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The mating-type locus *b* of the sugarcane smut *Sporisorium scitamineum* is essential for mating, filamentous growth and pathogenicity

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ABSTRACT

Sporisorium scitamineum is the causal agent of sugarcane smut, which is one of the most serious constraints to global sugarcane production. *S. scitamineum* and *Ustilago maydis* are two closely related smut fungi, that are predicted to harbor similar sexual mating processes/system. To elucidate the molecular basis of sexual mating in *S. scitamineum*, we identified and deleted the ortholog of mating-specific *U. maydis* locus *b*, in *S. scitamineum*. The resultant *b*-deletion mutant was defective in mating and pathogenicity in *S. scitamineum*. Furthermore, a functional *b* locus heterodimer could trigger filamentous growth without mating in *S. scitamineum*, and functionally replace the *b* locus in *U. maydis* in terms of triggering aerial filament production and forming solopathogenic strains, which do not require sexual mating prior to pathogenicity on the host plants.

1. Introduction

Sugarcane smut is spread worldwide and causes considerable yield losses and reduction in cane quality. The major symptom of the disease is a characteristic sorus from the top of plant cane that appears as a black whip. This whip-like structure consists of a central core of pithy plant cells and a layer of fungal cells, which can be from 20 mm to over 1 m in length (Singh et al., 2004). The sugarcane smut causal pathogen, *Sporisorium scitamineum*, has an interesting life cycle consisting of three cell types. Diploid teliospores formed in host tissues are disseminated mainly by wind or rain splashes and germinate to form four sporidia. The haploid sporidia grow by budding, and compatible ("+" and "−") sporidia fuse to develop pathogenic dikaryotic hyphae to infect plant canes, which leads to formation of diploid teliospores within the host tissues and completion of the pathogenic life cycle (Albert and Schenck, 1996).

S. scitamineum was previously known as *Ustilago scitaminea* belonging to the Basidiomycetes (Piepenbring et al., 2002). The life cycle of the sugarcane smut fungus is similar to that of the maize pathogen *Ustilago maydis*, which has been well studied as a model pathogenic fungus over the past 30 years due to the availability of the complete genome sequence, short period to complete life cycle, and rapid assessment of disease symptoms (Kamper et al., 2006; Schirawski et al., 2010). In *U. maydis*, the sexual mating process is regulated by two unlinked mating type loci, *a* locus and *b* locus (Fedler et al., 2009; Vollmeister et al., 2012; Wahl et al., 2010). Recognition of the opposite haploid sporidia and formation of conjugation tubes are regulated by the bi-allelic *a* loci that encode a pheromone/pheromone receptor system (Bölker et al., 1995). Because *S. scitamineum* is a bipolar species (Albert and Schenck, 1996; Que et al., 2014), the two mating type strains were more appropriately termed *MAT-1* and *MAT-2* following the proposed genetic nomenclature (Yoder et al., 1986).

After fusion of the sporidia, the maintenance of the dikaryotic filament and subsequent penetration of the host plant are controlled by the *b* loci, which encode homeodomain proteins that function as transcription factors (Gillissen et al., 1992; Kronstad and Leong, 1990; Schlesinger et al., 1997; Schulz et al., 1990). The *b* locus is multi-allelic and contains at least 25 alleles in *U. maydis* (Gillissen et al., 1992). In addition, *b* locus is composed of the *bE* and *bW* genes encoding a heterodimeric transcription factor, which is active only when *bE* and *bW* genes are derived from different alleles (Kamper et al., 1995). The variable domains present in

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bE and bW proteins are responsible for discrimination of self and non-self and therefore the fungus can maintain the noninfectious state in haploid cells (Bölker et al., 1995; Kamper et al., 1995). The bE/bW transcription factor acts through a regulatory cascade to affect various pathways in triggering pathogenic development, including cell cycle regulation, mitosis and DNA replication (Feldbrugge et al., 2004). A solopathogenic *U. maydis* haploid strain was constructed by introducing the chimeric alleles of *b*-locus originating from opposite mating types, to the original chromosomal position as the wild-type locus in an *a1* haploid, and inserting the pheromone gene derived from the *a2* allele into the resident *a1* allele (Bölker et al., 1995). The resultant solopathogenic strain is able to infect the plant without sexual mating (Bölker et al., 1995). Thus, a solopathogenic strain is really valuable in investigation of key factors involved in pathogenicity.

In order to understand the sexual mating mechanism of *S. scitamineum* in-depth and to unravel the molecular differences in sexual mating between *S. scitamineum* and *U. maydis*, we characterized the *b* mating type loci of *S. scitamineum* using reverse genetics, as well as by testing filamentous growth and pathogenicity of transgenic *S. scitamineum* or *U. maydis* haploid strains with *SsMAT-1 b locus* allele.

