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Arabidopsis JINGUBANG Is a Negative Regulator of Pollen Germination That Prevents Pollination in Moist Environments[OPEN]

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The molecular mechanism of pollen germination and pollen tube growth has been revealed in detail during the last decade, while the mechanism that suspends pollen grains in a dormant state is largely unclear. Here, we identified the *JINGUBANG* (*JGB*) gene by screening pollen-specific genes for those that are unnecessary for pollen germination. We showed that the pollen of the *jgb* loss-of-function mutant exhibited hyperactive germination in sucrose-only medium and inside the anther, while this phenotype was rescued by the transgenic expression of *JGB* in *jgb* plants. JGB contains seven WD40 repeats and is highly conserved in flowering plants. Overexpression of *JGB* inhibits pollen germination. These results indicate that JGB is a novel negative regulator of pollen germination. In addition, we found that jasmonic acid (JA) abundance was significantly elevated in *jgb* pollen, while exogenous application of methyl jasmonate rescued the inhibition of pollen germination in plants overexpressing *JGB*. Based on the molecular features of JGB and on the finding that it interacts with a known JA biosynthesis-related transcription factor, TCP4, we propose that JGB, together with TCP4, forms a regulatory complex that controls pollen JA synthesis, ensuring pollination in moist environments.

INTRODUCTION

Successful delivery of sperm cells to the embryo sac is a prerequisite for fertilization that is mediated by pollen tubes in higher plants. Sperm cells, carried within pollen tubes, are transferred through the stigma and style, guided and recognized by the female gametophyte, and eventually delivered into the embryo sacs. Thus, pollen germination and pollen tube growth are crucial events in sexual reproduction of flowering plants. During germination and pollen tube growth, pollen cells exhibit complex cellular and molecular dynamics, including constant modification of the cytoskeleton, establishment and retention of a tip-focused Ca^{2+} concentration gradient, active cellular trafficking, and biosynthesis of the cell wall. The molecular mechanisms underlying pollen tube guidance and reception have been reported in detail in recent years (Taylor and Hepler, 1997; Lord and Russell, 2002; Krichevsky et al., 2007; Okuda et al., 2009; Liu et al., 2013; Higashiyama and Takeuchi, 2015; Wang et al., 2016).

Because of the importance of the regulatory mechanisms influencing pollen germination and tube growth for sexual plant reproduction, these mechanisms have been the subjects of intensive research, which has revealed several genes and gene products involved in cell dynamics. For example, pollen germination and tube growth require Ca^{2+} and sucrose. The Ca^{2+} pump autoinhibited Ca^{2+} ATPases9 (Schiøtt et al., 2004) and *Arabidopsis thaliana* sucrose transporter (AtSUC1; Sivitz et al., 2008) are localized in the cell membrane and are necessary for normal pollen germination and tube growth. In addition, the cytoskeleton matrix of germinating pollen cells has a particular composition. A group of actin and tubulin binding factors, including microtubule-associated proteins (Cai et al., 2005), formin3 (Ye et al., 2009), Arabidopsis FIMBRIN5 (Wu et al., 2010), and VILLIN5 (Zhang et al., 2010), regulate cytoskeletal rearrangement and facilitate pollen germination. Proteins involved in active biosynthesis and cell trafficking in germinating pollen cells include Cd^{2+}-induced gene, a putative nucleotide-diphospho-sugar transferase (Li et al., 2012), and Subunit of Exocyst Complex (SEC8), a putative exocyst protein (Cole et al., 2005), which are required for pollen germination and tube growth in Arabidopsis.

Rho-related GTPases from plants1 (ROP1), a Rho-family small GTPase identified in plants, is an upstream regulator of pollen germination and tube growth that acts by regulating the Ca^{2+} concentration gradient and F-actin dynamics. Inhibition of ROP1 activity eliminates the Ca^{2+} concentration gradient (Li et al., 1999) and interferes with actin polymerization (Fu et al., 2001), thus impairing pollen tube growth. ROP1 activates the downstream factors ROP-interactive CRIB-motif-containing protein 3 (RIC3) and RIC4, which control the Ca^{2+} concentration gradient and F-actin polymerization (Gu et al., 2005). Changes in the Ca^{2+} concentration in pollen cells negatively regulate ROP1 activity via a feedback mechanism (Yan et al., 2009).

In addition to the regulatory factors that affect pollen Ca^{2+} concentration, sucrose, biosynthesis, cell trafficking, and the

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cytoskeleton, many other cellular factors are also necessary for pollen germination and tube growth. For example, inhibition of a phospholipase in Arabidopsis (Kim et al., 2011) and a ubiquitin ligase in lily (*Lilium longiflorum*) (Huang et al., 2006) strongly impair pollen germination and tube growth. It is clear that pollen germination and tube growth require multiple coordinated cellular processes, including genetic regulation and metabolism. Transcriptional surveys show that more than 22,000 Arabidopsis genes are expressed during pollen development (Honys and Twell, 2004) and more than 4000 mRNA-encoding genes are expressed specifically in germinating pollen and growing pollen tubes (Wang et al., 2008), demonstrating the complexity of these dynamic cellular processes.

The effects of phytohormones on pollen germination and pollen tube growth have been studied for decades. The effects of phytohormones on pollen germination are complex; hormones may have varying effects across species. In strawberry (*Fragaria x ananasa*), pollen germination was decreased when exogenous jasmonic acid (JA) was applied to the medium, but this effect was rescued by ethephon and 1-aminocyclopropane carboxylic acid (Yildiz and Yilmaz, 2002). Pollen germination of lily is independent of ethylene (Sfakiotakis et al., 1972). Brassinosteroids promote pollen germination in *Prunus armeniaca* (Hewitt et al., 1985) and Arabidopsis (Vogler et al., 2014). Methyl jasmonate (MeJA) inhibits pollen germination and pollen tube growth in *P. armeniaca* (Muradoğlu et al., 2010) and plays an essential role in anther dehiscence and pollen maturation in Arabidopsis (Ishiguro et al., 2001).

Although thousands of genes have been identified as specifically being expressed in pollen and pollen tubes (Honys and Twell, 2004; Wang et al., 2008), few genes have been associated specifically with pollen germination and pollen tube growth. In particular, genes identified to date that are involved in regulatory pathways are almost all positive regulators of cell dynamics, i.e., those required for pollen germination and tube growth. However, as mature pollen grains do not germinate until exposed to suitable conditions or until they come into contact with compatible stigmas, negative regulatory mechanisms that inhibit pollen germination must also exist. Further evidence for the negative regulation of pollen germination was provided in physiological studies that revealed hormone signaling pathways involved in pollen dormancy (reviewed in Šírová et al., 2011). Arabidopsis *inositol polyphosphate 5-phosphatases* (*At5PT12* and *13*) are among the few genes known to be involved in maintaining pollen dormancy (Wang et al., 2012). The genetic basis of pollen dormancy is difficult to study because the phenotypes of mutants harboring defects in pollen dormancy are hard to detect, impairing phenotype identification.

We screened for genes specifically expressed in pollen and pollen tubes with the aim of revealing the negative control mechanism necessary for proper pollen germination, with special attention to genes that were highly expressed in mature pollen and not required for pollen germination and tube growth. Here, we report our identification of an unknown Arabidopsis gene encoding a 51.4-kD protein containing seven WD40 repeats, which was designated *JINGUBANG* (*JGB*) and shown to play a critical role in ensuring proper pollen germination. Pollen grains of the *jgb* T-DNA insertion mutant exhibited hyperactive germination in distilled water and inside dehiscing anthers, but this phenotype was rescued by complementary expression of *JGB*. Furthermore, we demonstrated activation of pollen germination by MeJA and confirmed the interaction between JGB and the TCP4 transcription factor, which is involved in JA synthesis. These results indicate that JGB is a novel negative regulator of pollen germination that prevents pollination under excessively moist environmental conditions by inhibiting JA synthesis.

RESULTS

JGB Is a Single-Copy Pollen-Specific Gene Unnecessary for Plant Growth and Sexual Reproduction

To identify genes that may function as negative regulators of pollen germination, we analyzed available pollen transcriptome data (Honys and Twell, 2004; Wang et al., 2008) for unknown pollen-specific genes. Several parameters were used to limit the number of targets and increase the probability that genes regulating pollen germination were identified (Supplemental Figure 1). This search approach yielded 131 unknown genes with high expression levels in pollen and potential regulatory motifs or domains in their protein products, including At2g26490 (later termed *JGB*), a putative hydrophilic protein containing seven WD40 repeats. Mutant phenotype analysis confirmed that At2g26490/JGB was unnecessary for pollen germination and thus met the screening criteria.

Pollen-specific expression of *JGB* was confirmed using RT-PCR and *GUS* reporter analysis (Supplemental Figure 1). *JGB* mRNA was undetectable in the root, stem, and leaf of wild-type Arabidopsis. A GUS signal was identified in the pollen grains and pollen tubes of the transgenic plants carrying the *JGB* promoter sequence fused with *GUS* (JGB_{pro}-*GUS*). The change in GUS signal strength indicated accumulation of the reporter during pollen development; it was initially detectable in bicellular pollen and was later most abundant in tricellular and mature pollen and growing pollen tubes. These results matched well with reported microarray data (Honys and Twell, 2004; Wang et al., 2008), indicating that *JGB* is pollen specific and expressed in a development-dependent manner in mature pollen and pollen tubes.

Bioinformatic analysis identified *JGB* as an unknown single-copy gene in Arabidopsis with a genomic sequence of 1398 bp in length without introns (Figure 1A). *JGB* encodes a putative hydrophilic protein (JGB) of 51.4 kD in size (465 amino acids) containing seven WD40 repeats (Figures 1A and 1B). We obtained a T-DNA insertion mutant of *JGB* (SALK_018094C, T-DNA inserted 16-bp upstream of the stop codon; Figure 1A) from the ABRC and confirmed that the insertion disrupted *JGB* expression, resulting in undetectable levels of RNA and protein in the homozygous T-DNA insertion mutant plant (*jgb*) (Figure 1C). To assess *JGB* as a negative regulator of pollen germination, we examined the phenotype of *jgb*. *jgb* plants exhibited no abnormality in vegetative growth, flower morphology, pollen viability, pollen development, or seeding compared with Col-0 plants under standard growth conditions (22°C, 70% humidity, 16/8 h light/dark cycle) (Supplemental Figure 2). These results indicate that JGB is

a pollen-specific gene that is not required for plant growth or reproduction.

