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DEX1, a plasma membrane-localized protein, functions in microspore development by affecting *CalS5* expression in *Arabidopsis thaliana*

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Microsporogenesis in flowering plants plays important roles in sexual reproduction. It has been reported that *DEFECTIVE IN EXINE FORMATION1* (*DEX1*) is essential for exine pattern formation in *Arabidopsis thaliana*. However, the functions of DEX1 in regulating microspore development are largely not understood. In this study, we show that *DEX1* is strongly expressed in the tapetum by using RNA *in situ* hybridization. *dex1* microspores were degenerated and aborted after release from the tetrads. The callose wall in tetrads was thinner in the *dex1* mutant than in the wild type, suggesting that *DEX1* affects callose formation at the tetrad stage during anther development. RT-PCR and real-time PCR analyses showed that *CalS5,* which plays an important role in callose synthesis during microspore development, was greatly down-regulated in *dex1* plants. *DEX1* encodes a membrane protein with one transmembrane domain, one intracellular domain and one extracellular domain. Collectively, our results demonstrate that *DEX1* is essential for microspore development, possibly by regulating the expression of *CalS5*.

anther development, callose, *CalS5*, *DEX1*, microspore development

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The life cycle of flowering plants alternates between the diploid sporophyte generation and the haploid gametophyte generation. Male gametophytes develop in anthers where cell division, differentiation, and subsequent degeneration are essential for successful reproduction [1–3]. The *Arabidopsis* anther is usually a four-lobed structure consisting of highly specialized reproductive and non-reproductive tissues [2]. Anther development starts with the emergence of stamen primordia in the third whorl of the floral meristem, and terminates with the release of pollen grains from the mature anther at dehiscence [2].

In *Arabidopsis thaliana*, the anther development process can be classified into 14 stages based on the cellular events visible under the light microscope [4]. During meiosis, microsporocytes secrete β-1,3-glucan (callose) between the plasma membrane and primary cell wall. It has been proposed that the callose wall has multiple roles, including preventing microspores from fusing [5], serving as a molecular filter, and as a mold for primexine patterning [6]. At the tetrad stage, primexine matrix, a precursor to the exine layer, is deposited on microspores between the callose wall and the plasma membrane [7,8]. After the microspore is released from the callose wall, the main component of exine, sporopollenin, is secreted by the tapetum and deposited on the microspores following the patterning of the primexine [8,9].

Callose is a linear β-1,3-glucan with some 1,6 branches, differing from cellulose which is exclusively a β-1,4-glucan. Callose is synthesized by β-1,3 glucan synthase (callase) in plants at many locations during development and in response to biotic and abiotic stress [10]. *CalS5*, one of the 12 *CalS* gene family members in *Arabidopsis*, encodes a transmembrane protein with 16 transmembrane helices and multiple functional domains, and is required for callose formation and microgametogenesis [11]. Mutations in *CalS5* disrupt callose formation around developing microspores, causing aberrations in exine patterning, degeneration

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of developing microspores, and pollen sterility [11].

Several other membrane-associated proteins, including NO EXINE FORMATION1 (NEF1), RUPTURED POLLEN GRAIN1 (RPG1) and NO PRIMEXINE AND PLASMA MEMBRANE UNDULATION (NPU), have been shown to regulate anther development and male fertility in *Arabidopsis*. *NEF1* encodes a putative integral plastid envelope membrane protein that may play a role in maintaining the plastid envelope integrity. In the *nef1* mutant, microspores are aborted during postmeiotic development, and pollen grains are completely absent in the anther at anthesis [12]. RPG1 is an integral membrane protein essential for microspore cell integrity. In the *rpg1* mutant, microspores are ruptured and aborted during postmeiotic anther development, and pollen grains are almost absent in the mature anther [13]. NPU is also a membrane-associated protein essential for primexine formation and microspore plasma membrane undulation. In the *npu* mutant, the microspores are degenerated and aborted postmeiotically in anther development, and pollen grains are absent in the anther locules [14].

Previous studies have shown that *DEFECTIVE IN EXINE FORMATION1* (*DEX1*) is required for pollen exine patterning in *Arabidopsis thaliana* [8,15]. In this study, we studied the precise expression of *DEX1* during anther development. Microspores were degenerated and disrupted following release from the tetrads during anther development. Callose synthesis is severely disrupted, and *CalS5* expression is greatly down-regulated in the *dex1* plants. Furthermore, the DEX1 protein was confirmed to be localized to the plasma membrane. The possible roles of DEX1 in microspore development in *Arabidopsis thaliana* are discussed.