2. Materials and methods

2.1. Growth conditions and isolation of mating-type haploid strains

Teliospores of sugarcane smut were collected from the fields in Guangdong province of China. Teliospores were rinsed three times in sterile distilled water containing 100 μg/mL Ampicillin (Sigma–Aldrich, A9518) and plated onto YePSA medium (yeast extract 1%, peptone 2%, sugar 2%, agar 2%) supplemented with 100 μg/mL Ampicillin, incubated at 28 °C for 2 days. Serial dilutions of the resultant cultures were plated onto YePSA medium and incubated as mentioned above to get haploid colonies. Haploid colonies were then transferred to 400 μL of YePS medium (yeast extraction 1%, peptone 2%, sugar 2%, pH7.0) containing 100 μg/mL Ampicillin, incubated with shaking at a rotational speed of 200 rpm at 28 °C for 2 days. These cultures were arbitrarily designated and mating by random on YePSA plates. Compatible strains Umn9 and Umn10 of *U. maydis* were isolated by ZJ (unpublished) and were grown at 28 °C on YePS or YePSA medium. Mixtures of Umn9 and Umn10 of *U. maydis* were incubated on YePSA medium (that contains 1% activated charcoal) to induce mating.

2.2. Cloning of the b loci and generation of constructs for functional assays

An 459 bp genomic sequence (as partial sequences of *S. scitaminea MAT-1* and *MAT-2 b* loci, from regions conserved with *U. maydis b* locus (Albert and Schenck, 1996)) was obtained from PCR products generated using primers bE4: 5′-CGCTCTGGTTCAT CAACG-3′, and bE8: 5′-TGCTGTCGATGGAAGGTGT-3′. Three nested primers were designed based on the upstream or downstream sequences and combined with degenerate primers randomly. The left flank and right flank of a 459 bp genomic fragment were amplified using the hiTAIL-PCR (Liu and Chen, 2007), respectively. These amplified fragments were sequenced and joined to form the full length DNA sequences of *S. scitaminea b* locus. The full length of *b* loci was generated by PCR amplification with primers (B1: CATAAAGAACTGCGCCTCTG and B10: ATGTCAGCCTTTAACTCGTCCAT), and cDNA of *b* loci were generated with primers (WT18bEF: ATGACGCCGACCAACAGCT and WT18bER: TTAACCAAATGCAGCAGAAAGGCTG; WT17bEFFF: ATGGCGCAACACAGTAGCTTC and WT17bER: TTAACCAAATGCAGCAGAAAGGC), following RNA extraction with TRIzol (Life Technologies, 15596-018) and reverse transcription was performed with Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, K1621). Structures of mating type (*MAT-1* and *MAT-2*) of *b* loci in *S. scitaminea* were analyzed through bio-software (Vector NTI 9.0, Life Technologies). The tree is calculated with Maximum Likelihood using Mega ver. 6.0 software (http://www.megasoftware.net/), based on a ClustalX alignment.

A 1.5-kb flanking fragment of *b* locus (namely fragment A) was amplified by PCR using with primers bfF1: AAAGTTTAAACTGTGAG GAACTAACTGGTTTGCA and bfR22: AAAAAGCTT GATGCGCTGGTTGCGTCTTGT using genomic DNA of *MAT-1* strain of *S. scitaminea* as template. Fragment A was digested with *Pme*I/*Hind*III and inserted into vector pEX2 carrying Hygromycin resistance cassette and formed vector pEX2-bF. A 1.1-kb fragment flanking the other side of *b* locus (namely fragment C) was amplified by PCR using with primers brF55: AAAACTAGTGTTATG GAAATCTGAACCCCG and brR36: AAATTAATTAAGATCCACACCA GATCCATCAAAAC, and ligated to the other side of vector pEX2-bF digested by *Spe*I and *Pac*I. The resultant plasmid was used for knockout of *b* locus and named as pEX2-bFR.

A 6.6-kb fragment D (containing *b* locus flanked by 1.5-kb fragment A and 1.1-kb fragment C) was amplified by PCR using primers 17bF11: AAAACTAGTTGTGAGGAACTAACTGGTTTGCA and 17bR22: AAATTAATTAATCCACACCAGATCCATCAAAACA and genomic DNA of the *MAT-1* strain of *S. scitaminea* as template. Fragment D was then digested with restriction enzymes *Spe*I and *Pac*I and ligated with vector pEX2 to form new vector pEX2bMAT-1. A 1.2-kb fragment containing eGFP and GAP from vector pEX2-eGFP was ligated with vector pEX2bMAT-1 downstream of fragment D to form vector pEX2bMAT-1-eGFP. A 6.8-kb fragment containing *b* locus cloned from Umn10 strain of *U. maydis* was used to replace fragment D of pEX2bMAT-1-eGFP and the resultant vector was named pEX2-Um10b-eGFP.

The aforementioned four constructed vectors were transformed into *Agrobacterium tumefaciens* by electroporation (Main et al., 1995) and the positive transformants were allowed to grow on LB agar with spectinomycin (100 μg/mL) and rifampicin (75 μg/mL) at 28 °C for 2 d. Colonies were used for transformation of *S. scitamineum* haploid wild-type strain (Sun et al., 2014), or of *U. maydis* haploid strain (Ji et al., 2010). Transformants of *S. scitaminea* were verified by PCR or Southern blot analyses and subsequently used for mating and pathogenicity assays.

2.3. Pathogenicity tests

Fungal colonies incubated on solid medium were inoculated into 50 mL of YePS liquid medium at 28 °C, shaking at 200 rpm for 2 days. Fungal cells were collected by centrifuge at 4000 rpm for 5 min. Collected cells were washed twice with distilled water and re-suspended in 20 mL of distilled water. Conidia of mutants and opposite mating type of wild-type haploid were mixed at equal volume. One mL of mixture was inoculated onto sugarcane seedlings, with wild-type smuts as positive control and water inoculation as a negative control. Three replicates were performed for each combination.

