Competitive Ability of Maize Pollen Grains Requires Paralogous Serine Threonine Protein Kinases STK1 and STK2

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ABSTRACT *serine threonine kinase1 (stk1)* and *serine threonine kinase2 (stk2)* are closely related maize paralogous genes predicted to encode serine/threonine protein kinases. Pollen mutated in *stk1* or *stk2* competes poorly with normal pollen, pointing to a defect in pollen tube germination or growth. Both genes are expressed in pollen, but not in most other tissues. In germination media, STK1 and STK2 fluorescent fusion proteins localize to the plasma membrane of the vegetative cell. RNA-seq experiments identified 534 differentially expressed genes in *stk1* mutant pollen relative to wild type. Gene ontology (GO) molecular functional analysis uncovered several differentially expressed genes with putative ribosome initiation and elongation functions, suggesting that *stk1* might affect ribosome function. Of the two paralogs, *stk1* may play a more important role in pollen development than *stk2*, as *stk2* mutations have a smaller pollen transmission effect. However, *stk2* does act as an enhancer of *stk1* because the double mutant combination is only infrequently pollen-transmitted in double heterozygotes. We conclude that the *stk* paralogs play an essential role in pollen development.

KEYWORDS *Zea mays*; microgametophyte; reduced pollen transmission; protein serine threonine kinases; *bronze*

THE relative paternal transmission of the wild-type and mutant alleles of a gene in heterozygous plants is an exquisitely sensitive assay for the function of that gene in pollen development. This is particularly so in maize, a plant with strikingly long polystigmatic styles (“silks”) capable of receiving multiple pollen grains. Maize styles may reach a length of 30 cm, the longest known in the plant kingdom. Strong competition occurs when multiple pollen grains grow down the same style toward the embryo sac. Maize pollen tubes grow in the style at a rate of 1 cm/hr (Bedinger 1992), and any mutation adversely affecting this growth will show a

reduced pollen transmission (RPT) phenotype in a heterozygote because the mutant pollen tube will grow slower than the wild type, hence will be less likely to achieve fertilization and will be recovered at a reduced frequency (Nelson 1994).

Mutations in genes affecting development of the pollen can be conveniently identified by the aberrant segregation of markers linked to it (Arthur *et al.* 2003) and in cases where the mutations are caused by transposons that confer a phenotype, by the aberrant segregation of the causative transposon itself (Lalanne *et al.* 2004b; Boavida *et al.* 2009). In an extreme example of linkage in maize, a line was found to transmit an *Ac* (*Activator*) transposon normally through the female parent, but only rarely through the male. In that line, *Ac* is inserted in the *apt1* (*aberrant pollen transmission 1*) gene, causing mutant pollen tubes to grow short and twisted. The *apt1* gene is expressed only in pollen and the protein is targeted to the Golgi body, suggesting a role in vesicular trafficking during pollen tube elongation (Xu and Dooner 2006).

It has long been known that a gene or genes closely linked to *bz* (*bronze*) also affects pollen development in maize because several deficiencies that include *bz* do not show

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Mendelian male transmission (Mottinger 1970). A series of deletion mutants involving *bz* and multiple adjacent genes have been analyzed in our laboratory, and interestingly, all of those that show reduced male transmission have a deletion extending into the adjacent *stk1* (*serine-threonine kinase 1*) gene (Huang and Dooner 2012). *stk1* is the proximalmost gene in the gene-rich *Bz-McC* haplotype on chromosome 9S (Fu *et al.* 2001). Unlike *apt1*, the deletion mutations reduce, but do not abolish, pollen transmission. We have now isolated four *Ac* insertions in *stk1* and confirmed that a mutated *stk1* is the cause of the RPT phenotype.

A possible reason that the *stk1* mutations and deletions do not abolish male transmission is that another gene performing a redundant function is present in the genome. In this study, we have found that a paralogous gene, *stk2*, is also required for normal pollen transmission, though *stk2* mutations have a smaller pollen transmission effect than *stk1* mutations. The *stk1 stk2* double mutant combination is very poorly transmitted in double heterozygotes, confirming that the two genes play a redundant role in pollen development. STK1 and STK2 fluorescent fusion proteins localize on the pollen's vegetative cell membrane, and likely only do so upon hydration. An RNA-seq experiment identified 534 genes that are either underexpressed or overexpressed in *stk1* mutant pollen relative to wild type, indicating that *stk1* mutations can have both positive and negative effects on the expression of other genes in the maize genome. Particularly prominent among them were genes involved in translation, including many encoding ribosomal proteins.

Materials and Methods

Genetic stocks

The *stk1* and *stk2* mutations are described in detail in the *Results and Discussion*. The *stk1* mutations arose in a W22 inbred background and the *stk2* mutations were introgressed into the same background. Transformation of the hybrid line Hi-II (Armstrong *et al.* 1991) with a binary vector was performed according to the method of Frame *et al.* (2002). The Ti-plasmid pTF101.1 was kindly provided by the Plant Transformation Facility (Iowa State University, Ames, IA).

