vector to generate pSNA115. DNA fragment corresponding to amino acid residues 1–40 of Arabidopsis 2S1, which is the signal sequence, was amplified from the Arabidopsis genomic DNA using 2S1F and 2S1RFPR primer. DNA fragment corresponding to amino acid residues 1–237 of tagRFP was amplified from pTagRFP-C (Evrogen, Moscow, Russia) using tagRFPCDSF and tagRFPCDSR primers. DNA fragment corresponding to amino acid residues 129–455 of SUN2 with the C-terminal ER retention signal was amplified from pSNA115 using primers RFPSUN2F and SUN2HDELR. Total three amplified DNA fragments were ligated using the In-Fusion HD Cloning Kit and then cloned into the pENTR/D-TOPO vector to generate pSNA118. The SUNDN-Mut sequence contained point mutation of H434A and Y438F in SUN2 (Zhou et al., 2015b). These mutations were introduced by PCR amplification of pSNA118 using primers SUN2dMut-Fw and SUN2dMut-Rv primers, followed by self-ligation. The constructs for *pCOXIV-GFP*, *SUNDN* and *SUNDN-Mut* were introduced into pSW9 using LR clonase II (Invitrogen).

To generate transgenic lines, *Agrobacterium tumefaciens* stain GV3101 was introduced into Arabidposis plants using the floral-dip method (Clough and Bent, 1998). Transgenic plants were selected on MS agar plants containing 50μg/ml hydromycin and subsequently transferred to soil. Transgenic plants in the T_2 generation, which were homozygous for the transgene were selected by observation of FGs by fluorescent microscopy and used for further experiments.

Table 1. List of primers used in this study

Primer name	Primer sequence (5'→3')
loxP-H2BF-infusion	GCAGGTCGACTCTAGATAACTTCGTATAGCATACATTAT ACGAAGTTATATGGCACCAAGAGCCGAG
loxP-H2BR-infusion2	TGTTGATAACTCTAGGCTCCGCGGCCGCACTAGT
pENTR_H2B_F	CACCATGGCACCAAGAGCCGAGAAG
loxP-NOSter_R	ACTAGTATAACTTCGTATAATGTATGCTATACGAAGTTA TGATCTAGTAACATAGATGACACCGC
1g26795-F	GGCCAGTGCCAAGCTTTGATCTCTCTTTCAGTG
1g26795-R	GCAGGCATGCAAGCTGAGTGTGTTTTACTTTTA
pCOXIV-F	CACCATGCTTTCACTACGTCAATC
GFP-R	TTACTTGTACAGCTCGTCCATGCCGTGAGT
SUN2LmF	CACCAGGAAATTGACTTTGAAGGAT
SUN2LmR	TCACAATTCATCATGGTGGTGATGGTGATGCCCACCTCCA GCATGAGCAACAGAGACTGA
2S1F	GCCGCCCCCTTCACCATGGCAAACAAGTTGTTCCT
2S1RFPR	GCCCTTAGACACCATGCCTATGGGGTTAGTGGCGT
tagRFPCDSF	ATGGTGTCTAAGGGCGAAGAGCTG
tagRFPCDSR	ATTAAGTTTGTGCCCCAGTTTGCT
RFPSUN2F	GGGCACAAACTTAATAGGAAATTGACTTTGAAGGA
SUN2HDELR	GGCGCGCCCACCCTTTCACAATTCATCATGGTGGT
SUN2dMut-Fw	TGCATCTTCCGCTTCAGGGTT
SUN2dMut-Rv	AGTGGCTGAAGAGCTTCCATG

Heat treatment of pistils

At late stage 12 pistils were used for heat treatment. Isolated pistil was placed in 0.2 ml of PCR tube containing ovule culture medium (50 μl) (Gooh et al., 2015). Samples were heated from 30 to 40 °C in a thermal cycler (Dice Gradient, Takara, Bio). The heated pistils were incubated at 22°C under the dark conditions. Also, other heat treatment was conducted. Pistils were emasculated 4 h before then applied by immersing in water at 35°C. For repeated heat treatments, inflorescences were heated at 35°C for 5 min in three times (0 h, 16 h, and 24 h). Plants were then brought back to the growth room at 22°C and incubated under continuous light.

Microscopy

Ovules were dissected from pistils, mounted in a multi-well glass bottom dish containing 400 μl of ovule culture medium, and then analyzed by confocal laser scanning microscopy (CLSM). Whole ovules in pistils were fixed and cleared with ClearSee (Kurihara et al., 2015), then analyzed by CLSM. For CLSM, a Leica TCS-SP8 confocal microscope was used with a 20x multi-immersion objective lens (PL APO CS2 20x /0.75 IMM CORR HC; Leica Microsystems) or a 40x or 63x water-immersion objective lens (40x; PL APO CS 40x/ 1.10 W CORR HC, 63x; PL APO CS 63x 1.20 W CORR HC; Leica Microsystems). To analyze the GFP fluorescence, fluorescence images at 495–540 nm were captured after excitation at 488 nm with a solid-state laser. To analyze the tagRFP and chlorophyll fluorescence, fluorescence images at 560–650 nm (tagRFP) and 560–700 nm (chlorophyll) were captured after excitation with a 552 nm solid-state laser. The acquired images were processed using the LASX software

(Leica Microsystems) to create maximum intensity, then processed using Adobe Photoshop CC (Adobe Systems Inc., San Joes, CA).

Statistical analysis

All statistical analyses were performed using the R software (R 3.5.2; R Core Team, 2018).

Results

Development of a FG-specific gene induction system

In this study, I developed a new gene induction system that allows FG-specific expression of a target gene by heat treatment. The developed gene induction system is consisted of two gene constructs (Fig. 4), heat-inducible Cre-*loxP* recombination and *A. thaliana* FG-specific *EMBRYO SAC 2* (*ES2*) promoter (At1g26795) (Yu et al., 2005; Pagnussat et al., 2007). The driver construct consists of Cre recombinase coding sequence and *A. thaliana HEAT SHOCK PROTEIN18.2* (*HSP18.2*) gene promoter placed in downstream of Cre recombinase (Ogawa et al., 2015). The effector cassette includes *loxP*-flanked regions containing *HISTONE H2B-GFP* (*H2B-GFP*) coding sequence and *NOPALINE SYNTHASE* terminator (*NosT*). This sequence was located between the *ES2* promoter and the target gene. A short heat treatment was expected to induce Cre-*loxP* site recombination and result in removal of the *H2B-GFP-NosT* cassette. Continued expression of the target gene from the *ES2* promoter was expected to take place after the removal of heat. To examine the gene induction system, I used the gene for pCOXIV-GFP, which is a fusion protein between GFP and mitochondria target sequence of yeast cytochrome c oxidase subunit IV (Maruyama et al., 2015) as a target gene and generated transgenic lines.