3. Results

3.1. Isolation of mating-type haploid strains of S. scitamineum

Teliospores of sugarcane smut fungus are spherical and brown or black in color (Fig. 1A). Haploid cells are cigar-shaped and colorless (Fig. 1B). Mycelial dikaryotic colonies became visible after inoculation of teliospores on YePSA medium at 28 °C for 2 days (Fig. 1C). Yeast like haploid cells appeared after serial dilutions of

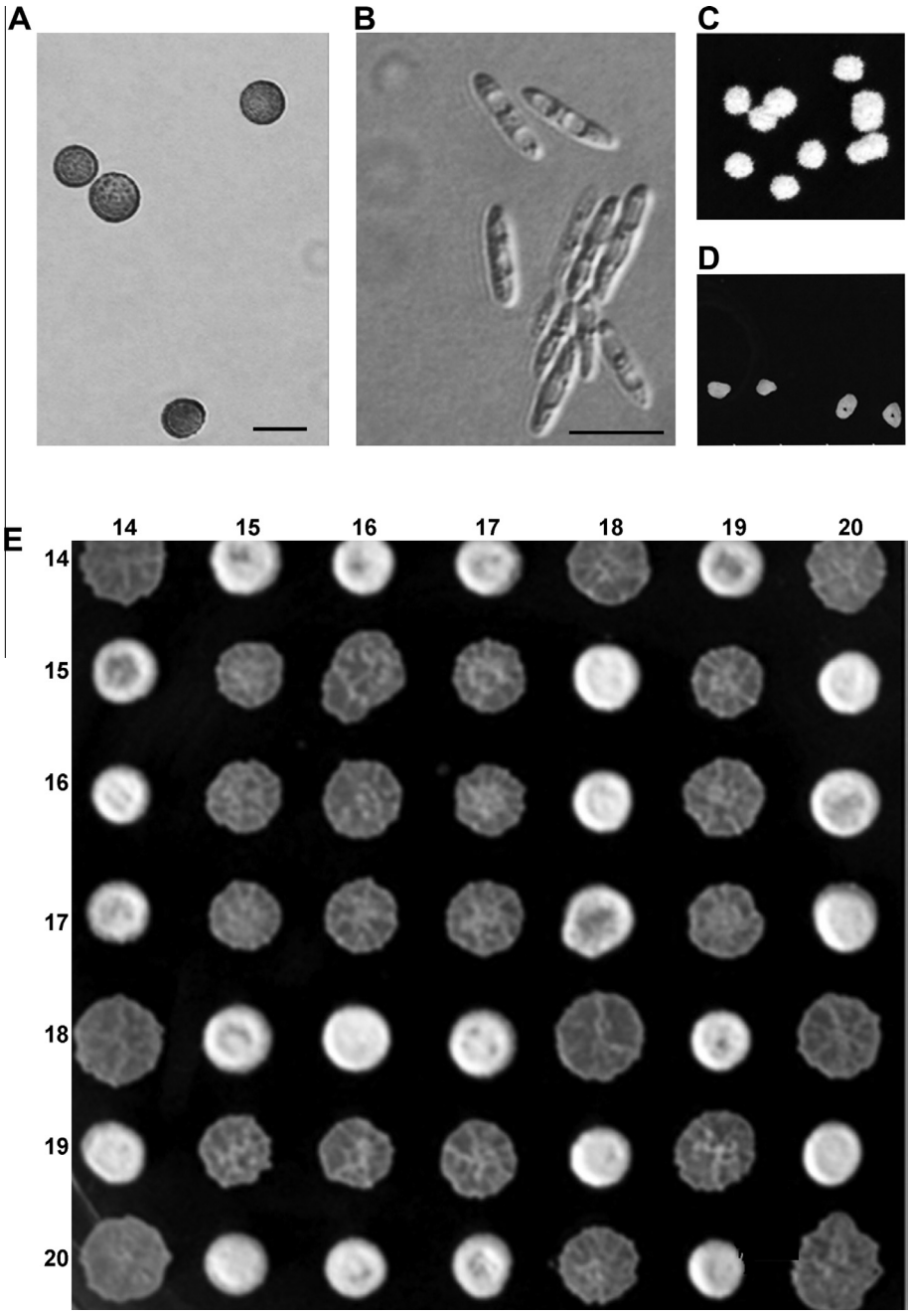

Fig. 1. Isolation and characterization of haploid strains of *S. scitamineum.* Cells were grown in YePS or YePSA medium and analyzed by microscopy (A and B). (A) Teliospores of *S. scitamineum*. (B) Haploid sporidia of *S. scitamineum*. (C) Dikaryon colonies of *S. scitamineum*. (D) Haploid colonies of *S. scitamineum*. (E) Random mating of different haploid strains.