1 Materials and methods

1.1 Plant material

Arabidopsis plants used in this study are in the ecotype Wassilewskija (Ws-2) background. Seeds were sown on vermiculite and allowed to imbibe for 3 d at 4°C. Plants were grown under long-day conditions (16 h of light/8 h of dark) in an approximately 22°C growth chamber.

1.2 Microscopy

Plants were photographed with a Nikon digital camera (Coolpix 4500). Flower images were taken using an Olympus dissection microscope with an Olympus digital camera (BX51). Alexander and aniline blue staining was performed as described [16,17]. Plant material for the semi-thin sections was prepared and embedded in Spurr's resin as described previously [18]. For scanning electron microscopy (SEM) examination, fresh stamens and pollen grains were coated with 8 nm of gold and observed under a JSM-840 microscope (JEOL). Confocal laser microscopy observation of DEX1: GFP protein localization was performed as described previously [19].

1.3 Protein structure prediction and phylogenetic analysis

The SOSUI, TMHMM, and SMART programs were performed to predict the transmembrane, intracellular, and extracellular regions of DEX1, respectively. The full-lengh amino acid sequence of DEX1 was used to search for homologous proteins through the BLASTp and tBLASTn programs. Multiple sequence alignment of full-length amino acid sequences was performed with ClustalX 2.0, and the alignment was displayed by BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic trees were constructed by MEGA 4.0 (http://www.megasoftware.net/) using the neighbor-joining method with bootstrap (1000 replicates) test.

1.4 RT-PCR and plasmid construction

Total RNA extraction, cDNA synthesis, RT-PCR and real-time PCR analyses were performed as described previously [18]. The sequences of the oligonucleotide primers used for the expression analysis of *CalS5* were: forward (5′-ATTATTGCAGCTGCTAGAGATG-3′), and reverse (5′-CTTGTTCAGAGGTTCTGGCTT-3′). To generate the GFP fusion construct, the full-length cDNA of *DEX1* without the stop codon was amplified using the primers DEX1-F (5′-GGTACCATGAAATCTCGAGCGAGGC-3′, *Kpn* I site is underlined) and DEX1-R (5′-GTCGACATTAAGTCCCACATGCCAGTT-3′, *Sal* I site is underlined), and cloned in-frame with the sequence encoding GFP into a modified plant expression vector pCAMBIA1300.

1.5 *In situ* hybridization

The DIG (for digoxigenin) RNA labeling kit (Roche) and PCR DIG probe synthesis kit (Roche) were used for the RNA *in situ* hybridization experiment. A DEX1-specific cDNA fragment of 313 bp was amplified with primer pair YW-F (5′-GAATTCATGGAGACAAGATGCCAGGTTGG-3′, *Eco*R I site is underlined) and YW-R (5′-GGATCC-GTTGCATTCGTTTGAGTGGTCGA-3′, *Bam*H I site is underlined), and cloned into the pSK vector. Antisense and sense DIG-labeled probes were prepared and *in situ* hybridization analysis was performed as described previously [19,20].

2 Results

2.1 *DEX1* is highly expressed in the tapetum

Previous results showed that *DEX1* is expressed at low, relatively equal amounts throughout the plant, with a slightly

higher level in flower buds [15]. To study the functions of DEX1 during microspore development in more detail, we initially examined the precise expression pattern of *DEX1* during anther development. RNA *in situ* hybridization analysis demonstrated that *DEX1* mRNA was initially detected at stage 5 anther (Figure 1(a)) and the levels increased in tapetal cells and meiocytes at stage 6 (Figure 1(b)). *DEX1* transcripts were highly abundant in stage 7 tapetal cells (Figure 1(c)), and then decreased during later stages of anther development (Figure 1(d)–(g)). The expression pattern of *DEX1* indicated that this gene may play an important role during the early stages of microspore development.

It has been shown that *DYT1* is expressed in the tapetal cells beginning at stage 5 during anther development [21]. To investigate whether *DYT1* regulates the expression of *DEX1*, we also examined the expression of *DEX1* in the anthers of *dyt1* mutant plants using RNA *in situ* hybridization. As shown in Figure 1, although there is very little difference in hybridization signals between *dyt1* and WT anthers at stage 5 (Figure 1(i) and (a)), the *DEX1* transcripts were barely detectable in *dyt1* anthers from stage 6 and later stages (Figure 1(j) and (k)). These data indicate that the expression of *DEX1* is dependent on the presence of functional DYT1 protein in the anther.