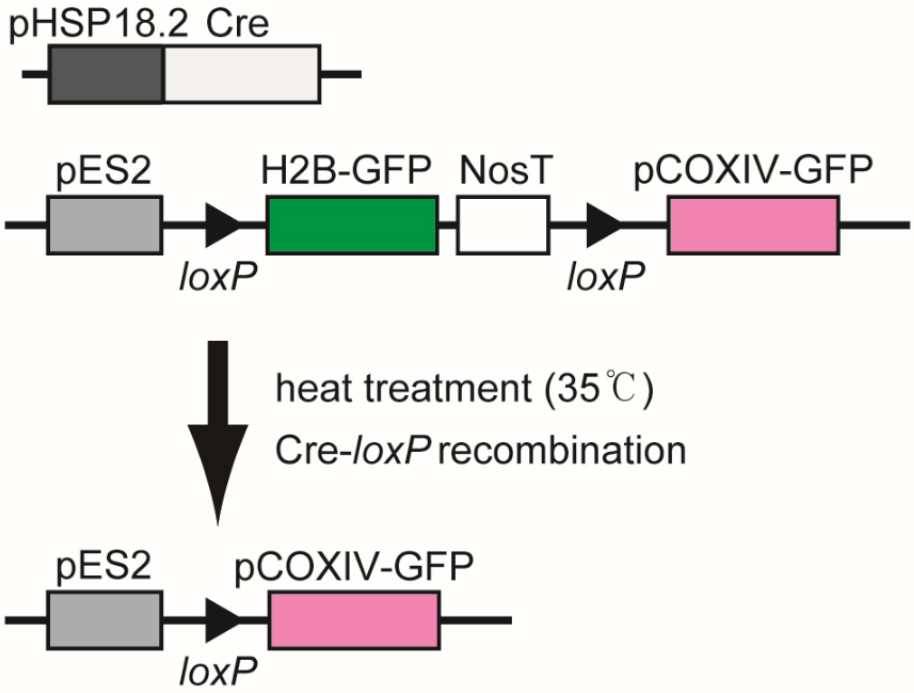

Figure 4. Schematic representation of the gene induction system

A sequence containing the *EMBRYO SAC2* promoter (*pES2*) driving the coding sequence of histone *H2B-GFP* fusion (*H2B-GFP*) and *NOPALINE SYNTHASE* terminator (*NosT*) flanked by two loxP sequences on either side in the same orientation, followed by the coding sequence of yeast cytochrome c oxidase subumit 4 (*pCOXIV*) fused to GFP (*pCOXIV-GFP*). This construct was introduced into the HS-Cre line expressing the Cre recombinase under the control of the Arabidopsis *HSP18.2* promoter (*pHSP18.2 Cre*). Ovules or pistils of transgenic plants were treated at 35°C for ~5 min induced Cre-*lox*P recombination, resulting in the expression of *pCOXIV-GFP* from the *ES2* promoter.

ES2 promoter allows gene expression from the FG4 stage

Ovules isolated from the transgenic plant were analyzed by CLSM. Expression of *H2B-GFP* from the *ES2* promoter was observed in the nuclei of developing FGs at between the FG4 and FG6 stages (Figure 5). This result suggests that gene induction is able to apply in developing FGs from the FG4 stage and also in mature FGs.