JGB Inhibits Hyperactive Pollen Germination

During our examination of pollen germination, we observed an interesting phenomenon: Mature pollen grains of *jgb* mutant plants germinated inside dehiscing anthers and eventually grew from the anthers when the flowering plants were periodically sprayed with water (Figures 1D to 1G). This result indicates that *jgb* pollen exhibits a strong tendency for hyperactive germination in comparison with Col-0 pollen, suggesting that *JGB* maintains pollen dormancy.

Because pollen grains of *jgb* exhibited hyperactive germination, we evaluated the germination rate of this pollen in vitro. Pollen grains undergo dehydration and rehydration before release and germination. Using the ratio of pollen width to pollen length as an indicator of the hydration state of pollen grains, we found no obvious difference between Col-0 and *jgb* pollen with regard to dehydration and rehydration (Supplemental Figure 3). In addition, several factors, including Ca^{2+}, Mg^{2+}, K^{+}, and H_3BO_3, are required for pollen germination in vitro; however, our examination showed that nearly half of *jgb* pollen (40.0% ± 5.0%; mean ± SD) germinated without these substances 3 h after imbibition (Figure 2A). By contrast, Col-0 pollen showed 2.3% ± 0.4% germination without Ca^{2+}, Mg^{2+}, K^{+}, and H_3BO_3 within the same time (Figure 2A). It is notable that the germination rate of Col-0 pollen 3 h after imbibition was 27.7% ± 1.4% when Ca^{2+}, Mg^{2+}, K^{+}, and H_3BO_3 were supplied (see Methods for concentrations). These results indicate that *JGB* knockout eases germination requirements and induces rapid pollen germination. Further germination tests were performed using germination medium to verify these effects on

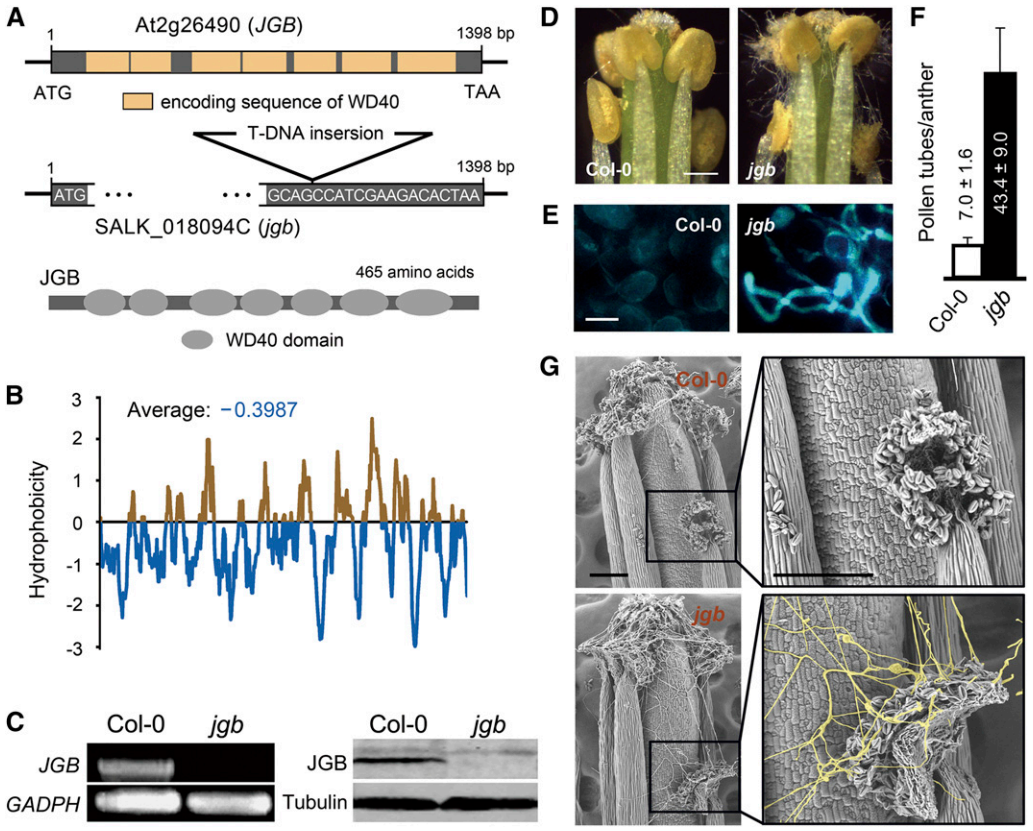

Figure 1. JGB Is an Unknown Hydrophilic Protein Containing Seven WD40 Repeats and Inhibits Improper Pollen Germination in Vivo.

(A) Diagrams of *JGB*, the *jgb* insertional mutant, and JGB protein containing seven WD40 repeats.
(B) The hydrophilic feature of JGB analyzed with ProtParam and ProtScale tools of ExPASy.
(C) *JGB* transcript was absent in the mature pollen grains of *jgb* plants (left panel, RT-PCR with *GAPDH* as a loading control). JGB was undetectable in *jgb* pollen using immunoblot analysis (right panel, with an anti-JGB antibody produced in this study and tubulin as a loading control).
(D) to **(G)** Improper germination of *jgb* pollen occurred when the flowering plants were periodically sprayed with water.
(D) Hyperactive germination of *jgb* pollen observed from the outside of the anther. Bar = 200 μm.
(E) Pollen tubes inside the anther visualized with aniline blue staining. Bar = 20 μm.
(F) Pollen tubes per anther in the flowers of **(E)**. Error bars represent SD (n = 5).
(G) Scanning electron microscopy showing the occurrence of hyperactive germination from the lower anther (pollen tubes highlighted in yellow). Panels in the right are magnifications of the boxed regions in the left panels. Bars = 200 μm.

jgb. Germination of *jgb* pollen grains occurred 30 min after imbibition (12.8% ± 2.0% germinated), whereas Col-0 pollen grains remained ungerminated (0% germinated) 30 min after imbibition and showed initial germination (0.6% ± 0.2% germinated) within 45 min (Figure 2B). Thus, the encoded protein factor JGB inhibits improper hyperactive pollen germination.

To confirm that the loosening of control for pollen germination is a single event caused by *JGB* mutation, we performed a transgenic complementation experiment in which the *JGB* sequence was transformed into a homozygous *jgb* plant. *JGB* transformation rescued the hyperactive germination phenotype of the mutant, confirming that loosening of pollen germination control was caused by *JGB* mutation (Supplemental Figure 4).

JGB Is a Negative Regulator of Pollen Germination

Examination of pollen phenotypes associated with excessive cellular accumulation of JGB was performed to illuminate the role of JGB in pollen germination. This assessment was achieved by applying a transgenic approach to the Col-0 plants, using the same construct as that used for *jgb RES* (Figure 3A). The assay showed the existence of native JGB and JGB-GFP in the transgenic line, indicating successful overexpression of *JGB* in the pollen cells (Figure 3B). The phenotype of the transgenic line showed that *JGB* overexpression markedly inhibited pollen germination (Figures 3C and 3D). The plants overexpressing *JGB* showed less than 10% pollen germination 10 h after imbibition, whereas the Col-0 and *jgb* pollen showed 75 and 94% germination, respectively, at this time point (see Figure 3D for the rates of germination at various time points). These results indicate that JGB restrains pollen germination. Therefore, JGB functions as a negative regulator of pollen germination.

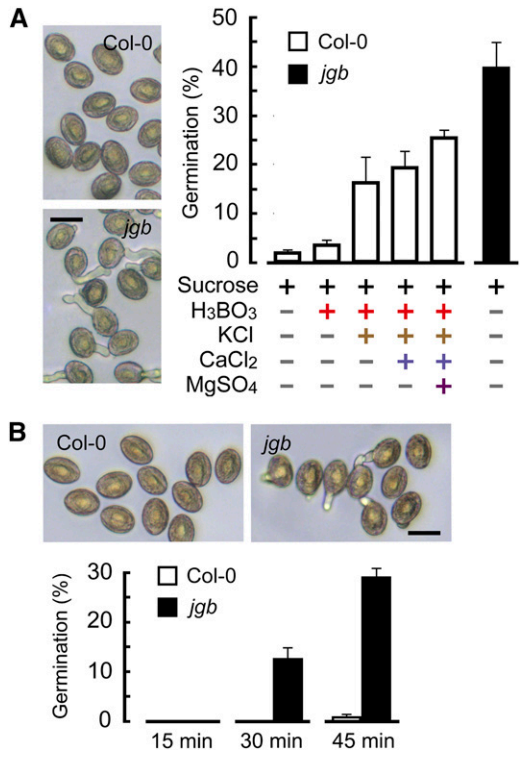

Figure 2. JGB Inhibits Pollen Germination in Vitro.

(A) *jgb* pollen germinated on sucrose-only medium. Images (left panel) were taken 3 h after imbibition on sucrose-only medium; germination rates of Col-0 pollen with partial and full components of the standard medium (containing H_3BO_3, KCl, $CaCl_2$, and $MgSO_4$) 3 h after imbibition (right panel) are shown for comparison. Error bars represent SD (n = 3). Bar = 20 μm.
(B) Hyperactive germination of *jgb* pollen on standard medium in vitro. Images (upper panel) were taken 45 min after imbibition and germination rates were compared at three time points (lower panel). Error bars represent SD (n = 3). Bar = 20 μm.

It was interesting that, for unknown reasons, the pollen grains of the homozygous transgenic lines overexpressing *JGB-GFP* had a range of fluorescence intensities (Figure 3E), which implied unequal accumulation of the fusion protein among the pollen population. The pollen was separated into two groups with high and low expression of the *JGB-GFP* fusion protein. The groups of pollen with high and low expression of *JGB-GFP* showed clear differences in germination (Figure 3E); 16.7% ± 1.9% of the low *JGB-GFP* expression group germinated 10 h after imbibition (n = 512), whereas no germination was observed in the high *JGB-GFP* expression group (0.0% ± 0.0%, n = 500). This observation suggests that JGB negatively regulates pollen germination in vitro.