2.2 Released *dex1* microspores degenerate during microsporogenesis

Previous studies have shown that *DEX1* is required for pollen exine pattern formation in *Arabidopsis thaliana* [15]. To further investigate the function of *DEX1* in anther development, we characterized the *DEX1* T-DNA insertion line CS3827 obtained from the ABRC [15]. The homozygous mutants were male sterile (Figure 2(a)) with no pollen formation, as indicated by microscopic examination of the stigma and the Alexander staining method [16]. Consistent with the previous report, we found that, as compared with the WT, there was no released pollen on the *dex1-1* stigma (Figure 2(b)) and no viable pollen grains were observed in the *dex1-1* anthers by Alexander staining (Figure 2(c)).

We then performed SEM analysis to examine the development of *dex1* microspores. Compared with WT, *dex1* microspores were observed to be aborted and collapsed (Figure 2(d) and (f)). Moreover, in contrast to the reticulate exine pattern of the WT microspores (Figure 2(e)), the aborted microspores in the *dex*1 mutant exhibited an incomplete or flawed exine pattern (Figure 2(g)), indicating that sporopollenin deposition and exine pattern formation were defective in *dex1*.

To investigate whether the defects in anther development lead to no pollen formation in *dex1*, we examined anther cross-sections from WT and *dex1* plants using light microscopy. The *dex1* anthers showed a pattern of development similar to that of the WT until stage 6 (Figure 2(h) and (o)). There appeared to be no obvious differences between the WT and *dex1* anthers at stage 7, when tetrads of the haploid microspores were formed (Figure 2(i) and (p)). In contrast, at stage 8 the WT microspores were angular in shape, while the *dex1* microspores showed a much rounder appearance (Figure 2(j) and (q)). Although the microspores within the WT anther at stage 9 became vacuolated and formed a basic exine (Figure 2(k)), the exine of the *dex1* microspores appeared abnormal, and the microspores began to degenerate (Figure 2(r)). During stages 10 and 11, the WT microspores generated a basic pollen wall (Figure 2(l)

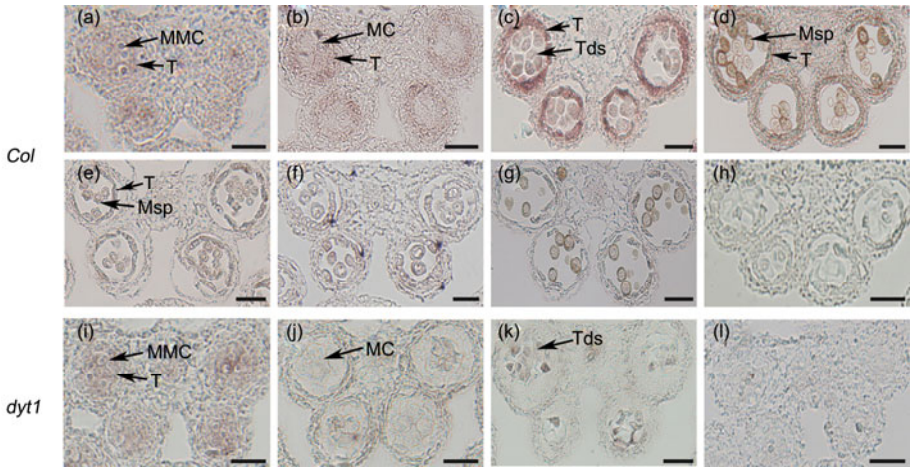

Figure 1 *DEX1* expression during anther development. *DEX1* mRNA was detected by *in situ* hybridization to sections taken from developing anthers of WT flowers using a gene-specific antisense probe and a control sense probe. Stages of anther development are according to Sanders et al. [4]. (a) Stage 5 anther with weak hybridization signal in microspore mother cells (MMC) and newly formed tapetum (T). (b) Stage 6 anther showing hybridization in the tapetum and meiocytes (MC). (c) In the stage 7 anther, maximal hybridization is detected in the tapetum; the signal in the tapetum weakens by stage 8 (d) and disappears by stage 11 (g). (h) Hybridization with the sense control probe produces no signal in stage 7 WT anther. (i) *dyt1* anther at stage 5, showing that signal can be detected in the tapetum and microspore mother cells; signal disappears in stage 6 (j) and 7 (k) anthers. (l) The sense control probe was hybridized against stage 6 WT anther, and only background signal is seen. Bars = 10 μm.