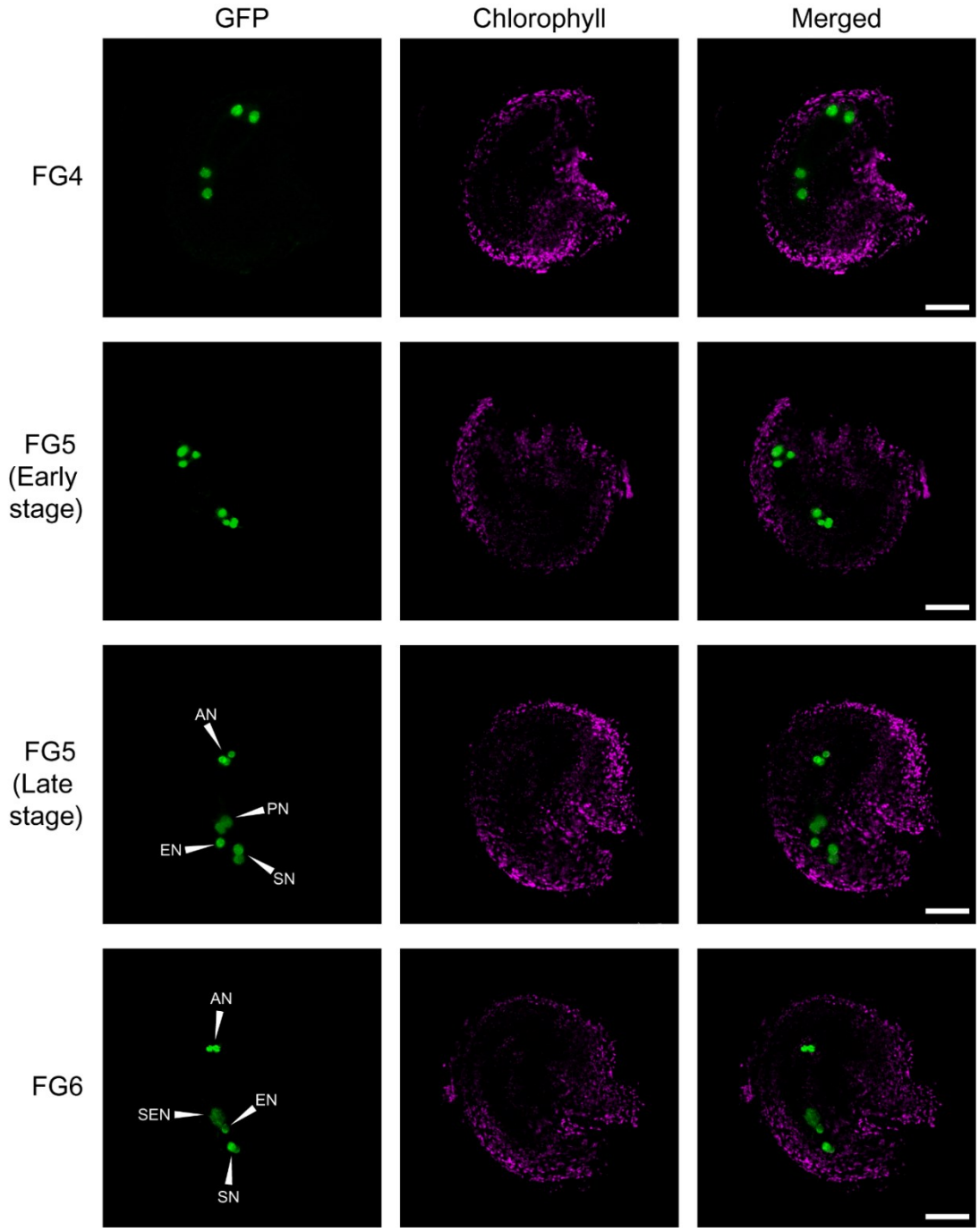

Figure 5. Images of ovules from the transgenic plants

Images of FG4, FG5, and FG6 ovules are shown. GFP fluorescence (green), chlorophyll autofluorescence (magenta), and merged images are shown. SN, synergid nuclei; PN, polar nuclei; SEN, secondary nucleus; EN, egg nucleus; AN, antipodal cell nuclei. Scale bar = 25μm

Mild heat treatment induce target gene expression in FGs

HSP 18.2 promoter expression is induced by heat treatment at 35–37°C (Takahashi et al., 1992; Ogawa et al., 2015). Pistils of the transgenic plants were heated by immersing whole inflorescences in distilled water at 35°C for 5 min. 16 h after the heat treatment, ovules were dissected from the pistils and analyzed by CLSM. Punctate GFP signals were observed in FG cells (Figure 6 D–F), which were not observed in ovules from pistils without heat treatment (Figure 6 A–C). At high magnifications, tubular GFP signals were observed, which are characteristic to mitochondria staining (Figure 7 D and F). These results indicate that the *pCOXIV-GFP* is expressed from the *ES2* promoter. These results also indicate that the expression of Cre protein expression after heat treatment led to the recombination in FG cells. In spite of the deletion of the *H2B-GFP* cassette by Cre-*loxP* site-specific recombination, GFP signal was observed in the nucleus. This was probably due to stability of the *H2B-GFP* fusion protein.

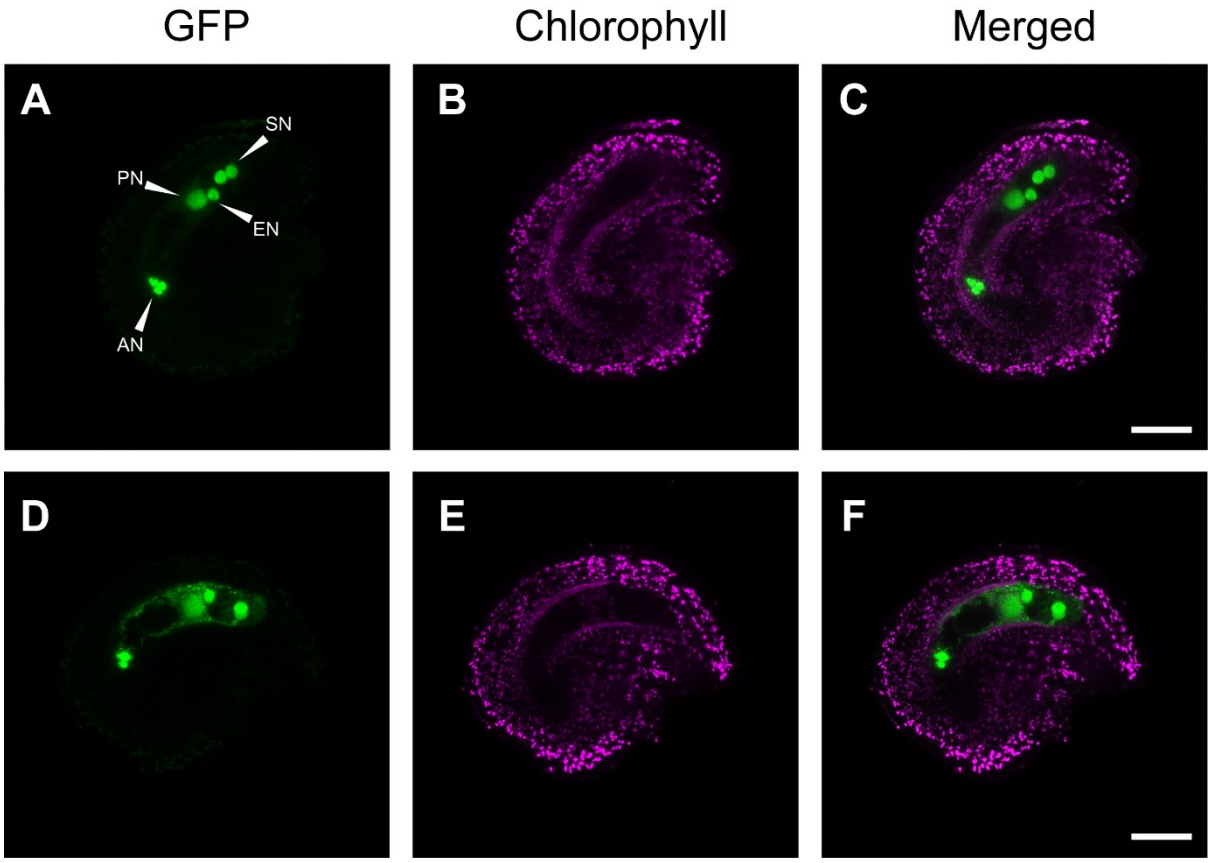

Figure 6. Induction of FG-specific gene expression by heat treatment

(A–C) Images of a transgenic FG6 ovule. (D–F) Images of a FG6 ovule isolated from a pistil which was heated at 35°C for 5 min and further incubated at 22°C for 16 h. GFP fluorescence (A and D, green), chlorophyll autofluorescence (B and E, magenta), and merged (C and F) images are shown. SN, synergid nuclei; PN, polar nuclei; EN, egg nucleus; AN, antipodal cell nuclei; Scale bar = 25μm