Following the observation above, we hand-pollinated stigmas of Col-0 flowers with pollen grains with different levels of JGB to assess the regulatory effect of JGB in vivo. It was interesting that Col-0 and *jgb* pollen germinated very rapidly after pollination (Figure 3F). Following pollination of stigmas, the rates of germination of Col-0 and *jgb* pollen appeared almost identical (no significant difference) (Figure 3H). These results indicate that the inhibitory effect of JGB on pollen germination can be reduced via stigma pollination. Among pollen grains overexpressing *JGB* (transgenic pollen termed *OX-LOW* and *OX-HIGH*, according to the abundance of the fused protein), *OX-LOW* pollen showed more rapid germination (Figure 3G), whereas *OX-LOW* and *OX-HIGH* pollen showed germination significantly slower (Student's t test, $P < 0.01$) than that of Col-0 (Figure 3H). These results indicate that JGB inhibits pollen germination in vivo with much reduced effectiveness in comparison with that observed in vitro.

JGB Stabilizes Pollen Tube Growth in Vivo

The strong GUS activity in the growing pollen tube (Supplemental Figure 1B) suggests that *JGB* exhibits persistent expression after pollen germination and implies that JGB plays a role in tube growth. We assessed these findings via transmission analysis using reciprocal crosses between Col-0 and *jgb*/*JGB* heterozygous plants. The experiments showed a significant reduction in transmission of the *jgb* gamete through the male lineage, but no abnormality in transmission through the female lineage (Table 1), indicating that the lack of JGB compromises the efficiency of male transmission. *jgb* pollen germinate normally on stigmas (Figure 3F); therefore, it is most likely that JGB supports proper pollen tube growth.

To establish how JGB functions in pollen tube growth, we inspected the ovaries of Col-0 flowers pollinated with *jgb* and Col-0 pollen. Using aniline blue staining, we showed that the leading

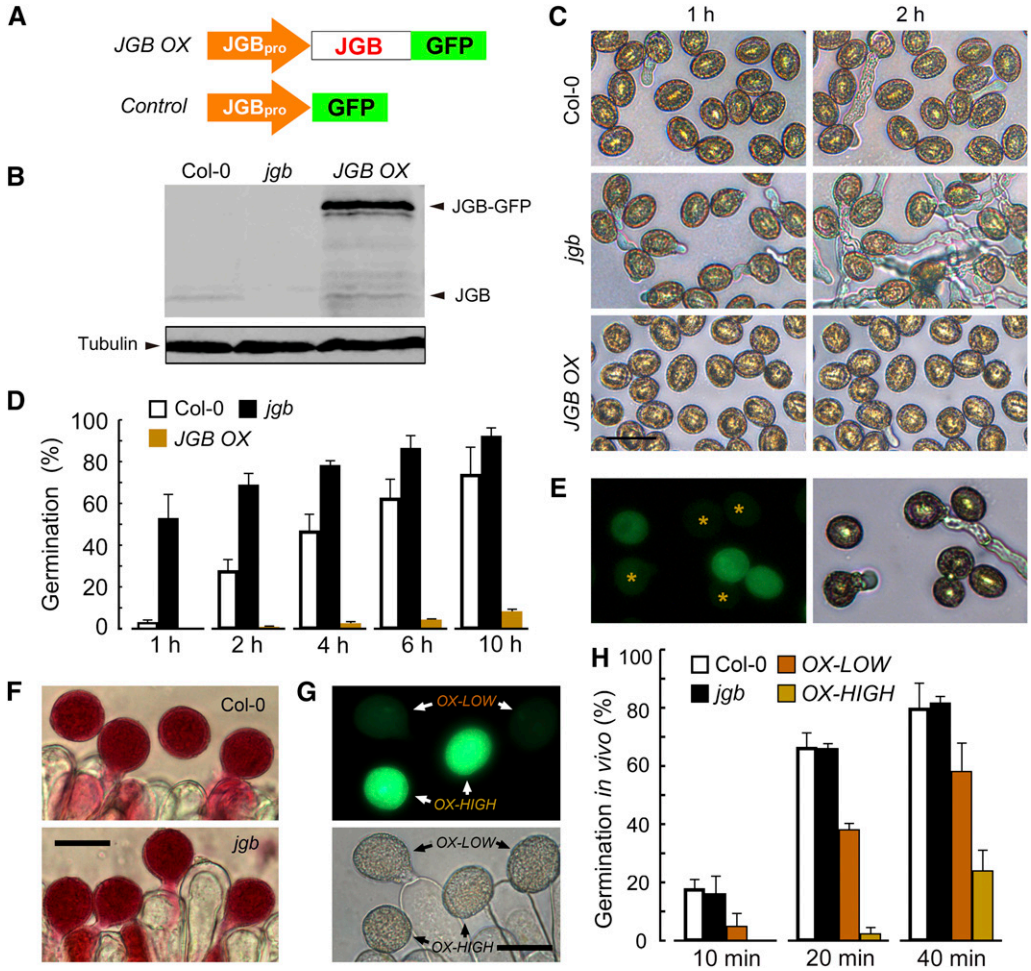

Figure 3. JGB Is a Negative Regulator of Pollen Germination.

(A) Construction of the transgenic vectors.
(B) Immunoblot showing the native level of JGB in Col-0, the undetectable level of JGB in *jgb*, and the native JGB and JGB-GFP levels in *JGB OX* pollen with tubulin as a loading control.
(C) to **(E)** Inhibition of pollen germination by excessive JGB in vitro.
(C) Images of Col-0, *jgb*, and *JGB OX* pollen 1 and 2 h after imbibition. Bar = 20 μm.
(D) Germination rates of Col-0, *jgb*, and *JGB OX* pollen 1, 2, 4, 6, and 10 h after imbibition. Error bars represent SD.
(E) *JGB OX* pollen exhibiting weaker JGB-GFP fluorescence (indicated by an asterisk) usually germinated.
(F) to **(H)** Inhibition of pollen germination by excessive JGB in vivo.
(F) Pollen grains of Col-0 and *jgb* 20 min after being pollinated to Col-0 stigmas (Alexander staining). Bar = 20 μm.
(G) Pollen grains of *JGB OX* 40 min after being pollinated to Col-0 stigmas (upper panel, JGB-GFP fluorescence; lower panel, bright field). Bar = 20 μm.
(H) The germination rates of Col-0, *jgb*, and *JGB OX* pollen when pollinated to Col-0 stigmas. Error bars represent SD.

pollen tubes of *jgb* were shorter than those of Col-0 6 h after pollination, whereas there was no difference between the pollen tubes 2, 4, and 8 h after pollination (Figure 4A), indicating that *jgb* pollen tubes may grow slower than those of Col-0 during the second phase of growth. By dividing the length of the leading pollen tubes by that of the ovaries, we found highly significant deviation (Student's *t* test, $P < 0.01$) in pollen tube efficiency 6 h after pollination (Figures 4B). These results show that the growth rate of *jgb* pollen tubes declines once they reach a length of ~600 μm compared with Col-0. The similar pollen tube efficiency of *jgb* and Col-0 pollen 2 and 4 h after pollination indicated that these pollen grains showed little or no difference in the germination and initial growth stages of the pollen tube (confirming the results shown in Figure 3F). Moreover, the *jgb* and Col-0 pollen tubes exhibited similar pollen tube efficiency 8 h after pollination, at which point they had reached the basal end of the ovary.

The reduced growth of *jgb* pollen tubes may reduce the probability of the pollen tubes reaching the ovules located in the bottom half of the ovary when competing with Col-0 pollen tubes; this difference in growth may explain the reduced male transmission of the reciprocal crosses (Table 1). To test this hypothesis, we again performed reciprocal crossing of Col-0 and *jgb*/*JGB*

Table 1. Analysis of Segregation in Reciprocal Crosses of Col-0 and *jgb/JGB*

Transmission	Cross (Female × Male)	*jgb/JGB*	Col-0	*jgb/JGB*:Total	TE (Expected)	χ^2 (P) for 100%
Male	Col-0 × *jgb/JGB*	129	203	38.9%	63.5 (100)	16.49** (<0.0001)
Female	*jgb/JGB* × Col-0	163	159	50.6%	102.5 (100)	0.05 (0.823)

jgb/JGB and Col-0 were detected by PCR in T1 seeds. TE (transmission efficiency of gametes) = number of *jgb/JGB*/number of Col-0 × 100%. Double asterisks indicate highly significant difference from the expected TE (100%; χ^2, $P < 0.01$).

plants with seeds harvested from the top, middle, and bottom parts of the siliques. Inspection of the *jgb* allele from each F1 seedling showed a clear reduction in the ratio of *jgb/JGB* seeds within the middle and bottom parts of the siliques in the Col-0 × *jgb/JGB* cross (female × male) (Figure 4C). This result verifies that the growth of *jgb* pollen tubes slows when they reach the bottom half of the ovary. Thus, JGB seems to stabilize pollen tube growth.

JGB Is Localized in the Nucleus and Cytoplasm

We next examined the subcellular localization of JGB in pollen expressing JGB-GFP fusion proteins. Confocal microscopy images showed GFP in the vegetative nucleus and vegetative cytoplasm (Figure 5A), implying that JGB localized to both of these areas in the vegetative cell. These results were confirmed with a *qrt/qrt* background transgenic line harboring *JGB-GFP/jgb*, which showed initial accumulation of JGB-GFP in bicellular pollen and a clear JGB-GFP signal in the vegetative nucleus/cytoplasm (two pollens in one tetrad served as the negative control) (Figure 5B). Further examination showed that the localization pattern of JGB did not change during pollen germination and pollen tube growth (Figure 5C).