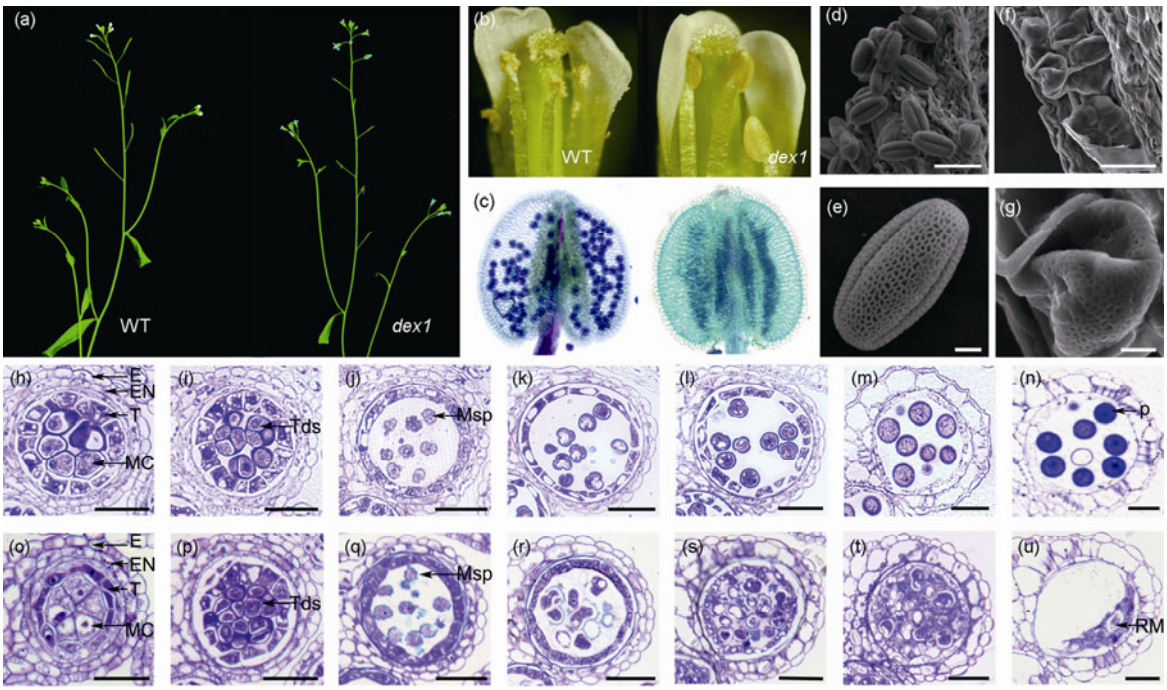

Figure 2 Phenotypic characterization of *dex1* mutants. (a) WT and *dex1* plants. (b) WT flower with pollen on the stigma, while *dex1* flower with no pollen on the stigma. (c) Alexander staining of the WT and *dex1* anther. SEM examination of dehiscent anther and pollen grains of the WT (d and e) and *dex1* (f and g) plants. (d) and (f) Dehiscent anthers. The WT anther contains numerous pollen grains, while the *dex1* anther is filled with degenerated microspores. Bars = 25 μm. (e) WT pollen grains with a regular reticulate exine patrern. (g) Malformed *dex1* microspores with defective exine pattern formation. Bars = 2 μm. Anther and microspore development in the WT (h–n) and *dex1* (o–u) flowers. (h) and (o) Stage 6 anther with well-differentiated anther cell layers. (i) and (p) Stage 7 anther. (j) and (q) Stage 8 anther. (k) and (r) Stage 9 anther. (l) and (s) Stage 10 anther. (m) and (t) Stage 11 anther. (n) and (u) Stage 12 anther. E, epidermis; En, endothecium; T, tapetum; MC, meiocyte; Tds, tetrads; Msp, microspore; P, pllen; RM, remnants of microspore. Bars = 10 μm.

and (m)). However, most *dex1* microspores were degenerated, and the microspore contents were disintegrated into the anther locules (Figure 2(s) and (t)). Finally, at stage 12, the WT anther contained mature pollen grains, while no pollen grains were formed in the *dex1* anther (Figure 2(n) and (u)). These results indicate that microspore development in the *dex1* mutant is abnormal after release from the tetrads.