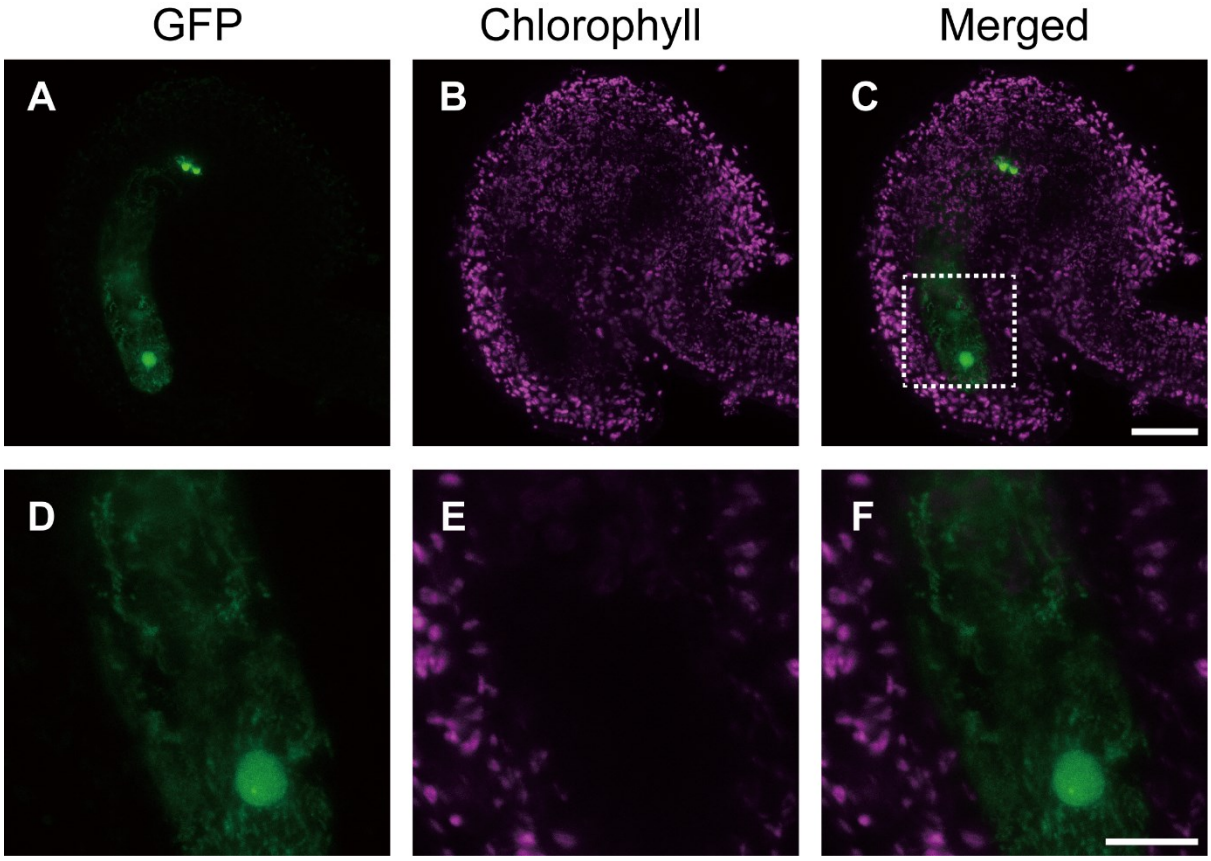

Figure 7. Images of a FG6 ovule isolated from a pistil that was heated at 35°C for 5 min and further incubated at 22°C for 18 h.

GFP fluorescence (A and D, green), chlorophyll autofluorescence (B and E, magenta), and merged (C and F) images are shown. (D-F) Magnification of a region shown as a box in (C). Scale bar; (A–C), 25 μm; (D–F), 10 μm

Efficient gene induction in FGs was achieved by short and mild heat treatment of pistils and inflorescences

Next, temperature and time dependency of the gene induction system was analyzed. The pistils were isolated from the transgenic plants at the late stage 12, immersed in the ovule culture medium (Gooh et al., 2015) and subjected to heat treatment using a thermal cycler. Then pistils were further incubated at 22°C, fixed and cleared by ClearSee (Kurihara et al., 2015). The *pCOXIV-GFP* expression was analyzed by CLSM. To analyze the gene induction of temperature dependency, pistils were heated at between 30–40°C for 1 min. Figure 8 A shows efficiencies of gene induction. Heat treatment at 35°C was the most efficient. At 35°C, 42 ± 13% of ovules express *pCOXIV-GFP* signals in the FG. The results represent that heat induction system is efficient at 35°C. This temperature was used to analyze the time dependency of gene induction.

Time dependency in gene induction system was analyzed using flower buds from the transgenic plants. The late stage 12 flowers were emasculated and heat-treated by immersing whole inflorescence in water at 35°C for 1–20 min. After heat treatment, pistils were isolated, fixed and analyzed by CLSM. Figure 8 B shows that gene induction efficiency did not increase by longer heat treatment. The most efficient time was 1 min; however, the short heat treatment (~5 min) was sufficient time for applying gene induction system. These results indicate that a short (~5 min) heat treatment at 35°C is sufficient for achieving efficient gene induction in this system.