the resultant cultures on YePSA medium at 28 °C for 2 days (Fig. 1D). Haploid colonies were then transferred to 400 μL of YePS medium for inoculation at 28 °C for 2 days, with shaking at a rotational speed of 200 rpm. These cultures were arbitrarily designated and randomly mated with each other on YePSA plates. Following 24–48 h incubation at 28 °C, the appearance of the "fluffy" phenotype and yeast-like colonies were recorded. Two strains are of different mating types (*MAT-1* and *MAT-2*) if they can mate together ("fluffy" colony), while they are of the same mating type (both are *MAT-1* or both are *MAT-2*) if they cannot mate (yeast-like colony). As shown in Fig. 1E, the haploid strains 14, 18 and 20 are of the same mating type, while the haploid strains 15, 16, 17, and 19 belong to the opposite mating type. We here designated mating type strains Ss15, 16, 17, 19 as *MAT-1* and mating type strains Ss14, 18, 21 as *MAT-2*. Haploid strain Ss17 displayed strong mating capability with Ss18 and therefore was chosen for genetic deletion of *MAT-1* via *Agrobacterium tumefaciens* mediated transformation (ATMT) (Sun et al., 2014).

3.2. Cloning of the b loci of S. scitamineum

As the genome sequence of *S. scitamineum* was not available by the time this study was initiated, we designed the primer pairs of bE4/bE8 based on the DNA sequence of *b* locus in *U. maydis* (Albert and Schenck, 1996) in order to amplify and clone the homologous locus from *S. scitamineum*. Two fragments of 450-bp were then

amplified using the genomic DNA from *S. scitamineum MAT-1* (Ss17) and *MAT-2* strain (Ss18) as templates. Three nested primers were designed based on the upstream or downstream sequences of such two fragments and combined with degenerate primers randomly. Therefore the left and right flanking sequences of 459 bp were amplified using hiTAIL-PCR (Liu and Chen, 2007). These amplified fragments were sequenced, joined and compared to the NCBI database using BLAST searches. (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) for identification of the full length DNA sequence of *S. scitamineum b* loci.

Sequence analysis revealed that the full-length DNA sequences of *b* loci isolated from *MAT-1* and *MAT-2 genes* were 3856 bp and 3886 bp, respectively, with about 89% identity to each other. The alleles contained two divergent open reading frames (ORFs) encoding peptides with various similarity to the bE and bW proteins of other fungal pathogens. We named these alleles S*sbW1* and S*sbE1* from *MAT-1* (Ss17) and S*sbW2* and S*sbE2* from *MAT-2* (Ss18). The amino acid sequences of SsbE1 and SsbW1 showed 51% and 41% identity, respectively, to bE1 and bW1 of *U. maydis*, 68% and 87% identity, respectively, to bE1 and bW1 of *S. reilianum*, and 52% and 38% identity respectively, to bE1 and bW1 of *U. hordei*. The amino acid sequences of SsbE2 and SsbW2 showed 52% and 38% identity, respectively, to bE1 and bW1 of *U. maydis*, 69% and 58% identity, respectively, to bE1 and bW1 of *S. reilianum*, and 52% and 40% identity respectively, to bE1 and bW1 of *U. hordei*. The position of introns was determined by sequencing the RT-PCR products using the total RNA from *SsMAT-1* and *SsMAT-2*, respectively, and the results showed that both the *bW* and *bE* genes contain only one intron (Fig. 2A).

Phylogenetic analysis (Fig. 2B) of the b proteins indicates that SsbE1 and SsbE2 proteins are more closely related to the bE proteins of *S. reilianum* than those of either *U. maydis* or *U. hordei*. The same holds true for SsbW1 and SsbW2 proteins (Fig. 2B). A recent publication of the sequence and comparisons of 3 *S. scitamineum* genomes (Taniguti et al., 2015) revealed that bE1/bE2 and bW1/bW2 of two opposing mating types, SSC39A and SSC39B, are more close related to each other than to the bE and bW proteins from related species. Our result here is consistent with Taniguti's finding that SsbE1 and SsbE2 are more close to each other than to bE proteins of other species. But SsbW1 is more close to SrbW1/2 proteins, than to SsbW2, of its opposing mating-type. We looked closer into the domain-arrangement of bE and bW proteins of *S. scitamiueum* strains of this study, and found that the b proteins of *S. scitamineum* contain a homeodomain that can be separated into two domains, i.e., a conserved C-terminal domain and a variable N-terminal domain (Fig. 3). The C-terminal domains of the SsbE1 and SsbE2 proteins are nearly 100% identical, whereas 95.9% identity was recorded between the C termini of SsbW1 and SsbW2; while the N-terminal regions of two bE or bW alleles showed 31.9% and 11.4% identity respectively (Fig. 3). We infer that the variable N-terminal domain of SsbW proteins may determine the phylogenetic relationship with other bW proteins. On the other hand, the *SsbE* gene is flanked by *c1d1*, whose sequence was only partially determined in this study. The *c1d1* gene encodes a putative nuclear regulator related to human C1D, which has been shown to be involved in transcription and DNA maintenance (Chen et al., 2004). The presence of (at least partial) *c1d1* gene was reported in *Sporisorium reilianum b1*, *b2* and *b3* loci, as well as in *U. maydis b1* locus, but not in *U. maydis b2* locus or *b* loci of other filamentous fungi (Bakkeren et al., 2008; Que et al., 2014).