Nucleic acid extraction, blotting, and hybridization

Leaf DNA for Southern hybridization was isolated by a urea extraction procedure (Greene *et al.* 1994). A modified CTAB extraction method (Huang and Dooner 2008) was used for the large number of DNA preparations for PCR and sequencing analysis. Restriction digested DNA (10 μ g) was resolved on 0.8% agarose gels and transferred to Hybond XL nylon membranes (Amersham Biosciences, Piscataway, NJ). Total RNA was extracted using TRIzol reagent according to the manufacturer (Life Technologies, Rockville, MD). Separation of total RNA in agarose gels and blotting onto Hybond XL membranes were performed according to the manufacturer's instructions (Amersham Biosciences). 32 P-labeled probes were generated with Ready-To-Go DNA labeling beads

(Amersham Biosciences). The *stk1* probe was amplified from a complementary DNA (cDNA) clone using primers in 3' UTR (AACAGGTACACGGCAATGGCAGAG) and exon 4 (ACAGCAACATCTCACGGTCACACG), and the *stk2* probe was amplified from a cDNA clone using primers in exon 5 (AGACGGGCATGCTGGGGGTGAAGT) and 3' UTR (ATGGC GCGACGGGCTGGTGT).

PCR and sequencing

PCR was performed according to the protocol of QiaTaq (QIAGEN, Valencia, CA). Long-strand DNA fragment amplification was performed according to the protocol of Roche Expand Long Range (Hoffmann-La Roche, Nutley, NJ). PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI) and transformed into XL-Blue competent cells. Plasmids were purified with a QIAGEN spin miniprep kit. DNA sequencing of plasmids or PCR products was carried out in an ABI 3730 sequencer (Perkin-Elmer, Torrance, CA) following the manufacturer's instructions.

Primer sequences were as follows:

stk1-1: CTGGCCATGGCACAACACTGAGAT
stk1-2: GCGGGTCCGTCGGCTGCTTGAA
stk2-P: TGGATTGGAGTGAGAGTGACA
stk2-2: AGCCGCTTCATCTCCGCCTCCATC
 Mu53: GCCTCYATTTCGTCCAATCC

In vitro maize pollen germination

Full, freshly dehiscent anthers were collected from *stk1* homozygous and wild-type plants. The 2X pollen germination medium is made up of 20% sucrose, 0.001% H_3BO_3 , 20 mM $CaCl_2$, 0.1 mM KH_2PO_4 , and 12% PEG 4000 (Schreiber and Dresselhaus 2003). Pollen was gently squeezed from the anther and evenly dusted on the surface of a plate with germination medium. Plates were incubated in the dark at 25°. Pollen tubes were photographed at serial time points of 15 min, 30 min, and 1 hr on a microscope using bright field illumination.

Pollen RNA-seq methods

Pollen from three different field-grown mutant (K3063: *bz-s39.8; stk2-2*) and control (K3064: *Stk1-McC Bz-McC/stk1 bz-s39.8; stk2-2*) sib plants was collected at noon after ~1 hr of shedding, immediately frozen in liquid nitrogen, and stored at -80° until processing. Ten micrograms of total RNA was extracted for each sample using TRIzol reagent (Invitrogen, Carlsbad, CA). mRNA was purified by MicroPoly(A)Purist Kit (Ambion) and checked with Bioanalyzer Pico RNA chip for rRNA <2.0% (Agilent Technologies).

One hundred fifty nanograms of mRNA was fragmented, converted to cDNA with dUTP, and A-tailed according to the protocol of New England Biolabs Next Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA). Libraries were made SOLiD-compatible by ligating P1-T and P2-T barcode adaptors and amplifying with SOLiD DNA library PCR Primers 1 and 2 (Life Technologies). Six samples

were barcoded, pooled, and evenly distributed for Emulsion PCR, and then loaded into two lanes to reduce potential experimental batch effect. Seventy-five base pair single-end sequence reads were generated using our in-house SOLiD 5500XL platform (Life Technologies) with an Exact Call Chemistry module, an optional kit that is used to further enhance sequencing accuracy and makes it possible to convert the color space data to nucleotide sequences prior to the mapping.

Adaptors were removed using the software cutadapt (Martin 2011). The minimum overlap length was set to 10 and error rate was set to 0.05. After removing the adaptor, low-quality bases were trimmed with an average score of 15 for five consecutive bases from the 3'-end.