The structural feature of JGB underlying its nuclear localization was unclear. No nuclear localization signal was found in JGB using NucPred (Brameier et al., 2007) or NLStradamus (Nguyen Ba et al., 2009). Interestingly, a nuclear exporting signal (NES) sequence

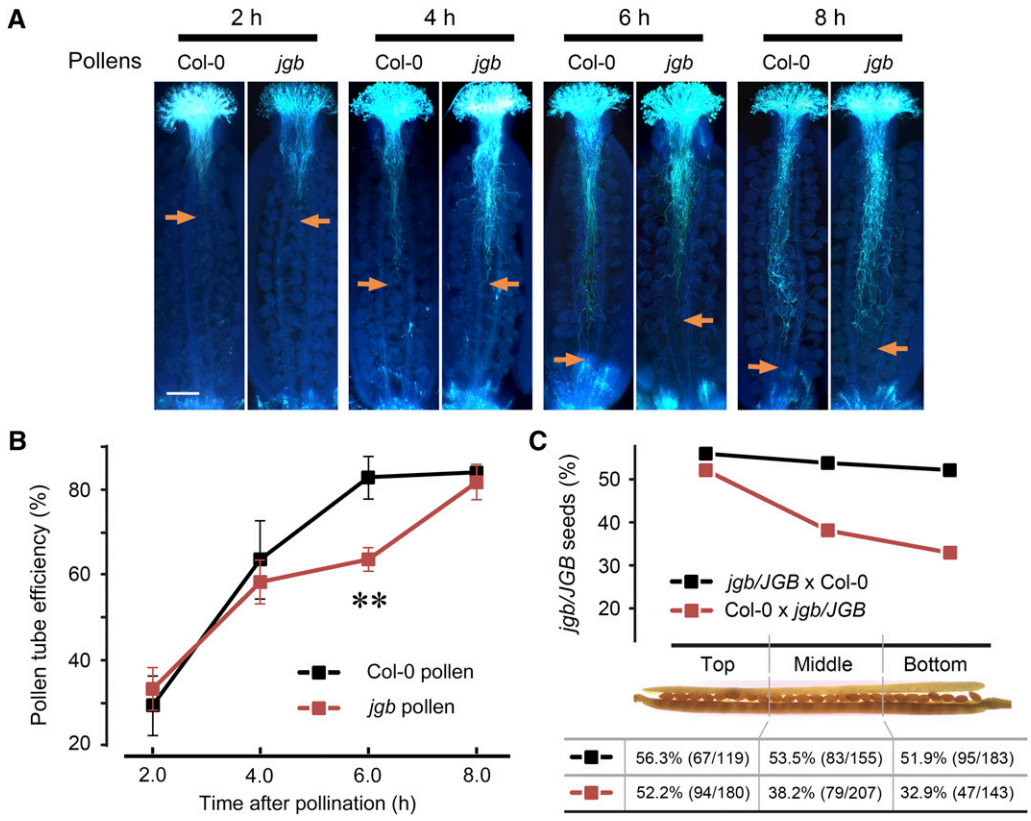

Figure 4. Pollen Tubes of *jgb* Plants Are Defective during Later Periods of Growth in Vivo.

(A) and **(B)** The slowdown of *jgb* pollen tube growth since 6 h after pollination.
(A) Fluorescence images of pollen tubes in vivo (aniline blue staining). Arrows indicate the position of leading pollen tubes. Bar = 500 μm.
(B) Pollen tube efficiency (length of the leading pollen tube/length of style) of pollen tubes in vivo. Error bars represent SD (Student's *t* test: **$P < 0.01$).
(C) The percentages of *jgb* progeny in the middle and bottom part of the siliques produced by the Col-0 × *jgb/JGB* cross.

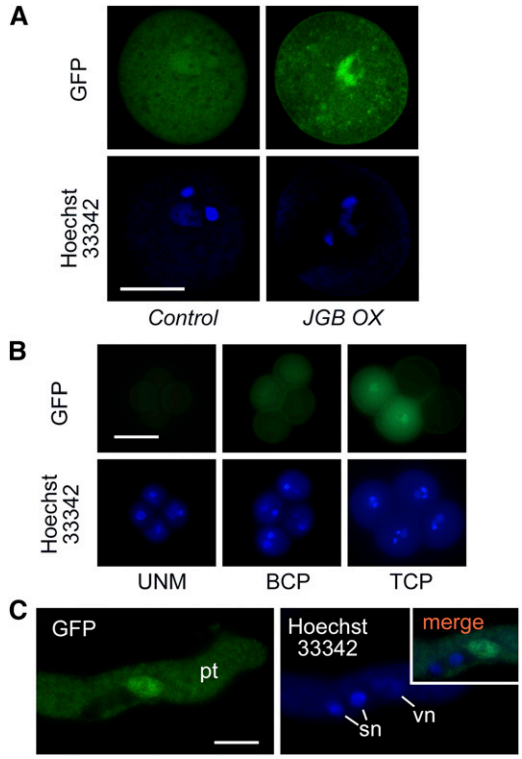

Figure 5. JGB Is Localized in the Nucleus and Cytoplasm of Vegetative Cells.

(A) Localization of JGB-GFP fluorescence in both the nucleus (Hoechst 33342 staining) and cytoplasm of vegetative cells. Bar = 20 μm.
(B) Appearance of JGB-GFP fluorescence from bicellular pollen (BCP) during pollen development. We show this finding with a *jgb/JGB qrt/qrt* plant having two pollen grains in a tetrad as the negative control. UNM, uninucleate microspores; TCP, tricellular pollen. Bar = 20 μm.
(C) Subcellular localization of JGB did not change during pollen germination and pollen tube growth. Panel in the upper right corner is the merged picture of the two original pictures. pt, pollen tube; sn, sperm nucleus; vn, vegetative nucleus. Bar = 20 μm.

(LEV, 344 to 346th amino acid) with high NES scores (1.026, 0.542, and 1.011) was predicted in JGB with the NetNES 1.1 server (http://www.cbs.dtu.dk/services/NetNES/). To determine whether the identified NES might underlie JGB localization, we transformed *jgb* plants with constructs containing *JGB^{AEV}-GFP* (i.e., *GFP* fused to *JGB* harboring a point mutation substituting A for L at amino acid 344; Figure 6A). Significant enrichment of nuclear JGB with *jgb RES^{AEV}* was observed (Figures 6B and 6C), implying that the predicted NES facilitates transportation of JGB into the cytoplasm.

Nuclear JGB Is Functional

As JGB shows dual localization in the cytoplasm and nucleus, we next examined how localization determines the function of JGB. We thus compared pollen germination and pollen tube growth for *jgb RES* and *jgb RES^{AEV}* plants. Our results showed complementation of *jgb* pollen germination phenotypes in the transgenic lines (Supplemental Figure 5), indicating that JGB^{AEV}-GFP rescues *jgb* phenotypes. This finding implies that the point mutation in the NES may not affect the function of JGB in regulating pollen germination. In addition, although a significant increase in the amount of nuclear JGB-GFP and a significant reduction in the amount of cytoplasmic JGB-GFP were achieved by mutating the NES (Figures 6A to 6C), the *jgb RES* and *jgb RES^{AEV}* plants showed no differences in the rates of pollen germination and pollen tube growth (Figures 6D to 6F). It is thus most likely that nuclear JGB, rather than cytoplasmic JGB, controls pollen germination and pollen tube growth. However, there was no obvious nuclear localization signal that could be mutated to analyze the function of cytoplasmic JGB and/or shuttling of JGB from the nucleus to the cytoplasm.

JGB Inhibits JA Synthesis in Pollen

To assess the mechanism by which JGB regulates pollen germination, we used transcriptome analysis to detect differences in gene expression between Col-0 and *jgb* pollen. We performed high-throughput mRNA sequencing (RNA-seq) using mRNAs purified from the mature anthers of Col-0 and *jgb* plants, after which differentially expressed genes were subjected to Gene Ontology (GO) annotation (Supplemental Data Set 1). The biological process term "jasmonic acid biosynthetic process" was highly enriched in the set of differentially expressed genes (Supplemental Figure 6), indicating that JGB may influence JA biosynthesis in pollen cells.

In line with this annotation, we examined pollen germination by applying MeJA to the germination medium, to assess whether exogenous MeJA would affect pollen germination. Surprisingly, supplying 500 to 1000 μM MeJA to the medium activated germination of Col-0 pollen (Figures 7A and 7B). After 1 h of imbibition, 85.4% ± 2.0% of the Col-0 pollen had germinated (500 μM MeJA supplied), which was more than 50 times the germination rate of the pollen not supplied with MeJA (1.6% ± 1.5%) and clearly higher than the germination rate of *jgb* pollen (53.0% ± 11.4%; Figure 3D). This germination activating effect of MeJA was also evident in *jgb* and *JGB OX* pollen (compare Figures 7C and 3D). These results indicate that control of pollen germination by JGB (also inhibition of pollen germination by excessive accumulation of JGB) can be prevented by adding exogenous MeJA to the pollen. Given that the GO annotation of the genes differentially expressed in the *JGB* and *jgb* transcriptomes suggests that JGB functions in JA biosynthesis, it is possible that JGB controls pollen germination by inhibiting JA biosynthesis in pollen.

To verify this possibility, we supplied diethyldithiocarbamic acid (DIECA), an inhibitor of endogenous JA biosynthesis (Farmer et al., 1994; Doares et al., 1995), to the germination medium to determine whether reducing the amount of JA in the pollen inhibited pollen germination. Col-0, *jgb*, and *JGB OX* pollen showed reduced rates of germination (Figures 7C and 7D), with that of *jgb* pollen reduced from 53.0% ± 11.4% (Figure 3D) to 10.6% ± 1.2% when supplied with 500 μM DIECA (Figure 7C). These results demonstrate that inhibition of endogenous MeJA synthesis inhibited pollen germination.