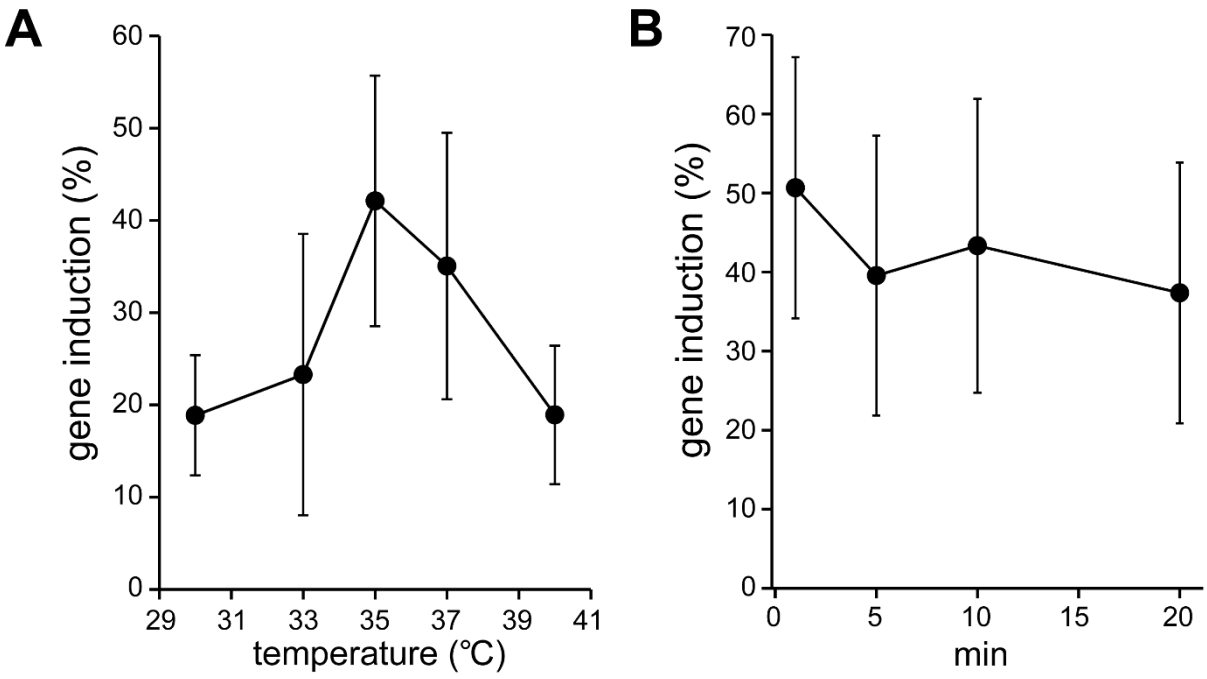

Figure 8. The temperature and time dependency of gene induction

(A) Pistils isolated from transgenic flowers at late stage 12 were heated at the indicated temperature (30, 33, 35, 37, and 40°C) for 1 min using a thermal cycler and further incubated at 22°C for 16 h. The percentage of ovules containing a *pCOXIV-GFP*-positive FG was scored for each pistil. Data represent mean ± standard deviation ($n \geq 6$ for each temperature)

(B) Transgenic flowers at late stage were emasculated and heated by immersing a whole inflorescence in water at 35°C for the indicated times (1, 5, 10, and 20 min). After heat treatment, plants were incubated at 22°C for 16 h. The percentage of ovules containing a *pCOXIV-GFP*-positive FG was scored for each pistil. Data represent mean ± standard deviation ($n = 8$ for each temperature).

Expression of a dominant-negative mutant of SUN proteins in developing FG causes defects in the fusion of polar nuclei

The constructed FG specific gene induction system was applied to the analysis of gene functions in FGs. I used this system to express a dominant negative mutant of SUN proteins in developing FGs. The dominant-negative approach is effective to analyze the protein function which is multiple genes such as *actin* (Kawashima et al., 2014). I chose SUN proteins from the following reasons. First, SUN proteins are encoded by multiple genes. Second, a dominant-negative mutant of SUN proteins is available (Zhou et al., 2015b). Figure 9 A shows that SUNDN consists of a N-terminal ER targeting signal sequence of Arabidopsis 2S albumin (SS), a tagRFP fluorescent protein, coiled-coil domain, SUN domains, and a C-terminal ER retention signal sequence (HDEL). Third, the yeast SUN protein Mps3/Nep98 was shown to be involved in nuclear fusion in yeast mating (Nishikawa et al., 2003), suggesting potential involvement of SUN proteins in nuclear fusion in Arabidopsis.

Using the gene induction system, transgenic plants expressing heat-inducible SUNDN in developing FGs were constructed. Figure 9 B–D shows the results of SUNDN induction by heat treatment. Early to middle stage 12 flowers of transgenic lines were treated at 35°C for 5 min; this treatment was repeated three times at 0, 16, and 24 h relative to the first heat treatment. 40 h after the third heat treatment, the ovules were dissected and observed by CLSM. Fluorescence signals of tagRFP (Figure 9 C and F) were observed in the FG of 65–80% of ovules in heat-treated pistils (Figure 10 A), indicating the induction of SUNDN by heat treatment in three independent lines. No tagRFP signal was observed in ovules of transgenic lines without heat treatment. The tagRFP-positive ovules containing unfused polar nuclei (Figure 9 B–D) and the secondary nucleus (Figure 9 E–G). Defective polar nuclear fusion was

observed in ~45% of tagRFP-positive ovules but not in tagRFP-negative ovules (Figure 10 B). No polar nuclear fusion defect was observed in pistils without heat treatment, suggesting a correlation between the polar nuclear fusion defect and SUNDN expression.