The processed reads were mapped to the reference genome *Zea mays* B73 v3 with BWA version 0.7.5-r404 (Li and Durbin 2009). Aligned reads in genes were counted with HTSeq framework, version 0.5.3p9. Mode "union" and mapping quality cutoff 20 were used for our analysis. Count-table was normalized so that all samples have a same level of total mapped reads. EdgeR (Robinson *et al.* 2010) and DESeq (Anders and Huber 2010) are two popular software tools in RNA-seq data analysis. Although they are similar in differential analysis, they are different in dispersion estimation. DESeq is more conservative (Robles *et al.* 2012) and EdgeR is more sensitive to outliers (Anders *et al.* 2013), so we decided to use both EdgeR and DESeq for our analysis. We used *q*-value 0.05 and fold change 2 as cutoffs to identify differentially expressed genes (DEGs).

Each DEG was annotated with functional annotations from Phytozome (<http://www.phytozome.net/>). Multiple resources, including KEGG, best *Arabidopsis* TAIR10 hits, and best rice hits, were combined in the functional annotations of *Z. mays* B73. Gene ontology (GO) enrichment analysis was done in the agriGO Singular Enrichment Analysis tool (Du *et al.* 2010; <http://bioinfo.cau.edu.cn/agriGO/analysis.php>), with customized annotation and customized annotated reference. The Fisher test was applied to calculate *P*-values and the Bonferoni multi-test adjustment method to calculate adjusted *q*-values. The significance level was set at 0.05. Generic GO slim was used to generate GO maps; MEME (<http://meme-suite.org/tools/meme>) to find significant motifs (Bailey and Elkan 1994), and KEGG (<http://www.kegg.jp/kegg/>) for pathway enrichment analysis (Kanehisa *et al.* 2016).

Data availability

All genetic stocks and sequence data are available upon request. The sequence of the *stk1-McC* allele has been deposited in GenBank (GenBank AAK73111.2). RNA-Seq data have been submitted to the National Center for Biotechnology Information (NCBI) as biosample submission SUB2927631.

Results and Discussion

stk1 mutations affect pollen transmission

stk1, the proximalmost gene of the *Bz-McC* haplotype gene-rich region (Fu *et al.* 2001), measures 3110 bp and contains a

Figure 1 Ac-induced *stk1* mutations show RPT. (A) *stk1* mutations induced by the transposon Ac. The blue triangles represent Ac insertions and the yellow arrows indicate their 5' to 3' orientation. The pentagon represents the *stk1* gene pointing in the direction of transcription, with the yellow rectangles standing for introns and orange ones for exons. *m1*, *m2*, and *m3* are unstable mutations and revert to wild type by Ac excision; *s4* is a stable mutation caused by a deletion (demarcated by brackets) that extended from a nearby Ac in *bz* to the *stk1* second exon, so it cannot revert to wild type by Ac excision. (B) Kernel progeny from an ear of a *bz* tester pollinated with pollen from a *Stk1/stk1-s4* heterozygote. *stk1-s4* is also a *bz* mutation as the deletion removes parts of both adjacent genes, so *bz* serves as a visual marker for segregation analysis. The ratio is 218 purple (Bz) to 110 bronze (bz) kernels or nearly 2:1, corresponding to a 33% RPT of the mutation.

189-bp 5' UTR, three introns, and a 292-bp 3' UTR (GenBank AAK73111.2). Its predicted 787-amino-acid protein has high similarity to receptor-like serine/threonine protein kinases, hence its name. Previous studies had suggested that *stk1* may be the gene closely linked to *bz* that affects pollen transmission because all of the *bronze* deletion lines that show RPT have deletions extending into *stk1* (Huang and Dooner 2012). To confirm that *stk1* is responsible for the RPT phenotype, we set out to isolate *stk1* mutations by mobilizing Ac into it from the *bz* gene located <1 kb away. The screen for such candidate mutations was based on the expectation that *stk1* mutants would also show RPT. Pollen transmission of the transposed Ac (*trAc*) element in 14 lines carrying *trAc*s closely linked to *bz* (<1 cM) was tested and four RPT candidates were found.

DNA of the four candidates was characterized by Southern blots, PCR, and sequencing. *stk1-m1* and *stk1-m2* have Ac insertions at different locations of the third exon, *stk1-m3* has an Ac insertion in the first exon, and *stk1-s4* has a 1.8-kb Ac adjacent deletion that includes the divergent *bz* and *stk1* promoters and extends into the second exon of *stk1* (Figure 1A). These mutations originated as follows: *stk1-m1* and *stk1-m3* are, respectively, *trAc1343* (H.K. Dooner,