To obtain conclusive evidence that JGB inhibits endogenous JA synthesis, we measured transcript levels of eight major genes involved in JA synthesis (*LIPOXYGENASE2* [*LOX2*], *ALLENE*

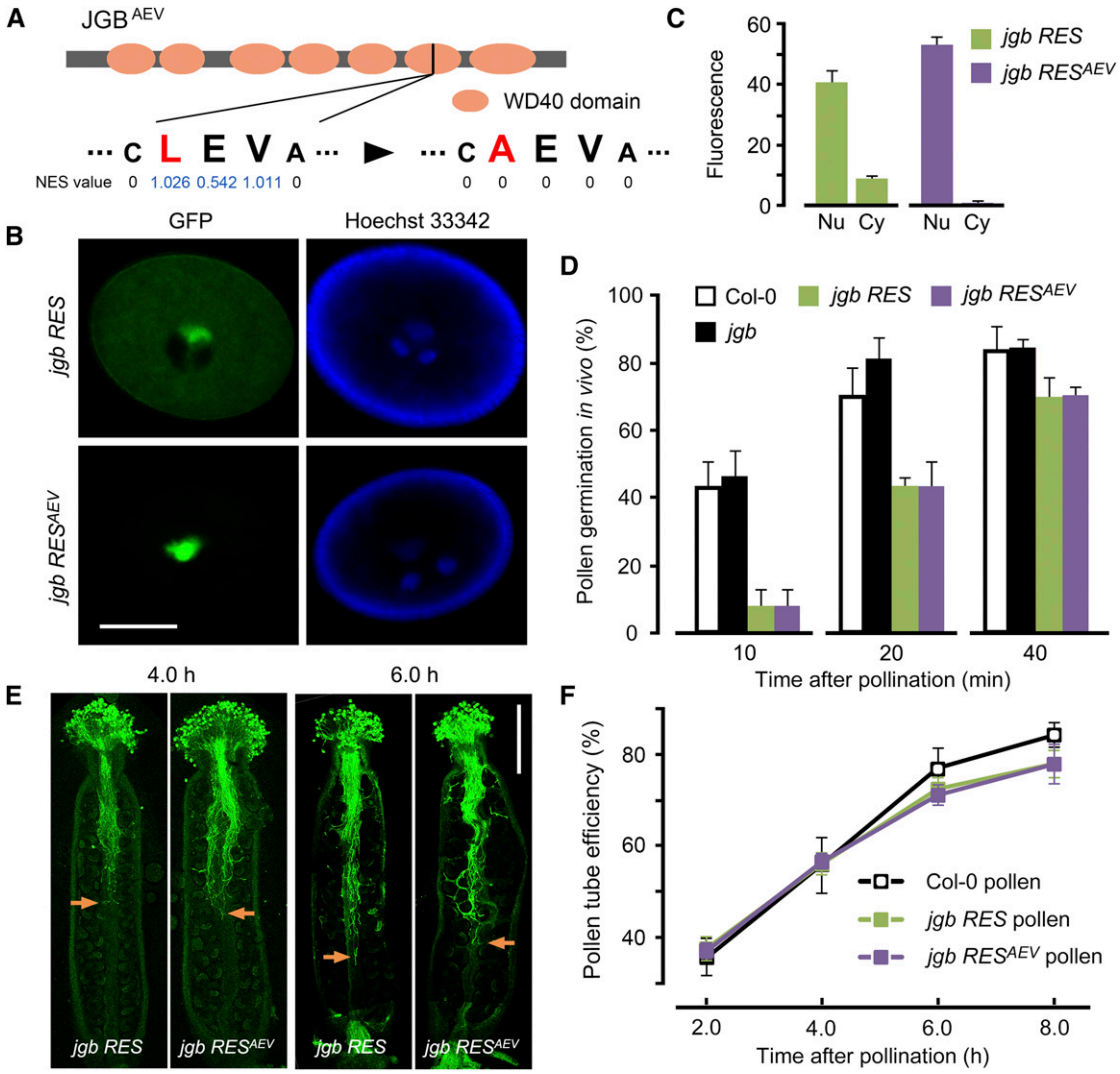

Figure 6. Enrichment of JGB in the Vegetative Nucleus Does Not Influence Pollen Germination and Tube Growth.

(A) Diagram of point mutation in JGB. Red indicates the change of the amino acid.
(B) and **(C)** Enrichment of JGB-GFP fluorescence in the nucleus by the point mutation.
(B) Fluorescence images of *jgb RES* and *jgb RES*^{AEV} pollen. Bar = 10 μm.
(C) Relative fluorescence intensity of the pollen. Nu, nucleus; Cy, cytoplasm.
(D) Pollen of *jgb RES* and *jgb RES*^{AEV} showed equal rates of (inhibited) germination in vivo (on Col-0 stigmas), implying excessive expression of JGB (JGB^{AEV}) in these transgenic lines and indicating that translocation of cytoplasmic JGB to the nucleus did not affect the function of JGB in controlling pollen germination. Error bars represent SD.
(E) and **(F)** The growth of pollen tubes showed no difference between *jgb RES* and *jgb RES*^{AEV}, indicating that translocation of cytoplasmic JGB to the nucleus did not affect the function of JGB in stabilizing pollen tube growth.
(E) Fluorescence images of pollen tubes 4 and 6 h after pollination. Arrows indicate the leading pollen tubes. Bar = 500 μm.
(F) Pollen tube efficiency of *jgb RES* and *jgb RES*^{AEV} pollen tubes. Error bars represent SD.

OXIDE CYCLASE4 [*AOC4*], *OPC-8:0 COA LIGASE1* [*OPCL1*], *ACYL-COA OXIDASE1* [*ACX1*], *ACYL-COA OXIDASE5* [*ACX5*], *ABNORMAL INFLORESCENCE MERISTEM1* [*AIM1*], *3-KETOACYL-COA THIOLASE2* [*KAT2*], and *JASMONIC ACID CARBOXYL METHYLTRANSFERASE* [*JMT*]) and quantified endogenous JA in Col-0, *jgb*, and *JGB OX* pollen. Real-time quantitative PCR experiments showed that *jgb* pollen had significantly higher transcript levels of each of these genes (Figure 7E), implying that JA synthesis was activated in *jgb* pollen. By contrast, the transcript levels of most of the analyzed genes were downregulated in *JGB OX* pollen. Most importantly, JA quantification using ultra-high-performance liquid chromatography-triple quadrupole mass spectrometry showed that the JA level of *jgb* pollen (345.5 ± 7.7 ng/gFW) was more than 17 times that of Col-0 pollen (20.0 ± 5.0 ng/gFW)

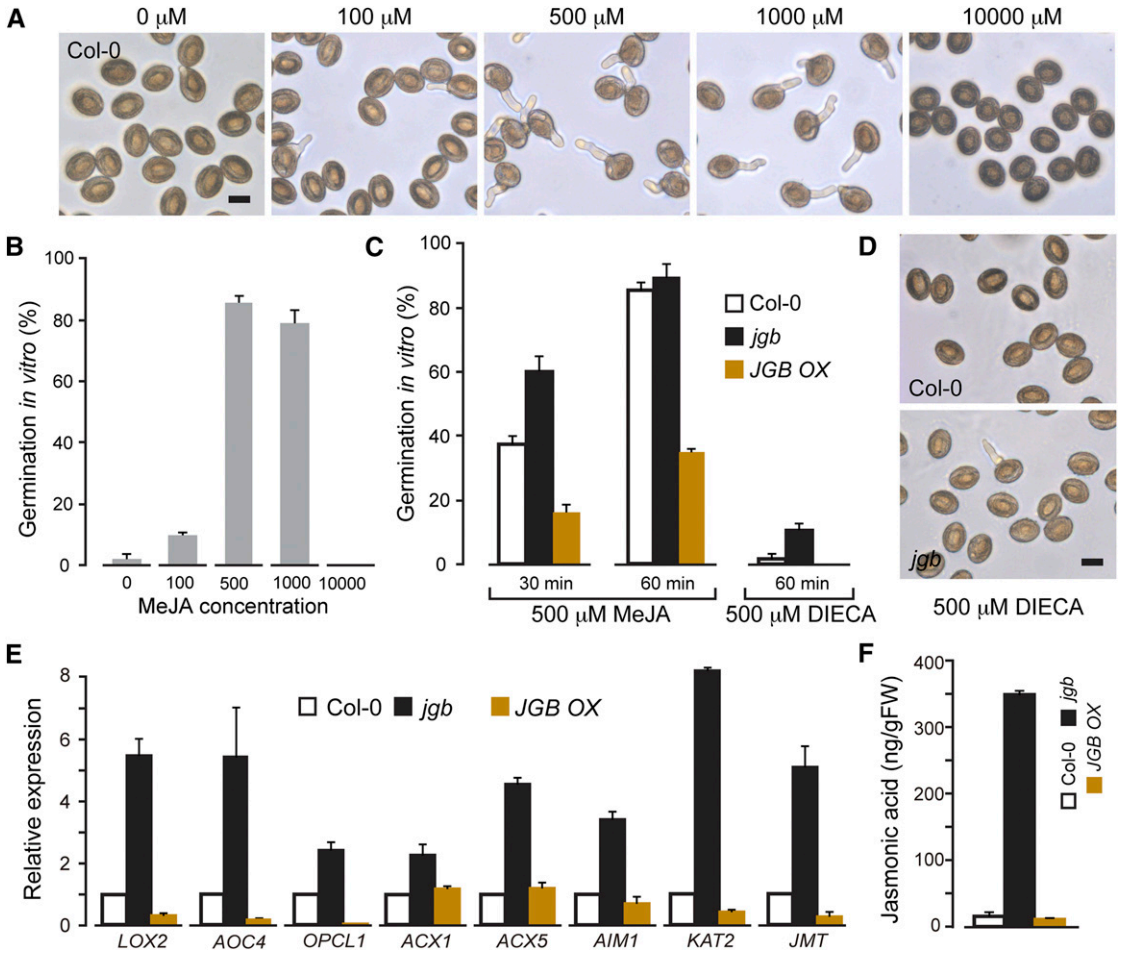

Figure 7. MeJA Activates Arabidopsis Pollen Germination in Vitro.

(A) to **(D)** Hyperactivated pollen germination by supplying MeJA in vitro.
(A) Images of Col-0 pollen germinated with MeJA (1 h after imbibition). Bar = 10 μm.
(B) The germination rates show clear dose dependency, with the most germination on the medium supplemented with 500 μM MeJA. Error bars represent SD.
(C) Effects of MeJA and DIECA on germination of Col-0, *jgb*, and *JGB OX* pollen. Error bars represent SD (n = 3).
(D) Images of Col-0 and *jgb* pollen germinated with DIECA (1 h after imbibition). Bar = 10 μm.
(E) *jgb* pollen has significantly higher transcript levels of genes (*LOX2*, *AOC4*, *OPCL1*, *ACX1*, *ACX5*, *AIM1*, *KAT2*, and *JMT*) encoding proteins in the JA synthesis pathway. Values were normalized to *ACTIN11* with the transcript abundance of Col-0 taken as 1. Error bars represent SD (n = 3).
(F) *jgb* pollen contains a significantly higher amount of JA. FW, fresh weight. Error bars represent SD (n = 3).

and 24 times that of *JGB OX* pollen (14.4 ± 1.0 ng/gFW) (Figure 7F), confirming that JGB inhibits JA synthesis and thereby controls proper pollen germination. In addition, we noted that the exogenous MeJA level that maximally promoted pollen germination was 1.0×10^5 ng/g medium (500 μM), which was 5000 times greater than the endogenous level of MeJA in Col-0 pollen and ~300 times greater than the endogenous level of MeJA in *jgb* pollen, indicating that a relatively large amount of exogenous MeJA is required to affect the endogenous MeJA level.