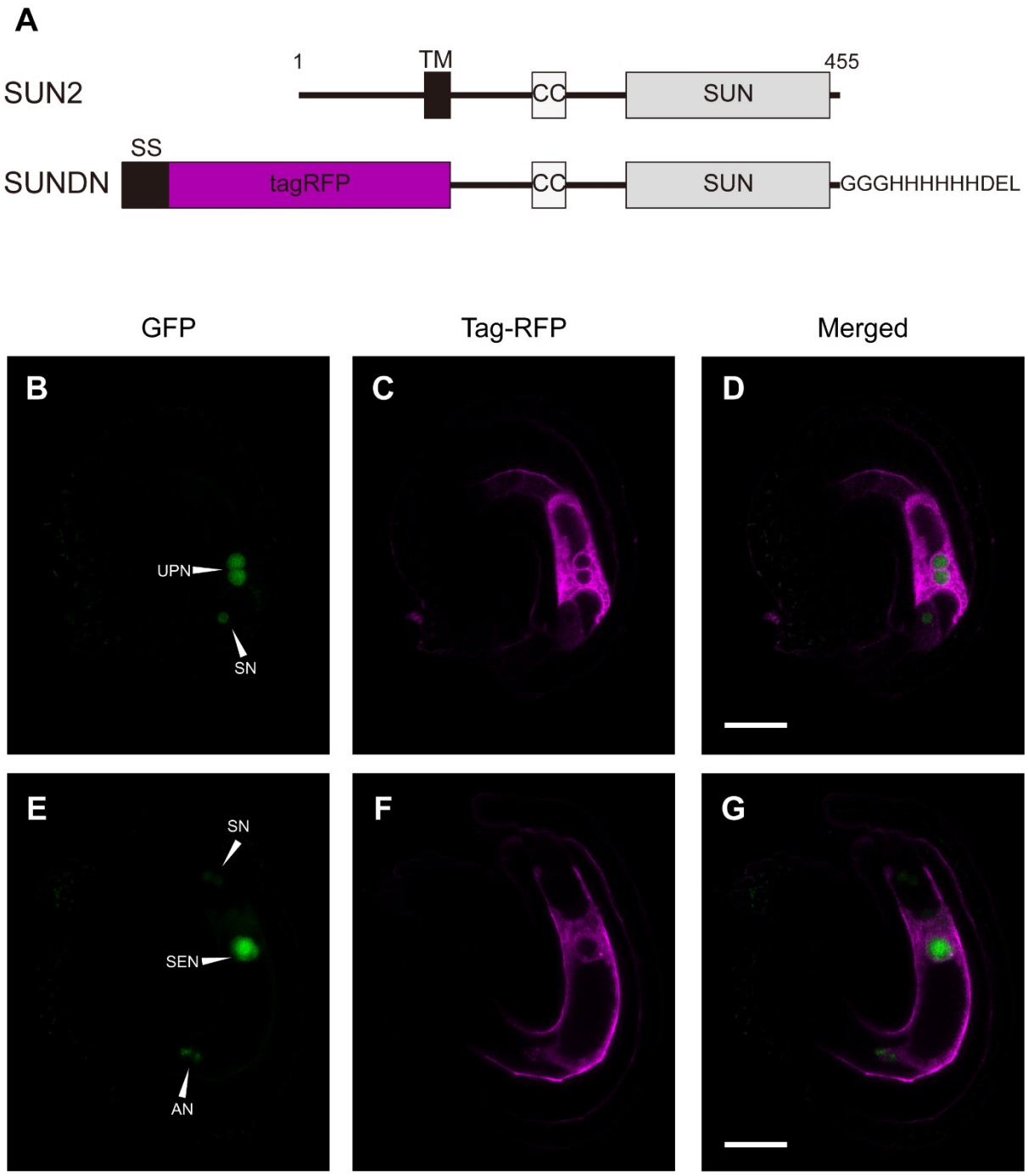

Figure 9. Expression of a dominant-negative mutant of SUN protein in developing FGs

(A) Schematic representation of Arabidopsis SUN2 and SUNDN proteins. Numbers above SUN2 indicate the first and last amino acid residues of SUN2. SUNDN is consisted of the signal sequence of Arabidopsis 2S albumin (SS) fused to tagRFP, the luminal domain of SUN2

containing the SUN domain (SUN), and coiled-coil (CC) domain tagged with the C-terminal ER retention signal (GGGHHHHHHDEL). TM shows the transmembrane region of SUN2.

(B–G) Representative images of an ovule after the induction of SUNDN in the developing FG. (B–D); polar nuclear unfusion; (E–G); polar nuclear fusion. Transgenic flowers at late stage 12, expressing SUNDN from the *ES2* promoter using heat-inducible Cre-*loxP*-based site-specific recombination, were emasculated and heat by immersing the whole inflorescence in water at 35°C for 5 min. Ovules were dissected from the heat-treated pistil at 16 h after the treatment and analyzed by CLSM. Images are shown as GFP fluorescence (green), tagRFP fluorescence (magenta), and merged. Scale bar = 25μm.

SN, synergid nuclei; UPN, unfused polar nuclei; SEN, secondary nucleus; AN, antipodal cell nuclei

Expression of SUNDN-Mut in FG did not cause the polar nuclear fusion defect

Introduction of two point mutations (H434A and Y438F) in the SUN domain impaired the binding activity between SUN2 and KASH proteins (Zhou et al., 2014). SUNDN-Mut, A mutant version of SUNDN containing these mutations, did not interact with the KASH proteins, WIP1, WIP2, and WIP3, and also lost the dominant-negative activity of SUNDN (Zhou et al., 2015b). I also generated two transgenic lines that express SUNDN-Mut in FG by heat treatment. The transgenic plants were heat treated as in the case of SUNDN transgenic lines. The efficiency of SUNDN-Mut induction in FGs by heat treatment was similar to that of SUNDN induction (Figure 10 A). However, unlike SUNDN, the expression of SUNDN-Mut did not cause the polar nuclear fusion defect (Figure 10 B). Only ~7% of tagRFP-positive ovules contained unfused polar nuclei. No polar nuclear fusion defect was observed in tagRFP-negative ovules. These results indicate that the short repetitive heat treatment at 35°C did not cause the polar nuclear fusion defect. Instead, the polar nuclear fusion defect was caused by the expression of SUNDN retaining its KASH-binding activity. Since SUN proteins function together with KASH proteins in various processes, our results strongly suggest the involvement of SUN proteins in polar nuclear fusion.