Table 1 Pollen transmission data for different *stk1* mutant alleles

Female parent	Male parent	Location	Sh seed	sh seed	N	RPT ^a
<i>sh1 Stk1</i>	<u><i>Sh1 stk1-m1</i></u> <i>sh1 Stk1</i>	Piscataway, NJ ^b	89	143	232	23.3
			86	197	283	39.2
			107	178	285	24.9
			48	109	157	38.9
			81	140	221	26.7
			99	230	329	39.8
<i>sh1 Stk1</i>	<u><i>sh1 stk1-m2</i></u> <i>Sh1 Stk1</i>	Piscataway, NJ ^a	510	997	1507	32.3
			170	101	271	25.5
			95	64	159	19.5
			129	61	190	35.8
			99	66	165	20.0
			175	83	258	35.7
<i>sh1 Stk1</i>	<u><i>Sh1 stk1-m3</i></u> <i>sh1 Stk1</i>	Piscataway, NJ ^a	668	375	1043	28.1
			105	190	295	28.8
			95	157	252	24.6
			120	189	309	22.3
			28	58	86	34.9
			25	52	77	35.1
<i>sh Stk1</i>	<u><i>sh1 stk1-s4</i></u> <i>Sh1 Stk1</i>	Piscataway, NJ ^a	108	152	260	16.9
			481	798	1279	24.8
			218	110	328	32.9
			131	57	188	39.4
			173	59	232	49.1
			522	226	748	39.6
<i>sh1 Stk1</i>	<u><i>Sh1 stk1-m1</i></u> <i>sh1 Stk1</i>	N. Vallarta, MX ^c	116	126	242	4.1
			101	145	246	17.9
			96	145	241	20.3
			50	79	129	22.5
			88	102	190	7.4
			68	83	151	9.9
			54	60	114	5.3
			573	740	1313	12.7

^a Pollen transmission of *stk1* mutant based on recovery of its linked *sh1* allele (a).
RPT = $(1 - 2a/N) \times 100$.

^b July average high temperature: 30°.

^c February average high temperature: 26°.

unpublished data) and *trAc6114* (Dooner and Belachew 1989) from *bz-m2(Ac)*; *stk1-m2* is *trAc2151* from *bz-m39(Ac)* (Dooner, unpublished data), and *stk1-s4* corresponds to *bz-s39.8(Ac)*, an adjacent deletion derivative from *bz-m39(Ac)* (Dooner and He 2014).

The three *Ac* insertion alleles showed pollen transmission deficiencies ranging from 25 to 32%, while the deletion allele *stk1-s4* showed ~40% RPT (Figure 1B and Table 1). This difference may be attributable to *Ac* excisions that restore gene function in the three insertion alleles, whereas the deletion mutant carries a nonrevertible *stk1* allele. The transmission ratio of wild-type and mutant *stk1* alleles can vary depending on the environment in which the plants are grown, as high temperatures during pollination ($\geq 30^\circ$) appear to enhance the RPT effect (Table 1). This correlation suggests that the mutant pollen is more heat sensitive than the wild-type pollen. Furthermore, the *stk1-s4* showed the same degree of RPT as previously analyzed multi-gene deficiencies, such as *sh-bz-X2* and *sh-bz-X3*, arguing that *stk1* is the sole factor responsible for the reduced transmission of the deficiencies. The *bz* RPT phenotype is only observed when

pollinating with *stk1* heterozygous mutant plants, not with homozygous mutants, and the *stk1* homozygous mutant ears have a normal seed set, indicating that the mutation causes a defect in the male but not in the female.

The genetic transmission analysis of *stk1* mutants suggests that the *stk1* gene plays an important role in normal pollen development. To look for differences in pollen tube growth rates, mature pollen from homozygous *stk1-s4* and wild-type plants was collected, germinated *in vitro* on pollen germination medium (Schreiber and Dresselhaus 2003) and examined under a light microscope under 50 $\times$ and 100 $\times$ magnification, but no differences could be detected (data not shown). Maize pollen tubes need to travel down strikingly long styles, measuring as much as 30 cm, to fertilize the ovules, but *in vitro* germinated pollen is susceptible to burst and tubes rarely grew longer than 1 cm. Possibly, the effect of the *stk1* mutation on pollen tube growth is not sufficiently adverse to be visualized under the light microscope. Alternatively, *stk1* may affect the pollen grain and pollen tube germination, rather than pollen tube growth. The observation that STK fluorescent protein signals can be readily detected in the pollen, but only faintly in the pollen tube (see section *The STK proteins localize to the plasma membrane in maize pollen*), supports this possibility.

Mutations in the homologous *stk2* gene also affect pollen transmission

Mutations of *stk1* reduce the transmission of mutant pollen in heterozygotes by as much as 40%, whereas mutations in a number of other pollen-specific genes, such as *apt1* in maize (Xu and Dooner 2006) and *SETH1* (Lalanne *et al.* 2004a) in *Arabidopsis*, almost completely abolish it. A possible reason for this difference is that the *stk1* gene is duplicated, whereas the others are not. The maize genome was queried with *stk1* and a gene with 86% similarity was identified on chromosome 4 (GRMZM2G301647) and designated *stk2*. The *stk2* gene is 3292 bp long and contains a 135-bp 5' UTR, a 294-bp 3' UTR, and five introns, and encodes a predicted 773-amino-acid protein.