JGB Interacts with TCP4 to Regulate JA Synthesis in Pollen

Exogenous application of MeJA to Col-0 pollen activated pollen germination, while *jgb* pollen exhibiting hyperactive pollen germination contained significantly more JA than pollen that did not. We thus conclude that endogenous JA promotes pollen germination in Arabidopsis. This finding contradicts previous studies, which showed that JA inhibits pollen germination (Yildiz and Yilmaz, 2002; Muradoğlu et al., 2010). More importantly, our results suggest that JGB inhibits endogenous JA synthesis in pollen.

To determine how JGB affects JA synthesis, we performed a yeast two-hybrid assay to screen a cDNA library of Arabidopsis transcription factors to determine whether any factors interact with JGB. The experiments yielded four TCP family members, TCP4, TCP8, TCP20, and TCP22, as candidates for JGB binding. To confirm the interaction between JGB and the candidate proteins, full-length *JGB* coding sequence (CDS) and *TCP* (*TCP4*,

TCP8, *TCP20*, and *TCP22*) CDSs were cloned into pDEST32 and pDEST22, respectively. Yeast cells cotransformed with JGB and TCP4 grew well on Trp-, Leu-, and His-dropout media (Figure 8A), indicating interaction between JGB and TCP4; GST pull-down assays further confirmed this interaction (Figure 8B). These results provide direct evidence for the manner in which JGB regulates JA biosynthesis. LOX2 catalyzes the first step in JA synthesis in plants, in which linolenic acid is converted to 13-(*S*)-hydroperoxylinolenic acid (Vick and Zimmerman, 1983). TCP4 activates *LOX2* expression by binding to its promoter (Schommer et al., 2008; Danisman et al., 2012).

Based on our results and the established role of TCP4 in regulating *LOX2* expression, we suggest a model for the mechanism by which JGB controls pollen germination (Figure 8C). In this model, JGB serves as a platform that associates transcription factor TCP4 into a transcriptional regulatory complex. In *jgb* pollen, activation of *LOX2* expression by TCP4 is increased because of the lack of JGB, resulting in excessive JA synthesis and hyperactive pollen germination. This basic model requires further assessment by future studies. Our result showing localization of JGB to the nucleus (functional localization) agrees with this model.

We performed bimolecular fluorescence complementation analysis in tobacco (*Nicotiana tabacum*) and Arabidopsis leaf cells to verify the interaction between JGB and TCP4. Unfortunately, these attempts failed because JGB was not detected in the cells. Through a transgenic approach using the *35S* promoter to drive expression of *JGB* in Arabidopsis, we found that JGB was also not detected in somatic cells (mRNA was successfully detected). We also tried to express JGB in cultured animal cells (driven by the *CMV* promoter in 293 cells), but this effort also failed. These results indicate that an unknown mechanism blocks production of JGB in plant cells that are not pollen cells.

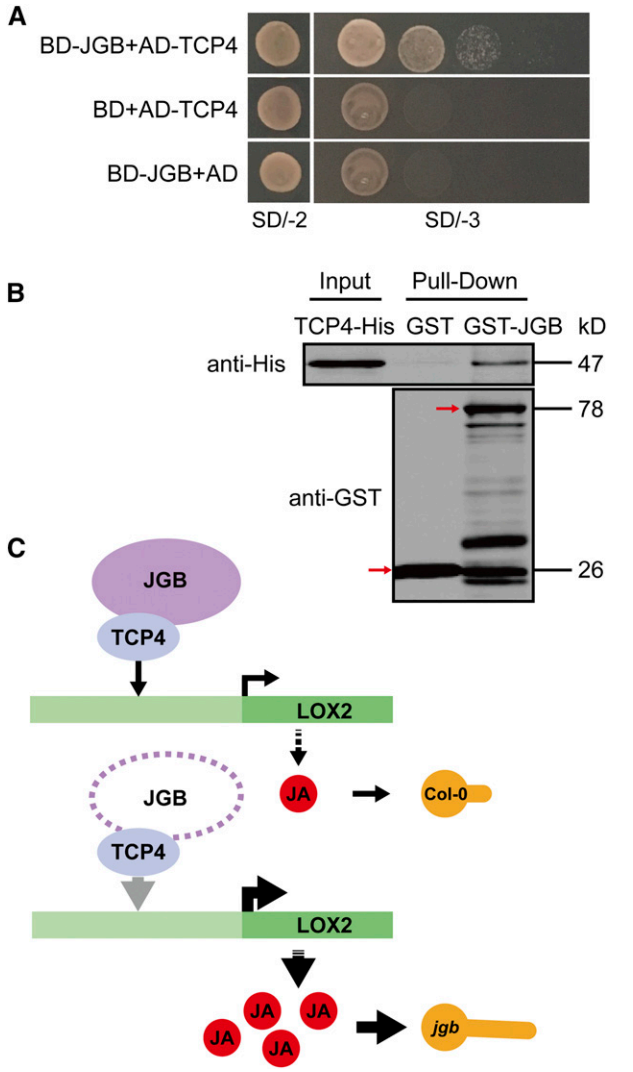

Figure 8. JGB Interacts with TCP4 and a Schematic Model Showing That JGB Inhibits Pollen Germination by Inhibiting JA Synthesis.

(A) Yeast were cotransformed with the plasmids as indicated on the left. Yeast were grown on SD/-2 (SD/-Leu-Trp) dropout media (left panel) and SD/-3 (SD/-Leu-Trp-His) dropout media (right panel). Cells grown on SD/-3 dropout media are indicative of the physical interaction between JGB and TCP4.

(B) In vitro pull-down assays for the interactions between JGB and TCP4. The fusion proteins were expressed in *E. coli* and used for GST pull-down assays, and then immunoblotted with anti-His or anti-GST antibody. GST protein was used as a negative control. The arrows indicate GST-JGB fusion protein (upper) and GST protein (lower).

(C) Working model of inhibition of pollen germination by JGB. TCP4 activates the JA biosynthesis gene *LOX2*. TCP4 and JGB regulate *LOX2*, affecting JA production and pollen germination.

DISCUSSION

JGB Is a Novel Regulator of Pollen Germination

A number of genes indispensable for pollen germination and tube growth have been identified by analyses of mutants. For example, plants lacking *DAYU* (*DAU*; Li et al., 2014) and *phospholipase* A_2 (PLA_2; Kim et al., 2011) show impaired pollen maturation and in vitro germination. *No Pollen Germination1* (*NPG1*; Golovkin and Reddy, 2003) and *Arabidopsis autophagy protein6* (*AtATG6*; Fujiki et al., 2007; Qin et al., 2007) have essential roles in pollen germination. Plants with mutant *lipid transfer protein5* (*LTP5*; Chae et al., 2009) exhibit ballooned pollen tubes and delayed pollen tube growth. A mutation in *MIRO1* (Yamaoka and Leaver, 2008) impairs pollen germination and tube growth. These studies illuminate the mechanisms underlying pollen tube germination; however, the mechanisms underlying pollen dormancy, which determine how and why pollen grains do not germinate until they come into contact with a compatible stigma, remain unclear. Previous studies demonstrate that expression of the whole transcriptome in pollen is increased dramatically during pollen germination and tube growth (Wang et al., 2008). This alteration implies that mature pollen grains are in a relatively metabolically quiescent state and suggests that transcription of genes for pollen germination and tube growth is significantly inhibited until the pollen comes into contact with the stigma. We believe that the mechanism of this inhibition and its reversal must involve a number of genes that may not be indispensable for pollen development and germination.

Previous studies focused on a few inhibitor-like genes and the mechanisms through which they control pollen germination. An

Arabidopsis mutant called *raring-to-go* (*rtg*) exhibits precocious pollen germination inside the anther as early as the bicellular stage (Johnson and McCormick, 2001); however, this mutant has not been subjected to gene mapping. Another mutant with a similar precocious pollen germination phenotype is the T-DNA insertion line *callose synthase9* (*cs9*), in which the loss-of-function pollen also shows a changed male germ unit and aberrant callose deposition in the bicellular stage (Xie et al., 2010). These mutants both show precocious pollen germination, indicating that the encoded genes are likely negative regulators of germination. However, considering that the pollen grains of both mutants germinate before reaching maturation, the genes altered in these mutants are likely to function as constructive elements for pollen development rather than regulatory factors in pollen germination. With regard to genes that inhibit pollen germination, the *5pt12* and *5pt13* mutants were found to produce functional mature pollen grains with precocious germination. Increased levels of Ins(1,4,5)P_3/Ca^{2+} in these mutants were found to end pollen dormancy and trigger early germination (Wang et al., 2012); however, this report did not assess whether pollen germination was inhibited by overexpressing *5PT12* and *5PT13* in pollen.

In this report, we identified *JGB* as a novel negative regulator of pollen germination. Based on assessments of Col-0, *jgb*, and *JGB OX* pollen germination (Figures 3C, 3D, and 3F to 3H), we found that a strong and negative association exists between the germination rate of a cell and the amount of JGB contained in the cell. *jgb* pollen germinates much rapidly than Col-0 does in vitro on standard culture medium (Figures 3C and 3D), as well as in sucrose-only medium (Figure 2A) and EGTA-containing medium (Supplemental Figure 7), while germination of *JGB OX* and *jgb RES* pollen was inhibited in vitro and in vivo (Figures 3D, 3H, and 6D; Supplemental Figure 4). Thus, JGB is a novel negative regulator of pollen germination. It is notable that the in vivo germination rates of Col-0 and *jgb* pollen were indistinguishable (Figure 3H), probably because the pollen may receive stimulation from the stigma, overcoming the inhibitory effect of JGB, as indicated in some other reports (Ge et al., 2009; Qin et al., 2011).