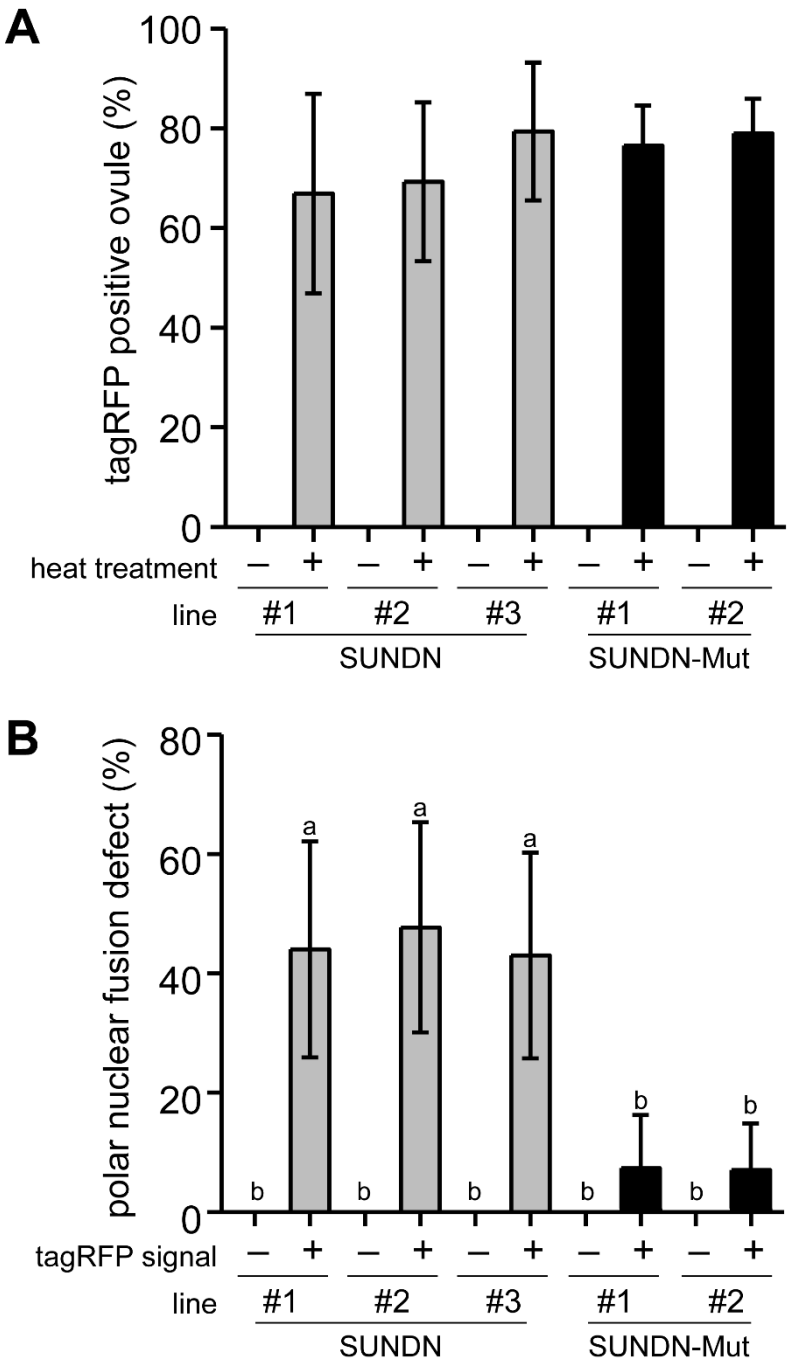

Figure 10. Expression of SUNDN in developing FGs inhibits polar nuclear fusion

(A) Percentages of tagRFP-positive FGs in a pistil; (B) Percentage of FGs containing unfused polar nuclei among tagRFP-positive and -negative FGs in pistil.

Whole inflorescences were heated at 35°C for 5 min three times (at 0, 16, and 24 h relative to the first heat treatment). Early to middle stage 12 flowers in the first heat treatment were emasculate at 20 h and fixed at 64 h after the first heat treatment. Control experiments without heat treatment were performed in parallel. Three or two independent transgenic lines expressing SUNDN or SUNDN-Mut (a mutant version of SUNDN containing mutation in the KASH-binding region), respectively, in the FG were analyzed. Statistical differences were calculated using the Tukey-Kramer method. $p<0.05$ is indicated by different letters. No polar nuclear fusion was observed in FGs in pistils without heat treatment ($n \geq 7$ for each line). Data represent mean ± standard deviation ($n \geq 7$).

Discussion

In this study, I developed a new method for the analysis of gene functions in female gametophyte using a heat-inducible Cre/*loxP* recombination and the FG-specific *ES2* promoter. Chemically inducible gene expression has been widely used for transgene induction in Arabidopsis using steroid hormone-based transactivators (Moore et al., 2006). This technique has also been used for the analysis of gene function in FGs. Kawashima et al. (2014) previously used this technique for the induction of a dominant-negative mutant of *actin* in the central cell of mature FGs, and demonstrated the role of F-actin in fertilization. The gene induction system developed in this study showed efficient FG-specific gene induction by short heat treatment at 35°C.

The FG specific promoter used in this study was the *EMBRYO SAC 2* (*ES2*) promoter. Transgene expression from this promoter is detected in the embryo sac from the FG3 to FG7 stages (Yu et al., 2005). However, in our gene expression system expression of *H2B-GFP* from the *ES2* promoter was detected in FGs from the FG4 stage (Figure 5). Since the FG4 stage corresponds to the four-nucleated FG stage, our gene induction system can be used for analysis of gene functions after the FG5 stage, and can be applied to analysis of SUN proteins in the polar nuclear fusion.

The Cre/*loxP* system is a flexible and simple recombination system, which has been used to identify gene functions in mice (McLellan et al., 2017). The Cre/*loxP* system is also used in plants for targeted insertion and deletion of a DNA fragment from the chromosome (Wang et al., 2005). Heat-inducible Cre/*loxP* recombination is also used in animals. For example, human K-RASG12D protein was expressed in zebrafish embryos by heat shock

treatment (Le et al., 2007). Heat treatment of transgenic embryos at 37°C for 30–120 min resulted in Cre expression from the heat shock protein (*hsp*) 70 promoter. Cre expression was the most effective by heat treatment of embryos after 4–5 hours postfertilization or 24–25 hours postfertilization without affecting viability or development (Le et al., 2007).