3.3. Functional study of the b loci of S. scitamineum

The entire *b* locus was deleted to test the role of the *b* loci of *S. scitamineum*. Transformation of *MAT-1* (Ss17) strain with the gene disruption cassette in pEX2-bFR yielded hygromycin-resistant fungal transformants that appeared yeast-like, similar to the untransformed wild-type strain. Ten transformants and wild-type *MAT-1* (Ss17) strain were chosen for PCR verification of the requisite disruption of the *b* locus. The amplification of a 4.6-kb fragment with primers bfF1/brR36 was indicative of the replacement of the *b* locus with the hygromycin cassette (Fig. S1A, lanes 1–10). The untransformed *MAT-1* (Ss17) strain gave a single 6.6-kb product (Fig. S1A, Lane 11). Clearly, the *b* locus was successfully deleted in these ten examined transformants. Therefore, we named these ten independent mutant strains as *SsΔMAT-1b* and characterized their mating and pathogenicity abilities in the following studies.

We first tested whether the *b* locus is required for sexual mating and/or pathogenicity in *S. scitamineum*. It was found that the *SsΔMAT-1b* mutants were defective in mating when mixed with the opposite mating type haploid *MAT-2* (Ss18) strain (Fig. 4A). In addition, the *SsΔMAT-1b* mutants were non-pathogenic to the host (Fig. 4B, number 2) after mixing with wild-type *MAT-2* strain,

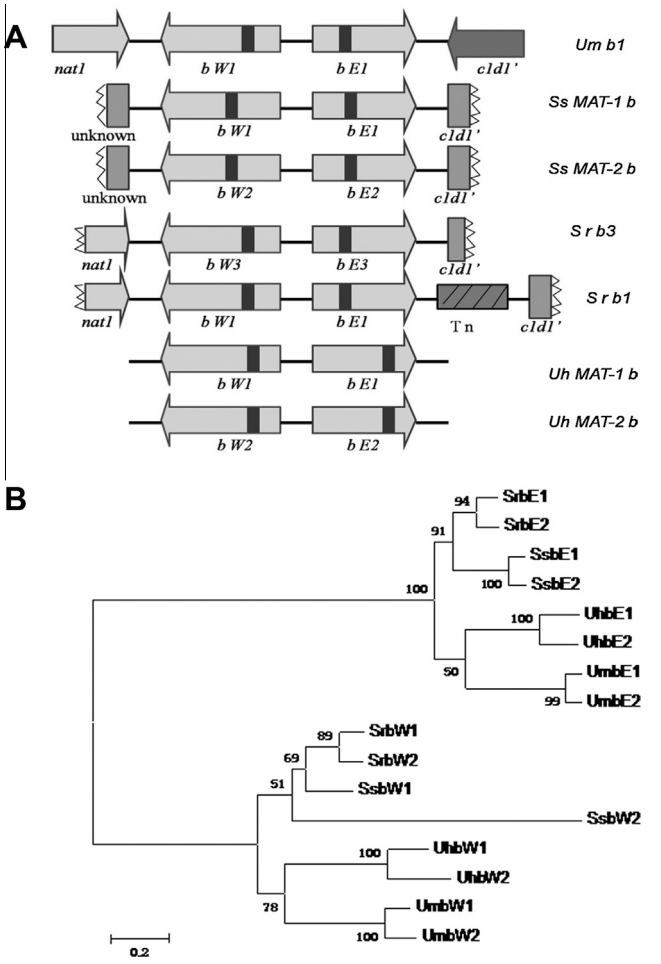

Fig. 2. The mating type *b* locus of *S. scitamineum* is highly conserved among filamentous and/or pathogenic fungi. (A) Schematic representation of the gene order in the *S. scitamineum b* loci in comparison with *U. maydis b1* and *S. reilianum b1* and *b3*, *b* loci of *U. hordei*. Open reading frames are shown as arrows; shaded bars indicate introns. (B) Phylogenetic analysis of bE and bW proteins of *S. scitamineum* and orthologues from other fungi. The tree is calculated with Maximum Likelihood using Mega ver. 6.0 software, based on a ClustalX alignment. The horizontal bar represents 20 mutations per 100 residues. The following proteins were chosen for comparison: SsbE1, SsbE2, SsbW1 and SsbW2 of *S. scitamineum*; bE and bW of the *U. maydis b* alleles 1 and 2 (Um; GenBank accession numbers EAK81226.1, AAA63554.1, EAK81227.1 and AAA34221.1); bE and bE of *S. reilianum b* alleles 1 and 2 (Sr; GenBank accession numbers CAI59728.1, CAI59732.1, CAI59727.1 and CAI59731.1); bE and bW of the *U. hordei b* alleles 1 and 2 (Uh; GenBank accession numbers CAA79218, CAA79216, CAA79219, and CAA79217).