2.3 *CalS5* expression is greatly down-regulated in the *dex1* mutant

Callose deposition onto the microspore wall is an important process for the formation of active pollen [11]. To investigate whether abnormal microspore development in *dex1* is related to the defects in callose formation, we performed an aniline blue staining assay. The anthers of the *dex1* and WT plants were stained and compared. As shown in Figure 3(a), the callose wall surrounding the microspores in the *dex1* tetrads was thinner than that in the WT.

It has been previously shown that *CalS5* plays an important role in callose synthesis during microspore development [11]. We further examined the expression of *CalS5* by RT-PCR and real-time PCR. Compared with the WT, the expression level of *CalS5* was greatly down-regulated in *dex1* (Figure 3(b) and (c)). These results indicate that *DEX1* affects callose synthesis, possibly by regulating *CalS5* gene expression during anther development.

2.4 DEX1 is localized to the plasma membrane

The *DEX1* gene encodes a predicted protein of 896 amino acid residues with unknown function. Consistent with a previous prediction [15], our bioinformatic analysis suggests that DEX1 localizes to the plasma membrane: residues 1–859 are predicted to be extracellular, residues 880–896 are predicted to be on the cytoplasmic side of the membrane, and amino acids 860–879 represent a potential transmembrane domain (Figure 4(a)). To confirm the subcellular localization of the DEX1 protein, we generated a *DEX1*-GFP fusion construct driven by the *Cauliflower mosaic virus* 35S promoter. This construct was introduced into tobacco leaf cells by *Agrobacterium*-mediated infiltration. As shown in Figure 4(b), the GFP signals were predominantly observed at the plasma membrane, strongly indicating that DEX1 is a plasma membrane-associated protein.

Pfam database (http://www.sanger.ac.uk/Software/Pfam/) analysis indicated that there is only one copy of the *DEX1* gene in *Arabidopsis*. Furthermore, homologs of the DEX1 protein were identified in various plant species and a few microbes by BLASTp and tBLASTn searches against the National Center for Biotechnology Information (NCBI)

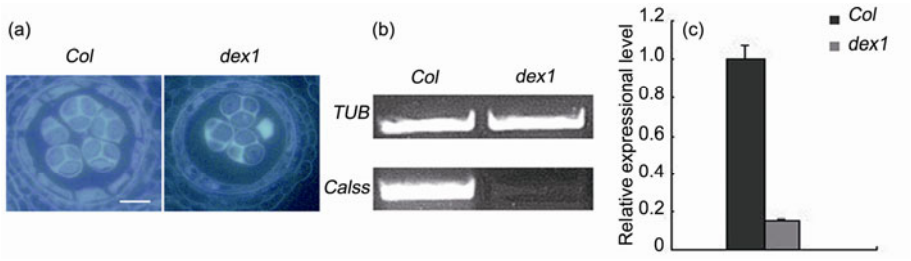

Figure 3 *CalS5* expression in the *dex1* plants. (a) Callose wall of *dex1* mutant was thinner around the microspores of the tetrad stage anther compared with that of the WT. Bar = 20 μm. (b) Semi-quantitative RT-PCR analyses of *CalS5* expression in WT and *dex1* flower buds of 32-d plants. *TUB*, *TUBULIN* expression as a control. (c) Real-time PCR analysis of *CalS5* transcript level in WT and *dex1* flower buds.

Figure 4 Characterization of the *DEX1* gene. (a) Predicted protein structure of DEX1. Grey box, plasma membrane; the extracellular region contains the putative seven integrin alpha repeats. (b) Subcellular localization of DEX1:GFP inflorescence in transient transgenic tobacco leaf cells. Green inflorescence indicates the localization of DEX1:GFP protein. The arrowhead indicates a plasmolyzed epidermis cell treated by 0.8 mol L^{-1} mannitol. Bar = 20 μm. (c) Unrooted phylogenetic tree of DEX1 and the homolog proteins. Protein sequences of DEX1 and its homologs were analyzed with the neighbor-joining method by MEGA 4.0 software. The numbers at the nodes represent percentage bootstrap values based on 1000 replications.

database (Figure 4(c)); these included proteins from castor bean (*Ricinus communis*), poplar (*Populus trichocarpa*), grape (*Vitis vinifera*), soybean (*Glycine max*), alfalfa (*Medicago truncatula*), sorghum (*Sorghum bicolor*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), purple falsebrome (*Brachypodium distachyon*), *Selaginella moellendorffii*, a moss (*Physcomitrella patens*), a small micro algae (*Micromonas pusilla*), a polar eukaryotic microalga (*Coccomyxa subellipsoidea*), volvox (*Volvox carteri*), a cellular slime mold (*Polysphondylium pallidum*), an amoeba (*Naegleria gruber*), and a trypanosome (*Trypanosoma brucei*). No DEX1 homologs were found in the animal kingdom. These data suggest that DEX1 is more likely to be plant specific.