In my study, I used the HS-Cre line (Ogawa et al., 2015), which express Cre from the *HSP18.2* promoter for heat-inducible Cre expression. Using the *GUS* gene as a reporter, expression from the *HSP18.2* promoter was detected after heat treatment at 35°C in tissues of the transgenic Arabidopsis plants including pistils (Takahashi et al., 1992). The use of *pCOXIV-GFP* (Maruyama et al., 2015) as a target gene resulted in no detectable Cre-*loxP* recombination in FGs in plants grown at 22°C, indicating that gene expression from the *HSP18.2* promoter is tightly repressed in the absence of heat treatment. After heat treatment of pistils or flowers for a short period of time (1–5 min), we observed efficient expression of *pCOXIV-GFP*; punctate and tubular GFP signals, which are characteristic to mitochondria staining, were observed in FG after heat treatment at 35°C for 5 min. This result also indicates that heat-inducible gene expression from the *HSP18.2* promoter and Cre/*loxP* site-specific recombination took place in FGs. In spite of the deletion of *H2B-GFP* cassette by Cre/*loxP* site-specific recombination, the nuclear GFP signals were observed. This was probably due to stability of the *H2B-GFP* fusion protein and indicates that analysis of nuclear dynamics is possible in FGs after heat induction of the target gene.

Analyses of temperature and time dependencies of gene induction showed that mild and short heat treatment of pistils or flowers resulted in efficient gene induction. The most effective temperature for gene induction was 35°C; 42 ± 13% (n = 7) of ovules showed expression of *pCOXIV-GFP* in FGs (Figure 8 A). The results are consistent with Takahashi et

al., (1992), where expression of the *HSP18.2* promoter-*GUS* fusion gene was maximal at 35°C. Gene expression from the *HSP* promoters of plants including maize, tobacco, soybean and other species was all under 40°C (Yoshida et al., 1995). Analysis of time dependency of gene induction showed that efficient Cre-*loxP* recombination after heat treatment of flowers for a short period of time (1–5 min). This indicates that the amount of Cre protein expressed in a cell following a short heat treatment was sufficient for the induction of recombination between the two *loxP* sites. Since mild and short heat treatment is sufficient for efficient gene induction, the inducible gene expression system developed in this study can be used for analysis of gene functions in FGs with minimal heat damages.

I applied the heat-inducible gene expression system to analysis of gene functions in developing FGs. Expression of SUNDN, a dominant-negative mutant of SUN proteins, in developing FGs inhibited polar nuclear fusion, suggesting the involvement of SUN proteins in this process. Not all tagRFP-positive ovules showed defects in polar nuclear fusion: ~45% of tagRFP-positive ovules contained unfused polar nuclei (Figure 10 B). This was probably due to the relatively long length of the FG5 stage in female gametogenesis. FG5 ovules are found in flowers in the period between mid-stage 12 to stage 13 (Christensen et al., 1997), whose duration is expected be more than one day (Smyth et al., 1990). It is possible that the polar nuclear fusion process does not proceed synchronously in a pistil. At the onset of SUNDN expression, a fraction of FGs in a pistil probably had proceeded to steps that do not require SUN protein functions. Nevertheless, ovules from untreated pistils and tagRFP-negative ovules from heat-treated pistils did not show the polar nuclear fusion defect, indicating a correlation between the polar nuclear fusion defect and SUNDN expression. Although the tagRFP signal was observed in the egg cell and synergid cells, the tagRFP signal was strongest in the central

cell (Figure 9 C), probably because expression from the *ES2* promoter is predominant in the central cell in mature FGs (Steffen et al., 2007; reported as the *DD9* promoter in this literature).

Analyses using SUNDN-Mut, which does not interact with KASH proteins, showed the importance of the KASH-binding activity of SUNDN for the inhibition of polar nuclear fusion. Expression of SUNDN-Mut in developing FGs did not cause the polar nuclear fusion defect (Figure 10 B), suggesting the involvement of SUN–KASH interactions during polar nuclear fusion. Recent analyses have identified three WIPs, four SUN-interacting nuclear envelope proteins (SINEs), and TIK as KASH proteins in Arabidopsis (Zhou et al., 2015a). However, the role of these KASH proteins in female gametogenesis has not yet been reported. Interestingly, Zhou and Meier (2014) reported that WIPs and their binding partners, WIT1 and WIT2, play important roles in male fertility. The *wip1 wip2 wip3* triple mutant and *wit1 wit2* double mutant plants show male fertility defects, which are associated with impaired pollen tube reception. Moreover, these mutants show impaired movement of the vegetative nucleus in the pollen tube and inefficient delivery of sperm cells. SUN–KASH interactions appear to be important for nuclear migration in pollen tubes. Expression of SUNDN in pollen tubes also results in male fertility defects similar to those observed in *wip* and *wit* mutants (Zhou et al., 2015b). By contrast, the movement of polar nuclei appeared not to be defective in FGs expressing SUNDN. These data suggest that SUN–KASH interactions are not required for the movement of polar nuclei. Alternatively, it is possible that defects in polar nuclear movement were not visible because of the short travel distance of polar nuclei. Detailed analyses in combination with live imaging will help determine whether the expression of SUNDN is required for the movement of polar nuclei.

The yeast SUN protein, Mps3/Nep98, is also required for nuclear fusion during mating. Nuclear fusion during yeast mating can be dissected into two processes: nuclear congression in yeast zygotes and nuclear membrane fusion (Kurihara et al., 1994). In zygotes lacking Mps3, nuclear congression is not affected; two haploid nuclei are in close contact but do not fuse (Rogers and Rose, 2015). This indicates roles of Mps3 in nuclear membrane fusion, suggesting that the function of SUN proteins in nuclear membrane fusion during reproductive processes is conserved between yeast and plants.

The gene induction system developed in this study could be used for the analysis of gene functions in FG development, provided dominant-negative mutants are available. Because efficient gene induction is possible *in planta*, the gene induction system developed in this study could be used for the analysis of gene function after FG development (e.g., during fertilization) by inducing the expression of a dominant-negative mutant in mature FGs. Another possible application of this gene induction system potentially includes stage-specific complementation of loss-of-function mutants defective in FG development. The heat-inducible Cre-*loxP* recombination system is not limited to the analysis of gene function in FG. Various cell-type specific promoters are available in Arabidopsis for the expression of transgenes (Schürholz et al., 2018). By replacing the *ES2* promoter with these promoters, cell-type specific induction of gene expression is possible in somatic cells. Region-specific induction of gene expression by IR-LEGO has also been reported in Arabidopsis and Marchantia (Deguchi et al., 2009; Nishihama et al., 2016). Targeted induction of a gene expression in a specific type of cell or tissue is a promising new tool for the analysis of tissue-specific gene functions in plants at the single cell level.

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