To identify mutations of *stk2*, we screened the Pioneer TUSC population (Meeley and Briggs 1995) for *Mu* (*Mutator*) insertion mutants. Three such *Mu* insertion mutations were found, and all bred true. All three insertions are in exons and produced a typical *Mutator* 9-bp target site duplication. *stk2-1* harbors a 4.9-kb autonomous *MuDR* element at the third exon, *stk2-2* has a nonautonomous 1.4 kb *Mu1* element in the fifth exon, and a 1.8 kb *Mu3* element is inserted at the fifth exon of a different location in *stk2-3* (Figure 2).

Unlike *stk1*, there is no visual marker closely linked to *stk2* to facilitate segregation analysis. To study *stk2* segregation in heterozygotes, 177 test-cross progeny plants from a single *Stk2/stk2-2* heterozygous male parent were analyzed by PCR to score the wild-type and *stk2-2* *Mu* insertion alleles. The ratio of wild-type vs. insertion mutant alleles was 200:154 (PCR data not shown), which differs significantly from the expected 1:1 ($\chi^2 = 5.98$, 1 df, $P < 0.02$). This deviation corresponds to an RPT phenotype of 13%, milder than

Figure 2 *Mu*-induced *stk2* mutants. The blue triangles represent *Mutator* elements, the pentagon represents the *stk2* gene pointing in the direction of transcription, with the yellow and orange rectangles standing for introns and exons, respectively; the black line denotes intergenic regions.

that of *stk1*. Therefore, a wild-type *Stk2* allele appears to be needed for normal pollen transmission, though we cannot exclude the formal possibility that the *stk2* RPT effect is due to a linked mutation in the TUSC line.

stk1 and *stk2* are expressed in mature tassels and pollen

Northern blot analysis shows that *stk1* and *stk2* are expressed only in pollen and mature tassels and are either not expressed or expressed at a very low level in other tissues (Figure 3). Semiquantitative RT-PCR suggests that these two genes are expressed at similar levels (data not shown), a result supported by RNA-seq data (Davidson *et al.* 2011). Possibly, *stk1* and *stk2* perform redundant functions, and if so, the *stk* double mutant should exhibit a much stronger male transmission reduction. Therefore, we set out to isolate the double mutant.

Synthesis and analysis of an *stk* double mutant

The *stk2* mutants come from a *Mutator* insertion collection in a heterogeneous genetic background. To introgress the *stk2* mutation into a common genetic background, we used SSR marker-assisted selection. Of the 96 SSR markers tested on the two mutant parents, *stk2-2* and *stk1-s4*, nine were selected based on chromosomal locations: *umc1305* (bin 1.01), *umc2116* (bin 1.08), *umc1256* (bin 2.09), *umc1117* (bin 4.04), *umc1511* (bin 4.05), *umc2060* (bin 5.03), *umc1805* (bin 6.05), *bmc1375* (bin 9.07), *umc1930* (10.04). The *stk2-2* mutant was back crossed to *stk1-s4* for two generations. Because *stk1-s4* is a deletion affecting *stk1* and *bz*, it can be tracked by its stable bronze phenotype. Thirty-two BC2 bronze individuals were screened with SSR markers *umc1117* (bin 4.04) and *umc1511* (bin 4.05) to select for cross-overs carrying the *stk2-2* mutant allele (bin 4.06) in a W22 chromosome 4. Eight positive plants were back crossed again to *stk1-s4* and 32 of their progenies were scored with the seven remaining SSR markers. Plants stkd.11 and stkd.30, carrying all W22 markers but one, were then reciprocally crossed.

A PCR screen was used to look for homozygous *stk1 stk2* double mutants since there is no visual marker closely linked to *stk2*. DNA from 196 unselected kernels was analyzed from the crosses of plants stkd.11 and stkd.30 to identify *stk1/stk1*; *stk2/stk2* homozygous plants. One exceptional plant showing homozygous *stk1* and *stk2* bands was found. The double mutant was pollinated with its own pollen first and with *Bz-McC* pollen 7 days later. At harvest, the twice-pollinated ear showed a distinct pattern: the lower half had only six bronze kernels resulting from the self-cross, whereas

Figure 3 *stk1* and *stk2* expression pattern. Blots containing RNA from different maize tissues were hybridized to an *stk1* probe (top: data from Fu *et al.* 2001) and an *stk2* probe (bottom). Top lanes: 1, young leaf; 2, mature leaf; 3, young root; 4, mature root; 5, immature ear; 6, mature ear; 7, immature tassel; 8, mature tassel. Bottom lanes: 1, 14-day seedling; 2, 7-cm ear; 3–6, four stages of tassel development, from immature to mature; 7–8, pollen; 9, young leaf; 10, mature leaf; 11, young root; 12, mature root.

the top half had 65 purple kernels resulting from the cross to the wild-type line. This result shows that female gametophyte development is not affected in the *stk* double mutant.