3 Discussion

In this study, we have characterized the roles of the *DEX1* gene in microspore development in *Arabidopsis thaliana*. *DEX1* is highly expressed in stage 7 tapetal cells during anther development (Figure 1(c)). In the *dex1* mutant, microspores are degenerated and aborted during postmeiotic development. *CalS5* expression was greatly down-regulated, resulting in decreased callose layer formation in the *dex1* tetrads (Figure 3). These results indicate that *DEX1* functions in the early determination of microspore formation, likely through its effect on *CalS5* expression.

Our study demonstrated that microspore abortion in *dex1* plants is apparently due to rupture of the microspores. This indicates that *DEX1* is required for the cell integrity of microspores. Generally, plant cells are bound by a rigid cell wall that prevents cellular migration and maintains cellular integrity [22]. In microsporogenesis, a common cell wall is replaced by callose in the microspores during meiosis; the callose wall serves as a mold for primexine patterning, and subsequently, sporopollenin deposits onto the microspore to form pollen exine [6]. In the *dex1* mutant, callose wall for-

mation was severely impaired, which may affect primexine and sporopollenin deposition on the microspores. Therefore, microspore rupture may be due to the reduction in callose wall formation. Our results indicate that proper development of the callose wall may be required for the microspore cell integrity.

The DEX1 protein is predicted to have one extracellular region, one transmembrane region, and one intracellular region (Figure 4(a)). Its subcellular localization to the plasma membrane was confirmed in our study (Figure 4(b)). DEX1 functions sporophytically in anther development, because the WT and *dex1* male sterile phenotype segregated 3:1 in an F_2 population. This is in agreement with its expression at the transcriptional level in tapetal cells during anther development (Figure 1(c)). The tapetum is a sporophytic cell layer highly specialized for the biosynthesis and secretion of maternally-derived pollen wall components, such as sporopollenin. The contribution of the tapetum to exine initiation and sporopollenin deposition starts while the microspores are still enclosed in tetrads, and continues through the vacuolated stages until the first pollen mitosis is almost completed [23]. Furthermore, the phenotype associated with loss of *DEX1* function in the *dex1* mutant first appears at stage 7 (Figures 2 and 3), consistent with defects in deposition of the secreted sporopollenin component(s) leading to defective microspores. These microspores, when released from tetrads in stage 8 anthers, fail to develop normal exine and are aborted in development by stage 9. Therefore, it is possible that DEX1 is required for transport of sporopollenin constituents and deposition in the early steps of exine formation.

In the knockout mutant of *DEX1*, callose wall formation around the tetrad microspores was decreased (Figure 3(a)). It is hypothesized that the synthesis, secretion and deposition of callose is controlled by the meiocytes and individual microspores [5,24]. It has also been reported that callose is composed of polysaccharide materials [10]. Another membrane integral protein, NPU, which is involved in exine patterning in *Arabidopsis*, was predicted to function as a sugar transporter [14]. Therefore, as a membrane-localized protein, DEX1 may be involved in transporting from the tapetum the materials that are essential for callose formation in meiocytes and microspores. *CalS5* was reported to be responsible for the synthesis of callose deposited at the primary cell wall of meiocytes and microspores of tetrads [11]. *In situ* hybridization results also show that *DEX1* is initially expressed in meiocytes, with its highest level of expression at the tetrad stage (Figure 1). The expression pattern of *DEX1* is similar to that of *CalS5* during anther development. It has been reported that plant cells usually maintain relatively stable sugar concentration levels, and a high or low level of sugar may inhibit or activate the expression of some other genes [25]. In the *dex1* mutant, the block of primexine material might lead to a low level of sugar inside microspores and/or microsporocytes, which could inhibit the expression of *CalS5* in the mutant. However, there might be other mechanisms that explain how the knockout of *DEX1* affects *CalS5* expression. The down-regulation of *CalS5* expression further affects callose synthesis, as shown in Figure 2. In addition, it has been reported that with the release of free microspores, dissolution of the callose wall would afford an important and convenient source of soluble carbohydrates for microspore ripening in the anther [5]. Therefore, the low level of callose accumulation in the *dex1* mutant might also affect microspore development.

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