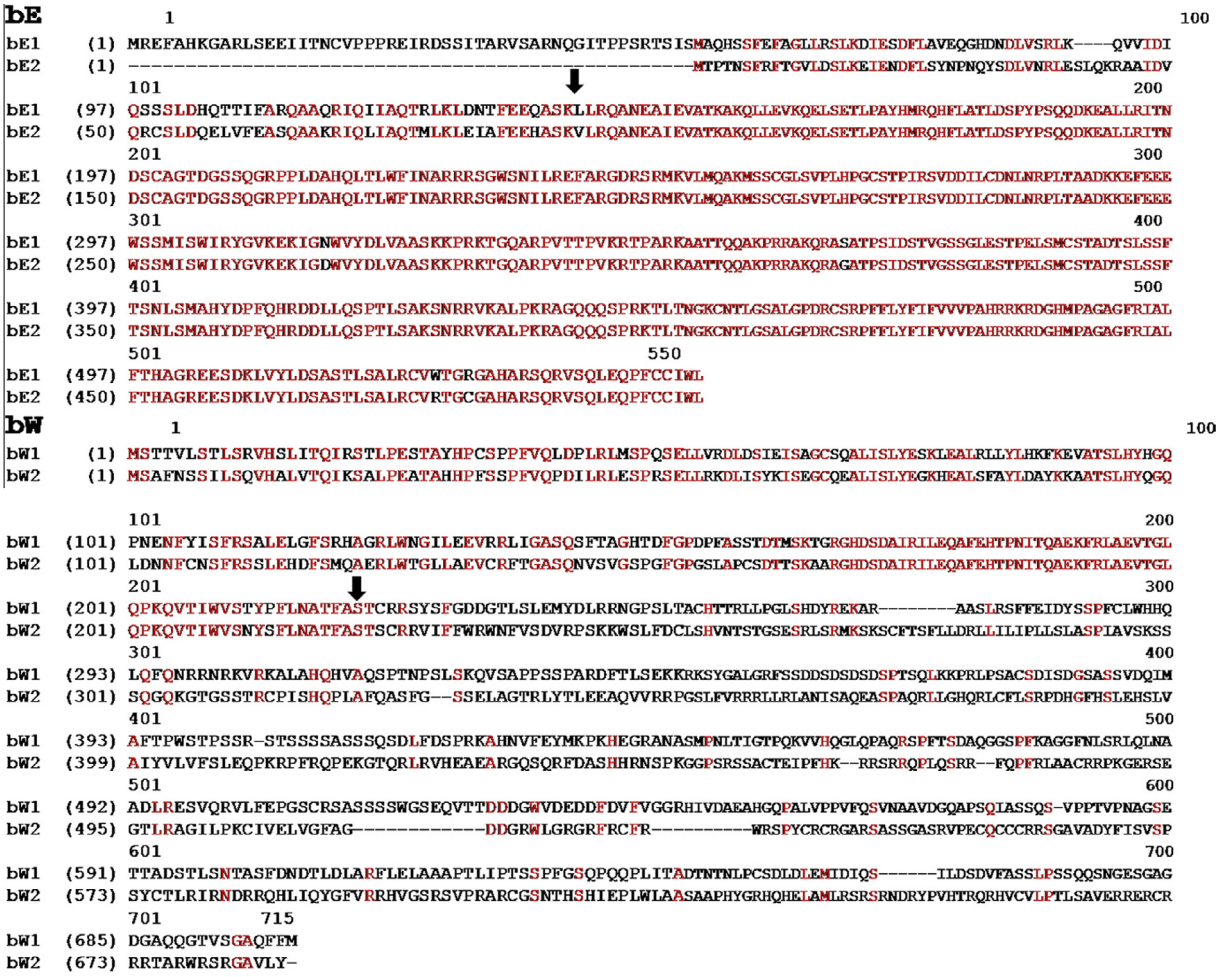

Fig. 3. Comparison of amino acid sequences of b proteins of *MAT-1* and *MAT-2* strains in *S. scitamineum*. Sequences were compared and aligned between SsbE1 and SsbE2 proteins (upper panel) and between the respective bW proteins (lower panel). Identical amino acids are in red. The beginning and the end of the variable domain were arbitrarily assigned (vertical arrow) for amino acid comparisons of bE and bW, respectively.

whereas wild-type mixture of *MAT-1* (Ss17) and *MAT-2* (Ss18) was pathogenic and induced the characteristic symptom of black "whip" within 90 days (Fig. 4B, number 1 and inset, arrow). Injection of sterile water served as a negative control (Fig. 4B, number 4). We conclude that *b* locus is essential for mating and pathogenicity in the sugarcane smut pathogen.

The bE/bW heterodimer is regarded as the major regulator of hyphal growth and pathogenic development. In *U. maydis*, manipulation of the mating type loci *a* and *b*, generates a solopathogenic strain, which contains compatible alleles of the *a*-locus and *b*-locus and is able to infect the plant without undergoing sexual mating with a mating partner (Bölker et al., 1995). To test the function of *S. scitamineum b* locus, a 6.6-kb fragment containing the entire *b* locus from *S. scitamineum MAT-1* (Ss17) strain was introduced into the *MAT-2* (Ss18) strain of *S. scitamineum*. The ectopic single insertion of the cloned *b* locus was confirmed by PCR amplification (Fig. S1B) and Southern blotting (Fig. S1C). As expected, haploid *MAT-2* (Ss18) strain was yeast-like (Fig. 5A, upper panel) while *MAT-1* and *MAT-2* mating mixture initiated filamentous growth (Fig. 5A, lower panel). *Ss*Δ*MAT-1b* mutant formed yeast-like colonies similar as un-mating haploids (Fig. 5B). The *MAT-1b*-carrying *MAT-2* (Ss18) strain was able to grow mycelia in YePSA from a single spore (Fig. 5C) without sexual mating. The filaments were able to branch and form septa (Fig. 5I), similar to the mating (mixed) *MAT-1* and *MAT-2* strains (Fig. 5H), which was absent in haploid *MAT-2* (Fig. 1B). This suggests that a functional b heterodimer could trigger fungal filamentation without mating. To test the pathogenicity, the *MAT-1b*-carrying Ss18 strain was injected into sugarcane seedling, and it failed to induce the black "whip" symptoms (Fig. 4, number 3). Therefore, we conclude that ectopic expression of bE/bW heterodimer is sufficient for inducing filamentous growth without mating, although the resulting fungal mycelia were non-pathogenic on sugarcane. This indicates that the pheromone response pathway mediated by the compatible *a*-locus is not only required for induction of mating, but continues to function in the dikaryotic phase for proper pathogenic development.