In a second approach to synthesize double mutants (Figure 4), *Stk1 Bz/stk1-s4(bz)*; *Stk2/stk2-2* heterozygotes were self-pollinated and the purple seed progeny was screened by PCR for plants that were homozygous for *stk2*, but heterozygous for *stk1-s4* (*bz*), so that we could use bronze as a visual marker for identification of the double mutant in the next generation. Three hundred seventy-two purple kernels (*i.e.*, retaining at least one wild-type *Stk1* allele) from four ears were analyzed (Table 2). Both *stk1* and *stk2* show significant deviations from the expected Mendelian segregation, proving that both genes affect male transmission. Consistent with previous findings, the *stk1* mutation shows a stronger RPT phenotype than *stk2*. The frequency of pollen transmission of *stk* mutant alleles (*p*) in the self-mating can be estimated from the ratio of *Stk/Stk* homozygotes to *Stk/stk* heterozygotes since that ratio should be equal to $1/2 \times (1-p)/1/2$ or, simply, $1-p$. From the data in Table 2, the frequency of pollen transmission of *stk1* is 0.09 and that of *stk2* is 0.28. Thirty-six plants with genotype *Stk1/stk1*; *stk2/stk2* were found. Selfing these plants has allowed us to generate the double mutants from a plant carrying a wild-type allele (*Stk1/stk1*; *stk2/stk2*), rather than from a homozygous double mutant plant (*stk1/stk1*; *stk2/stk2*). *stk1 stk2* homozygotes from heterozygous *Stk1/stk1* ears (generation 3 in Figure 4) can be generated and recognized by crossing *Stk1 Bz-McC/stk1-s4(bz)*; *stk2-2* ear parents (generation 2 in Figure 4) with *stk1*; *stk2* pollen parents and selecting for bronze seeds in the following generation. In the absence of pollen competition, these ears segregate purple and bronze kernels in a 1:1 ratio.

Relationship between *stk1* and *stk2*

Maize is considered to be an allotetraploid that arose not more than 10 MYA (Helentjaris *et al.* 1988; Gaut and Doebley 1997; Swigonova *et al.* 2004). With 86% nucleotide identity and 68% amino acid identity throughout the entire sequence, one would reasonably assume that *stk1* and *stk2* are orthologous

Figure 4 Flow chart for *stk* double mutant isolation. Generation 1: *Stk1 Bz/stk1-s4(bz)*; *Stk2/stk2-2* heterozygotes were selfed. Generation 2: The purple (*Bz*) progeny from Gen1 was screened by PCR to identify plants homozygous for *stk2* but heterozygous for *stk1*, and these plants were selfed. Generation 3: The double mutants were readily identified as bronze kernels segregating in the selfed ear.

genes derived one each from the diploid ancestors of maize. To determine the relationship between *stk1* and *stk2*, we performed a phylogenetic analysis of their predicted protein sequences. Figure 5 shows a phylogenetic tree of STK homologs from *Z. mays* subsp. *mays*, *Oryza sativa*, *Sorghum bicolor*, and *Arabidopsis thaliana*. Both STK1 and STK2 are more closely related to the counterparts in sorghum than to each other, suggesting that both genes were present in the common progenitor of sorghum and the two maize ancestors, which split trichotomously ~11.9 MYA (Swigonova *et al.* 2004). Each STK also has a remarkably closer counterpart in *O. sativa*, which diverged from maize 50 MYA. The respective STK proteins in rice are 83% identical to STK1 and 78% identical to STK2. Hence, we conclude that the gene duplication happened before the maize–rice speciation event and that the *Zm-stk1* and *Zm-stk2* genes are paralogs, not orthologs (Fitch 2000; Dehal and Boore 2005; Zhang and Cohn 2008). In other words, they are not derived from a speciation event, but by a duplication event from a single sequence in the common ancestor of maize and rice. As expected, the two most closely related *Arabidopsis* genes, which are also uniquely expressed in pollen (Becker *et al.* 2003), are more distantly related to the grass genes.

stk1 and *stk2* genes may have evolved different functions. Paralogs are often found to evolve novel functions and may have a mechanistically distinct, but biologically related, function (Kuzniar *et al.* 2008). Our data show that even though the two genes express at a similar level in the pollen, the

single mutations show different RPT rates. Nevertheless, their biological functions are related: they are both highly expressed in mature tassel and pollen and their joint loss results in poor pollen transmission from double heterozygotes (1/196).