JGB Is Specifically Conserved in Flowering Plants

To assess the mechanism through which JGB regulates pollen germination and tube growth, we performed bioinformatic analysis using the deduced amino acid sequence of JGB. However, we failed to find any protein homolog with a molecular mechanism studied in plants, confirming that JGB is a novel regulatory factor of pollen-related processes. However, BLAST analysis of the JGB sequence identified JGB homologs in 28 flowering plants (including dicotyledons and monocotyledons) for which genomic information was available, with one copy of the encoded gene per plant genome in most cases (Supplemental Table 1 and Supplemental Figure 8). We found the highest identity among Brassicaceae (96.7 $\pm$ 2.0, n = 4), moderate identity within other dicotyledon families (71.4 $\pm$ 4.5, n = 17), and relatively low identity in Poaceae (59.8 $\pm$ 1.4, n = 6), a representative monocotyledon family (Supplemental Table 1). It is notable that the identity of the matches was rather low in Gymnospermae (30.0 $\pm$ 0.0, n = 2) and Cryptogamae (21.2 $\pm$ 1.8, n = 4), indicating that *JGB* is specifically conserved in flowering plants.

Using the bioinformatic tool Phyre^2 for protein modeling (Kelley et al., 2015), we acquired a possible 3D structure for Arabidopsis JGB as a typical seven-bladed β-propeller structures (100% confidence) (Supplemental Figure 9). It is interesting that, except for the cases of failure in modeling owing to the lack of amino acid templates for eight of the flowering plant species, we identified seven-bladed β-propeller structures (100% confidence) for the deduced JGB molecules of the other twenty species that were assessed (Supplemental Figure 9 and Supplemental Data Set 2). These results imply that JGB homologs in flowering plants have a highly conserved amino acid sequence and similar 3D structure. In addition, by cloning the promoter and encoding sequences of *JGB* from rice (*Oryza sativa*; $OsJGB_{pro}$ and *OsJGB*), constructing a transgenic vector harboring $OsJGB_{pro}$:*OsJGB-GFP*, and successful transformation, we obtained transgenic *JGB* overexpressing rice plants, in which JGB was localized to the vegetative nucleus and cytoplasm, as in Arabidopsis (Supplemental Figure 9). These results imply that JGB molecules in flowering plants retain similar functions in controlling pollen germination and stabilizing tube growth. However, the pollen grains of transgenic *JGB* overexpressing plants showed no significant inhibition of germination for unknown reasons. This is possibly because multiple members of the JGB family exist with redundant functions (the rice genome possesses three JGB homologs).

JGB Probably Functions as a Scaffold for Protein-Protein Interaction

The WD40 repeat is a short structural motif of ~40 amino acids that often terminates in a tryptophan-aspartic acid (W-D) dipeptide (Neer et al., 1994). Tandem copies of WD40 repeats typically fold together to form a propeller-like structure called the WD40 domain (Stirnimann et al., 2010). The WD40 domain is one of the most abundant domains and top interacting domains in eukaryotic genomes. The WD40 domain and its propeller-like structure serve as a relatively rigid platform or scaffold for protein-protein interaction and thus regulate downstream events such as histone methylation and ubiquitination (Stirnimann et al., 2010; Xu and Min, 2011).

The structure, function, and mechanism of WD40 proteins in regulating biological processes are well studied in animals. WDR5 has seven WD40 repeats and provides a structural platform in humans for absent, small, or homeotic-like 2 (Ash2), retinoblastoma binding protein 5 (RbBP5), and a histone H3 lysine 4 (H3K4) methyltransferase, which together form the COMPASS-like complex, which catalyzes H3K4 methylation (Couture et al., 2006; Dou et al., 2006; Han et al., 2006; Ruthenburg et al., 2006; Schuetz et al., 2006). The same functional homolog complexes exist in yeast, in which SWD3, a homolog of WDR5, plays a platform role in catalyzing H3K4 methylation (Miller et al., 2001; Schneider et al., 2005; Jiang et al., 2011). In fact, WD40 proteins act as scaffolds for protein-protein interaction in many cases; WDR36, with 14 WD repeats grouped into two tandem seven-bladed β-propeller regions, acts as a scaffold protein that tethers Gαq G-proteins and PLCβ in a signaling complex (Cartier et al., 2011).

In contrast to WD40 proteins in animals, the functions and mechanisms of WD40 proteins are less understood in plants. The Arabidopsis genome is estimated to encode 269 WD40 proteins that contain at least one copy of the WD motif (van Nocker and

Ludwig, 2003). It is interesting that an Arabidopsis homolog of human WDR5, having seven WD40 repeats and termed WDR5a, functions as a structural platform to catalyze H3K4 methylation (Jiang et al., 2011), implying that the structure, function, and mechanism of WD40 proteins are highly conserved in plants and animals. Given the structural similarity to WDR5 and SWD3, we speculate that JGB, containing seven WD40 repeats and likely having a typical seven-bladed β-propeller structure, serves as a scaffold for protein-protein interaction.

JGB Prevents Pollination in Moist Environments

Plants lacking *JGB* (*jgb* plants) exhibit hyperactive pollen germination when periodically sprayed with water; therefore, the biological significance of *JGB* could be presumed to be keeping pollen in a viable and ungerminated state for as long as possible in moist environments so that they can germinate when spread on a suitable pollination target. This function of *JGB* likely increases the likelihood of successful sexual reproduction and thus perpetuation of the population.

A humid chamber containing a Petri dish with wet filter paper (Supplemental Figure 10) was used to simulate a moist environment to assess whether JGB increased Arabidopsis fertility. When treated within the wet chamber, *jgb* pollen began to germinate 3 h after treatment, after which numerous pollen tubes grew from the anther after 6 to 12 h of incubation in the chamber (Supplemental Figure 10). We then used excessive amounts of the treated pollen to manually pollinate the flowers of Col-0 plants to assess whether the moist pollen treatment influenced the rate of seeding. Flowers pollinated with the treated *jgb* pollen gave rise to remarkably thin and short siliques with marked vacancies (Supplemental Figure 10); those pollinated with 12-h-treated *jgb* pollen produced on average 13.1 ± 8.4 (mean ± SD) seeds, which was significantly fewer than the number of seeds produced by flowers pollinated with 12-h-treated Col-0 pollen (Supplemental Figure 10). These results indicate that JGB improves the ability of pollen to spread and pollinate (at least 12 h for Arabidopsis in a moist environment), which may confer a significant reproductive advantage.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as a wild-type plant material and for transgenic screening. Seeds were surface-sterilized with 70% ethanol for 1 min, exposed to 5% (v/v) sodium hypochlorite containing 0.5% (v/v) Tween-20 for 1 min, rinsed four times with sterile water, and germinated on half-strength Murashige and Skoog medium (Sigma-Aldrich) plates with or without antibiotics. For transgenic screening, the plates were supplemented with kanamycin or hygromycin to a final concentration of 50 mg/L. Seeds were vernalized in darkness at 4°C for 2 d before being transferred to a greenhouse kept at 22°C with 70% humidity and a 16-h-light/8-h-dark cycle (100 μmol m^{-2} s^{-1}).

Constructs and Plant Transformation

Construction of the JGB Promoter-GUS/GFP Reporter

A 2350-kb fragment upstream of the ATG start codon was amplified by PCR with JGB-pro-S/JGB-pro-A primers (all primers are listed in Supplemental Table 2) from Col-0 genomic DNA and ligated into pEASY-Blunt Simple (TransGen Biotech) for sequencing. The verified fragment was subcloned into pBI121, generating pBI121-JGB_{pro}-GUS. The 1.8-kb *GUS* gene was replaced by *GFP* with *Spe*I and *Nru*I, generating pBI121-JGB_{pro}-GFP. Plants were stably transformed with *Agrobacterium tumefaciens* strain GV3101 via the floral dipping method as previously described (Clough and Bent, 1998).

Construction of the JGB Genomic Clone for JGB Overexpression and Genetic Complementation

The genomic fragment of *JGB* was amplified from Col-0 genomic DNA with KOD -Plus- (Toyobo) and primers JGB-S and JGB-A. The obtained fragment was sequenced and subcloned into pBI121-JGB_{pro}-GFP using *Sma*I and *Spe*I to yield pBI121-JGB_{pro}-JGB-GFP. Transgenic Col-0 plants harboring the plasmid were designated *JGB OX*. Subsequently, the JGB_{pro}-JGB-GFP fragment was cleaved by *Hind*III and *Nru*I and inserted into pWM101 to generate pWM101-JGB_{pro}-JGB-GFP, which was stably transformed into *jgb* and yielded *jgb RES* for complementation.

Construction Used for JGB Site-Directed Mutagenesis

PCR-based site-directed mutagenesis was performed with primers JGB^{AEV}-S/JGB^{AEV}-A according to the instruction manual included with the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). The altered *JGB* fragment (LEV to AEV) was cloned into pBI121-JGB_{pro}-JGB-GFP using *Sma*I and *Spe*I to generate pBI121-JGB_{pro}-JGB^{AEV}-GFP, which was stably transformed into *jgb* and yielded *jgb* RES^{AEV}.

Construction of the $OsJGB_{pro}$-OsJGB-GFP for OsJGB Overexpression and Subcellular Localization

The promoter and CDS of *OsJGB* were amplified from rice (*Oryza sativa*) genomic DNA and cDNA with KOD -Plus- (Toyobo) and primers OsJGB-pro-S/OsJGB-pro-A and OsJGB-S/OsJGB-A. The obtained two fragments were sequenced and subcloned into pBI121-JGB_{pro}-GFP using *Hind*III and *Xba*I and *Spe*I, respectively, to yield pBI121-$OsJGB_{pro}$-OsJGB-GFP. Transgenic rice plants harboring the plasmid were designated *OsJGB OX*.

Spatiotemporal Expression Analysis

The spatial expression pattern of *JGB* was determined by RT-PCR. Total RNA was extracted from different tissues (root, stem, leaf, and newly opened flower) from Col-0 plants using TRIzol reagent (Invitrogen). Then, 1 μg of total RNA was reverse-transcribed with the PrimeScript first-strand cDNA synthesis kit (TaKaRa) according to the manufacturer's instructions. An 834-bp fragment of *JGB* was amplified with primer pair RT-JGB-S/RT-JGB-A to confirm the expression level of *JGB*. The primers spanned the T-DNA insertion site in the gene. *GAPDH* was used as an internal control. PCR products were checked by 1% agarose gel electrophoresis.