3.4. S. scitamineum b locus is functionally conserved with U. maydis homolog

To test whether the b heterodimer of *S. scitamineum* can be functional in *U. maydis*, we introduced the intact 6.6-kb *MAT-1 b* locus from Ss17 into *U. maydis* Umn9 and Umn10 strain, respectively. A solopathogenic strain of *U. maydis* was generated by transformation of the 6.8-kb *b* locus of Umn10 into Umn9, and served as a positive control. The *MAT-1b*-carrying Umn9 or Umn10 strains were verified by Southern blot analysis (Fig. S1D). The non-mating haploid *U. maydis* Umn9 or Umn10 colonies (Fig. 5D, upper a panels) were yeast-like and distinct from mating Umn9 and

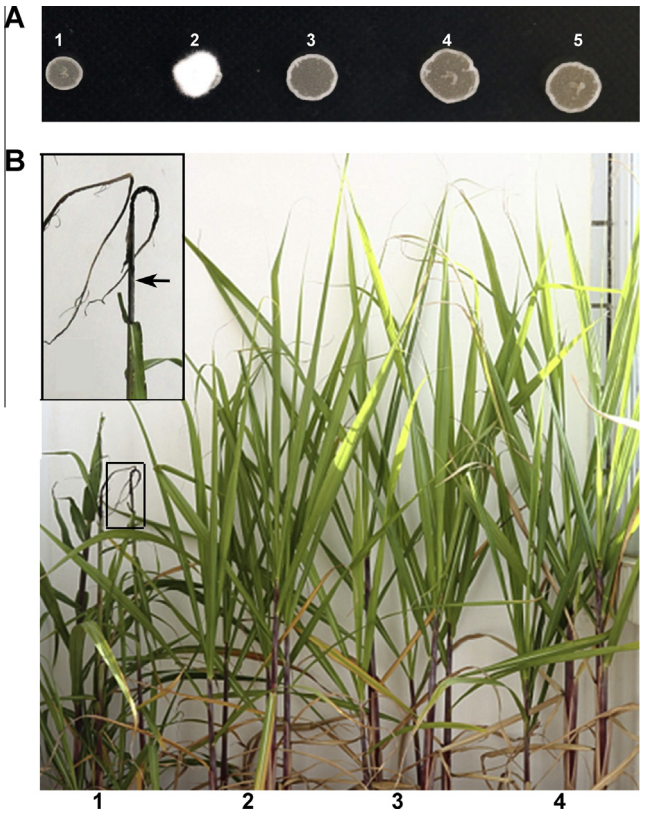

Fig. 4. Mating and pathogenicity assays with *Ss*Δ*MAT-1b* mutants. (A) Three transformants were first grown in liquid culture YePS, and then mixed with *MAT-2* (Ss18) strain respectively, and spotted on YePSA plate. Lane 1: Wildtype *MAT-1* (Ss17) strain, 2: Wild-type mixture of *MAT-1* and *MAT-2*, 3-5: *Ss*Δ*MAT-1b* mutants mating with *MAT-2* (Ss18) strain. (B) Sugarcane variety ROC22 susceptible to smut fungus *S. scitamineum* was inoculated with mixed fungal cells through injection at seedling stage. 1: WT control treatment infected with a mixture of compatible wild-type *S. scitamineum* strains *MAT-1* and *MAT-2* resulted in black "whip" formation within 90 days. Inset shows the enlarged boxed region. Arrow denotes typical black "whip" caused by *S. scitamineum* infection. 2: *Ss*Δ*MAT-1b* mutant strain mixed with *MAT-2* strain. 3: Mycelia haploid cells from *MAT-1* carrying *MAT-2* strain. 4: inoculation with water as negative control.