The STK proteins localize to the plasma membrane in maize pollen

To determine the subcellular localization of the STK proteins in the pollen, STK1-CFP and STK2-YFP fluorescent fusion protein (FP) constructs were generated (Mohanty *et al.* 2009), transformed into maize (Frame *et al.* 2002), and the localization site of the fusion proteins was examined under a fluorescent microscope (Figure 6). Fluorescent proteins in the pollen could only be detected in the mature anthers (Figure 6, B and D). Both STK1-CFP and STK2-YFP are targeted to the periphery of the pollen, suggesting that they are located on the plasma membrane (Figure 6, E, H, K, and L), and likely only do so upon hydration, as indicated by time-course *in vitro* germination experiments. The strongest fluorescent signal was detected around the pore, the site of pollen tube germination, and only trace amounts were detected in the cytoplasm and in pollen tubes (Supplemental Material, Figure S1 and Figure S2 in File S1).

AT2G24370, the closest homolog to STK1 in *Arabidopsis*, is identified in TAIR as being expressed in pollen and located in the plasma membrane (<http://www.arabidopsis.org/servlets/TairObject?type=locus&id=34726>). Analysis of its protein sequence by programs predicting transmembrane (TM) α -helices (<http://aramemnon.botanik.uni-koeln.de/>) identifies two TM spans of 21 amino acids each: one at 532–552 and another one at 664–684. Application of two such programs (TmPred and DAS) to the maize STK1 protein also identifies two TM domains (at 502–522 and 637–657) and a BLAST comparison of the *Arabidopsis* and maize STK1 proteins reveals that the two domains are highly conserved (Figure S3 in File S1). Alternatively, like some receptor-like kinases in plants and animals, STK1 and STK2 may associate with the inner surface of the plasma membrane via myristoylated N termini (Lin *et al.* 2013). Based on the above considerations and our experimental observations, we conclude that the maize STK proteins are plasma-membrane-localized and may play a critical role in pollen tube germination.

DEGs in *stk1* mutant pollen

Plant protein serine/threonine kinases have been equated to a “central processor unit,” accepting input information from receptors that sense environmental conditions, phytohormones, and other external factors, and converting it into appropriate outputs such as changes in metabolism, gene expression, and cell growth and division (Hardie 1999). Therefore, they can be expected to affect many different kinds of transcripts. To investigate which genes might be over- or underexpressed in the *stk1* mutant and to minimize genetic background and environmental effects, we conducted an RNA-seq experiment with pollen collected from field-grown *Stk1/stk1*

Table 2 Analysis of 372 *Stk1*⁻ individuals from the selfing of an *Stk1/stk1*; *Stk2/stk2* double heterozygote

	<i>stk1</i> analysis			<i>stk2</i> analysis			
	<i>Stk1/Stk1</i>	<i>Stk1/stk1</i>	Total	<i>Stk2/Stk2</i>	<i>Stk2/stk2</i>	<i>stk2/stk2</i>	Total
ear1	38	57	95	36	45	14	95
ear2	40	52	92	35	41	16	92
ear3	50	45	95	30	51	14	95
ear4	49	41	90	26	40	24	90
Total	177	195	372	127	177	68	372
Expected	124	248	372	93	186	93	372
Chi-squared	7.00	11.30	18.30 ^a	12.43	0.44	6.72	19.59 ^b

^a *P* (1 df) <0.001.^b *P* (2 df) <0.001.

heterozygous and *stk1/stk1* homozygous mutant sib plants that were also homozygous mutant for *stk2*. Because of this scheme, half of the pollen from the heterozygote is genetically double mutant.

Pollen from three *Stk1* heterozygous (*Stk1-McC Bz-McC/stk1 bz-s39.8*; *stk2-2*) and three *stk1* homozygous (*bz-s39.8*; *stk2-2*) field-grown plants was collected and processed as outlined in *Materials and Methods*. The six cDNA libraries were sequenced in a SOLiD 5500XL platform and the reads were processed and mapped to the B73 reference genome *Z. mays* B73.v3 (Schnable *et al.* 2009). Over 145 million reads were mapped to the genome, with little variation in percent of mapped reads among the six samples (Table S1). The RNA-seq data were analyzed with both DESeq and EdgeR software, using a *q*-value of <0.05 and a >twofold change as cutoffs for DEGs. Genome-wide gene expression patterns of mutant and wild-type pollen samples differ, with biological replicates aggregating together. The number of differentially expressed DEGs was 401 with DESeq and 497 with EdgeR, for a total (union) of 534 genes, of which 364 were common to the two sets (intersect). A top hit among the DEGs in wild type is the *stk1* gene itself, which is highly expressed in pollen and serves as positive control; peculiarly, another high hit is

an unspliced version of *stk1* in the reverse orientation that is likely an annotation artifact in the maize database. Each DEG was functionally annotated using the following resources: Phytozome, KEGG, best *Arabidopsis* TAIR10 hits, and best rice hits. The complete data are shown in Table S2. Out of 534 genes meeting our criteria for differential expression in the presence/absence of *Stk1*, 295 genes are associated with one or more GO terms, with translation being the most significant term.