Histochemical staining for GUS activity was performed using the standard 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) solution (100 mM sodium phosphate buffer, pH 7.0, 0.1% Triton X-100, 1 mg/mL X-Gluc, 1 mM potassium ferricyanide, and 1 mM potassium ferrocyanide). The samples (JGB_{pro}-*GUS*) were vacuum-infiltrated in X-Gluc solution for 10 min, treated with fixative (ethanol:acetic acid = 9:1) for 4 h at room temperature, and cleared in 95 and 70% ethanol. Images of the stained tissue samples were taken using a Leica dissecting microscope (Leica EZ4 HD) equipped with Leica Application Suite EZ.

Quantitative Real-Time PCR

Total RNA was extracted from mature pollen from Col-0, *jgb*, and *JGB OX* plants using TRIzol reagent (Invitrogen), after which 1 μg of total RNA was reverse-transcribed with the PrimeScript first-strand cDNA synthesis kit (TaKaRa) according to the manufacturer's instructions. Real-time qPCR was performed on a LightCycler (Roche) using SYBR *Premix Ex Taq* (TaKaRa). The PCR conditions were 95°C for 15 s, followed by 45 cycles of 95°C for 5 s, 55°C for 20 s, and 72°C for 30 s. Three technical replicates were performed with each cDNA sample. The results were normalized using *ACTIN11*. The primers used for real-time PCR are listed in Supplemental Table 2 online.

Characterization of the T-DNA Insertion Mutant

A T-DNA insertion mutant (*jgb*, SALK_018094C) was obtained from the ABRC. A homozygous line was identified by classical PCR-based genotyping using primers jgb-LP, jgb-RP, and LBa1 (the latter was near the right border of the T-DNA insertion). In addition, immunoblotting was performed to confirm knockout of *JGB* in homozygous *jgb* pollen. Anti-JGB serum was made using HIS-tagged JGB as an antigen to immunize rabbits. Anti-JGB polyclonal antibodies were obtained via antigen-antibody affinity purification. Using IRDye 800CW donkey anti-rabbit IgG (H+L) (Odyssey; 92632213) as the secondary antibody (1:5000), hybridization to protein bands was detected using the Odyssey scanning system (Li-Cor Biosciences) according to the manufacturer's instructions.

Phenotypic Analyses of Pollen and Pollen Tubes

The development and viability of mature pollen from Col-0 and *jgb* plants were investigated by Hoechst 33342 and Alexander staining for 5 min using an inverted microscope (Leica DMI6000 B) equipped with an epifluorescence UV light filter set. Digital images were collected with a CCD camera (Leica DFC420) using the Leica Application Suite (V4.2).

For in vitro pollen germination, mature pollen was isolated from newly opened flowers and placed onto pollen germination medium, which consisted of 0.01% (w/v) H_3BO_3, 5 mM KCl, 5 mM $CaCl_2$, 1 mM $MgSO_4$, and 22% (w/v) sucrose solidified with 1.0% (w/v) low gelling temperature agarose (pH 7.5) (Boavida and McCormick, 2007). To determine the effects of MeJA (Sigma-Aldrich) on pollen germination, various concentrations of MeJA were included in the germination medium. The plates were sealed by Parafilm to maintain moisture and pollen grains were incubated at 22°C for germination. To calculate the germination percentage of each sample, at least 1000 pollen grains were counted in each experiment. To determine pollen tube length in vitro, pollen tubes were measured using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij).

For in vivo pollen germination, 40 to 60 pollen grains from newly opened flowers were dabbed onto the surfaces of pre-emasculation Col-0 stigmas. After 10, 20, and 40 min, stigmas with pollen were stained with Alexander's stain and examined under an inverted microscope to allow germinated pollen to be counted. To view the elongation efficiency of pollen tubes inside the transmitting tract, pre-emasculated Col-0 flowers were pollinated with Col-0 and *jgb* pollen. After 2, 4, 6, and 8 h, pistils were collected and fixed in ethanol:acetic acid (3:1) for 1 h at room temperature. The fixed pistils were hydrated with an ethanol series (70, 50, and 30%), softened in 8 M NaOH overnight, washed several times with distilled water, and stained in 0.1% (w/v) aniline blue solution. Fluorescent images were obtained with an inverted microscope under UV light.

Yeast Two-Hybrid Assay

The *JGB* CDS was amplified from pollen cDNA with primers Bait-JGB-S/Bait-JGB-A and fused to the GAL4 DNA binding domain in bait vector pDEST32, which was a kind gift from Li-Jia Qu (Peking University). The bait construct was tested for autoactivation as described by the manufacturer (Clontech) and then transformed into yeast strain AH109 by electroporation. One transformant was designated as AH109-pDEST32-JGB and used for the mating to the Arabidopsis transcription factor cDNA library. The library was composed of Y187-pDEST22 with the known transcription factors. Four clones were obtained 1 week after transformation on SD/-2 and SD/-3 medium at 30°C. Yeast plasmid DNA was isolated from yeast cells using the TIANprep Yeast Plasmid DNA Kit (Tiangen) and sequenced with the pDEST22-S primer. The candidates were TCP4, TCP8, TCP20, and TCP22. To verify the interactions between JGB and TCP4/8/20/22, the CDSs of *TCP4*, *TCP8*, *TCP20*, and *TCP22* were amplified from pollen cDNA with primers Prey-TCP4/8/20/22-S/Prey-TCP4/8/20/22-A, cloned into Gateway-compatible pDEST22 (AD), and cotransformed with pDEST32-JGB into yeast strain AH109. Positive clones were selected and cultivated on SD/-3 medium. Plasmids without JGB or TCPs were used as negative controls. All primers are listed in Supplemental Table 2 online.

Determination of JA

Fresh mature pollen of Col-0, *jgb*, and *JGB OX* plants was collected from newly opened flowers by shaking the whole flower in isopropanol. Next, the pollen grains were centrifuged and dried. Extraction and quantification of JA were performed as described previously (Glauser and Wolfender, 2013).

RNA-Seq Experiment

RNA-seq was performed on the HiSeq Illumina Hi-Seq 2000 platform in the Biodynamic Optical Imaging Center (BIOPIC) of Peking University. The raw data were cleaned by Cutadapt and mapped to the reference genome using TopHat, after which the FPKM of each gene was calculated and DEGs between *jgb* and Col-0 were detected using Cufflinks.

Bioinformatics and Phylogenetic Analysis

TAIR (http://www.arabidopsis.org) and Phytozome (https://phytozome.jgi.doe.gov) were used to analyze the genomic, cDNA, and protein sequences of JGB homologs. The alignment used for phylogeny was produced with ClustalW program at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/msa/) using default values. The alignment has been made available in FASTA format (Supplemental Data Set 2). The phylogeny of the aligned sequences was generated with the neighbor-joining algorithm (Saitou and Nei, 1987) using MEGA5 software (Tamura et al., 2011), and bootstrap analysis was conducted with 1000 replications.

In Vitro Pull-Down Assay

The coding sequence of *JGB* was cloned into pGEX-4T-1 to generate a fusion construct with GST. The coding sequence of TCP4 was cloned into pET-28a to generate a fusion construct with a His tag. The fusion constructs were transformed into *Escherichia coli* strain BL21. Transformants were grown to a concentration of OD_{600} = 0.6 in a shaker at 37°C and induced to express the fusion protein by incubation in LB broth supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside for 6 h at 37°C. The cells expressing GST-JGB were lysed in PBS, after which the supernatant was incubated with 80 μM Glutathione-Sepharose 4B (GE Healthcare) for 2 h, followed by extensive washing with PBS. The supernatant from cells expressing TCP4-His was incubated with the above sepharose at 4°C for 3 h, washed with PBS three times, boiled in SDS loading buffer for 10 min, subjected to SDS-PAGE, and immunoblotted with anti-GST (B-14) (Santa Cruz; sc-138, lot # I1009, 1:1000) and anti-His (MBL; D291-3, lot. 006, 1:5000) antibodies.

Accession Numbers

The Arabidopsis Genome Initiative accession numbers for the genes and gene products mentioned in this article are as follows: *JGB* (At2g26490),

LOX2 (AT3G45140), *AOC4* (AT2G35690), *OPCL1* (AT2G35690), *ACX1* (AT4G16760), *ACX5* (AT2G35690), *AIM1* (AT2G35690), *KAT2* (AT2G33150), *JMT* (AT1G19640), and *TCP4* (AT3G15030).

Supplemental Data

Supplemental Figure 1. *JGB* Is Specifically Expressed in Pollen Grains and Tubes.

Supplemental Figure 2. *jgb* Plants Are Normal under Standard Growth Conditions.

Supplemental Figure 3. Dehydration and Rehydration of *jgb* Pollen Are Normal Compared with Col-0.

Supplemental Figure 4. *jgb RES* Rescued the *jgb* Phenotype.

Supplemental Figure 5. *jgb* RES^{AEV} Rescued the *jgb* Phenotype.

Supplemental Figure 6. GO Analysis of Col-0 and *jgb* Mature Pollen Transcriptomes Showed that JGB Is Related to JA Biosynthesis.

Supplemental Figure 7. Hyperactive Germination of *jgb* Pollen on EGTA-Containing Medium.

Supplemental Figure 8. Alignment of JGB Proteins in 28 Species of Flowering Plants.

Supplemental Figure 9. JGB Is Highly Conserved in Flowering Plants.

Supplemental Figure 10. *jgb* Flowers Lose Fertility in a Humid Environment.

Supplemental Table 1. JGB Is Specifically Conserved in Flowering Plants.

Supplemental Table 2. Primers Used in This Article.

Supplemental Data Set 1. GO Term Enrichment for the RNA-Seq Results.

Supplemental Data Set 2. Sequences Used for Alignment in Supplemental Figure 8 and the Phylogeny Analysis Presented in Supplemental Figure 9B.

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AUTHOR CONTRIBUTIONS

Y.J. and L.G. performed the experiments, analyzed the data, and prepared this article. Q.C., F.M., and Q.-Y.Z. performed some experiments. Q.Z. conducted the experiments. S. conceived the project and revised the article.

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