Roughly 60% of the transcripts are significantly higher in the pollen derived from the *stk* heterozygote (317 out of 534 genes) whereas 40% (217) are higher in the *stk1* homozygous mutant pollen. GO enrichment analysis of transcripts with higher expression in the mutant identified four significantly overrepresented categories: translation (GO:0006412, *P* = 2.4e⁻⁰⁷, 22 genes), translational elongation (GO:0006414, *P* = 6.4e⁻⁰⁶, 6 genes), response to metal ion (GO:0010038, *P* = 4.2e⁻⁰⁵, 13 genes), and response to temperature stimulus (GO:0009266, *P* = 4.1e⁻⁰⁵, 12 genes). More detailed inspection, using GO and KEGG tools, showed that a large number of ribosomal proteins (16) are overexpressed in the *stk1* mutant (Figure S4 and Figure S5 in File S1). We extracted 1-kb sequences upstream of the ATG in all 16 ribosomal

Figure 5 Phylogenetic analysis of *stk*. The *stk* homologs compared are from *Z. mays* subsp. *mays* (*Zm-STK1*, ABP57732.1; *Zm-STK2*, NP_001309776), *S. bicolor* (*Sb-STK1*, XP_002437997; *Sb-STK2*, XP_002452944.1), *O. sativa* (*Os-STK1*, XP_015643441.1; *Os-STK2*, XP_015627356.1), and *A. thaliana* (*At-STK1*, AT2G24370; *At-STK2*, AT4G31230). *Os-STK3* (XP_015626483), a GenBank sequence that shares the highest identity with *Os-STK1* and *Os-STK2*, is used as an out-group. Evolutionary history was inferred using the Maximum Likelihood method based on the Jones-Taylor-Thornton matrix-based model. The tree with the highest log likelihood is shown, with bootstrap support at the branches. The tree is drawn to scale, with branch lengths proportional to number of substitutions per site. Evolutionary analyses were conducted in MEGA7 (Felsenstein 1985).

Figure 6 Subcellular localization of the STK proteins. STK1-CFP and STK2-YFP fluorescent fusion protein (FP) constructs were transformed into maize and their localization examined by fluorescence microscopy. (A–D) Pollen from hemizygous STK1-CFP (A and B) or STK2-YFP (C and D) under transmitted (A and C) or excitation (B and D) light. Bar, 10 μm . (E–H) Pollen magnified 630 $\times$ under excitation light. Bar, 40 μm . (E and H) Forty-micrometer z-stacked images of STK-FP expression of a single pollen. (F and I) The surface of a pollen expressing FP. (G and J) Forty micrometers below the surface. (K and L) Confocal microscopy images of STK1-CFP (K) or STK2-YFP (L) pollen, 15 min after being placed on a plate with germination medium. Bar, 25 μm .

protein genes and found a common significant -GGC- box motif in 11 of these 16 proteins (Figure S6 in [File S1](#)), with an E -value of 3.5×10^{-7} . The JASPAR CORE database shows that AP2 domain transcription factors bind -GGC-box motifs. In our DEG list ([Table S2](#)), an AP2 transcription factor (GRMZM2G076602) is also significantly overexpressed in the *stk1* mutant.

The presence of DEGs either overexpressed or underexpressed in the mutant pollen suggests that the *stk1* gene can have both positive and negative effects on other genes in the maize genome. Interestingly, several genes in the list have been found to be highly or specifically expressed in anthers (Sekhon *et al.* 2011). In fact, most of the genes differing in expression by at least fivefold between wild type and mutant

([Table S3](#)) were expressed at a mid to high level in anthers or immature tassels in that study (http://bar.utoronto.ca/efp_maize/cgi-bin/efpWeb.cgi?dataSource=Sekhon_et_al_Atlas), pointing to the importance of *stk1* in the overall RNA make-up of the pollen.

Conclusions

To sum up, the maize paralogous genes *stk1* and *stk2*, which are uniquely expressed in pollen, are major players in determining the competitive ability of a maize pollen grain. The plasma membrane localization of the STK1 and STK2 proteins and their concentration around the germ pore upon hydration suggest a role in the initiation of pollen tube germination. Like other protein kinases, STK1 and STK2 most

likely act to transfer phosphates to other proteins, serving as switches for protein activation or inactivation. The pleiotropic effect of the mutants on multiple pollen transcripts suggests that the proteins may function in a signal transduction cascade that eventually turns on and off other genes in the genome involved in the key developmental step of germinating a pollen tube. It is also possible that the proteins function independently of a phosphorylation cascade and directly activate effector proteins that regulate gene expression (Lin *et al.* 2013).

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