Umn10 cell mixture initiating filamentous growth (Fig. 5D, lower panel). *MAT-1b*-carrying Umn9 and Umn10 strains developed "fluffy" hyphae when grown on YePSA (Fig. 5F and G) as solopathogenic *U. maydis* does (Fig. 5E). The haploid Umn9 and Umn10 colonies were composed of sporidia, with no filaments or branches (Fig. 5J and K). In contrast, the filaments of *MAT-1b*-carrying Umn9 and Umn10 strains branched and contained septa (Fig. 5L and M). When inoculated into maize seedlings, infected maize by solopathogenic *U. maydis* or by *MAT-1b*-carrying Umn 9 or Umn10 developed tumors on stems within 7 days post infection (Fig. 6, number 2, 3 and 4, arrows). Inoculation with water failed to induce tumors (Fig. 6, number 5) and served as negative control. Infection with a mixture of compatible wild-type *U. maydis* Umn9 and Umn10 resulted in tumor formation (Fig. 6, number 1, arrow), and served as a positive control. This showed that the b proteins encoded by the *S. scitamineum b* locus are functionally similar to the orthologous counterpart in *U. maydis*.

4. Discussion

The life cycle of the phytopathogenic fungus *U. maydis* is governed by the two unlinked mating type loci, *a* and *b* (Banuett and Herskowitz, 1989; Schirawski et al., 2005). The *a*-locus genes encode pheromones and pheromone receptors while at the *b* locus two subunits of a heterodimeric transcription factor (bE/bW) are encoded. *U. maydis* has a tetrapolar mating system, in which *a* and *b* loci are on separate chromosomes and segregate independently during meiosis (Bakkeren and Kronstad, 1994). Up to now, two mating type alleles of *a* locus *a1* and *a2* have been identified in *U. maydis* (Froeliger and Leong, 1991), while 25 mating type alleles were found at *b* locus (Fedler et al., 2009; Wahl et al., 2010). *S. reilianum*, another tetrapolar fungus, contains 3 alleles at *a* locus and 5 mating type alleles at *b* locus (Schirawski et al., 2005). In contrast, species such as *U. hordei* display a bipolar mating system composed of a single mating gene complex with two alternate specificities, previously called "a" and "A" (Bakkeren and Kronstad, 1993; Lee et al., 1999). The two mating type strains were renamed as *MAT-1* and *MAT-2*, based on the genetic nomenclature as proposed (Yoder et al., 1986). However in *S. scitamineum*, strains of different mating types were only known as "+" or "−" previously (Albert and Schenck, 1996). Our screen for the mating type genes in *S. scitamineum* isolates based on *a* locus and *b* locus according to the method described (Schirawski et al., 2005) failed although repeated attempts were made. Recently, *S. scitamineum* has been reported as bipolar species similar as *U. hordei*, that possesses *MAT-1 a* and *b* locus structure located on the same chromosome (Que et al., 2014). However, *MAT-2* was not previously identified. To avoid confusion in mating type gene nomenclature, we propose to designate the *S. scitamineum* Ss17 strain as *MAT-1* and Ss18 strain as *MAT-2*, according to *b* loci sequence and the classification of Yoder et al. (1986). The presence of pheromone receptor gene *PRA1* was reported by Que et al. (2014), but the full length genomic DNA sequence of *PRA1* was not reported. During our BLAST search in *S. scitamieum* genome (taxid: 49012) with *U. hordei PRA1* gene (GenBank: U07939.1) as bait, we were able to obtain a fragment from Chromosome 2 (860833-859805, – strand), with high similarity to *UhPRA1* gene, in the region of 225–1269 in bait sequence (from start codon to middle of 3rd exon). We manually identified the remaining C-terminal sequence of *SsPRA1*, including part of exon 3, the intron 3, and the exon 4, until the stop codon (Fig. S1A, underlined), based on the similarity between *UhPra1* protein and the deduced protein sequence of *SsPRA1* coding region. We verified the cDNA of *SsPRA1* by 3'RACE PCR (primers for 3'RACE PCR were listed in Supplementary Table 1). The deduced SsPra1 protein is composed of 357 amino acids and is highly similar to Uh Pra1 (Fig. S2B). However, we failed to identify *SsPRA2* (the pheromone receptor gene of *MAT-2* haploid, due to lack of genome sequence of this mating type). The pheromone precursor genes, *MFA1* and *MFA2*, have not yet been identified in *S. scitamineum*. We believe that the identification and functional analysis of the *S. scitamineum a*-locus would lead to comprehensive understanding of sexual and pathogenic development in this important phytopathogenic fungus.

In this study, we have identified, for the first time, two different mating types (*MAT-1* and *MAT-2*) *b* loci of *S. scitamineum*. Both alleles show high homology to the *b* locus of *U. maydis*, *S. reilianum* and *U. hordei* at the level of gene arrangement and the encoded polypeptides. Sequence analysis showed that the gene *c1d1* encoding a nuclear regulator is adjacent to *bE* in *S. scitamineum*, similar to the *c1d1* gene in *U. maydis*, *S. reilianum* and the *MAT1* in *S. scitamineum*. However, an unknown sequence of about 1.6 kb flanking the *bW* gene in *S. scitamineum* showed very low similarity to *bW* gene from *U. maydis*, *S. reilianum* and *MAT1* of *S. scitamineum* (Que et al., 2014). Unfortunately, we did not obtain information about the *b* locus of *S. scitamineum* in more detail due to technical difficulty in sequencing the genomic DNA from the diploid fungal hyphae and incomplete genome sequence (http://www.ncbi.nlm.nih.gov/nuccore/?term=PRJNA240344).

We have shown that the intact *b* locus of 6.6 kb from *S. scitamineum MAT-1* (Ss17) strain is capable of inducing filamentous

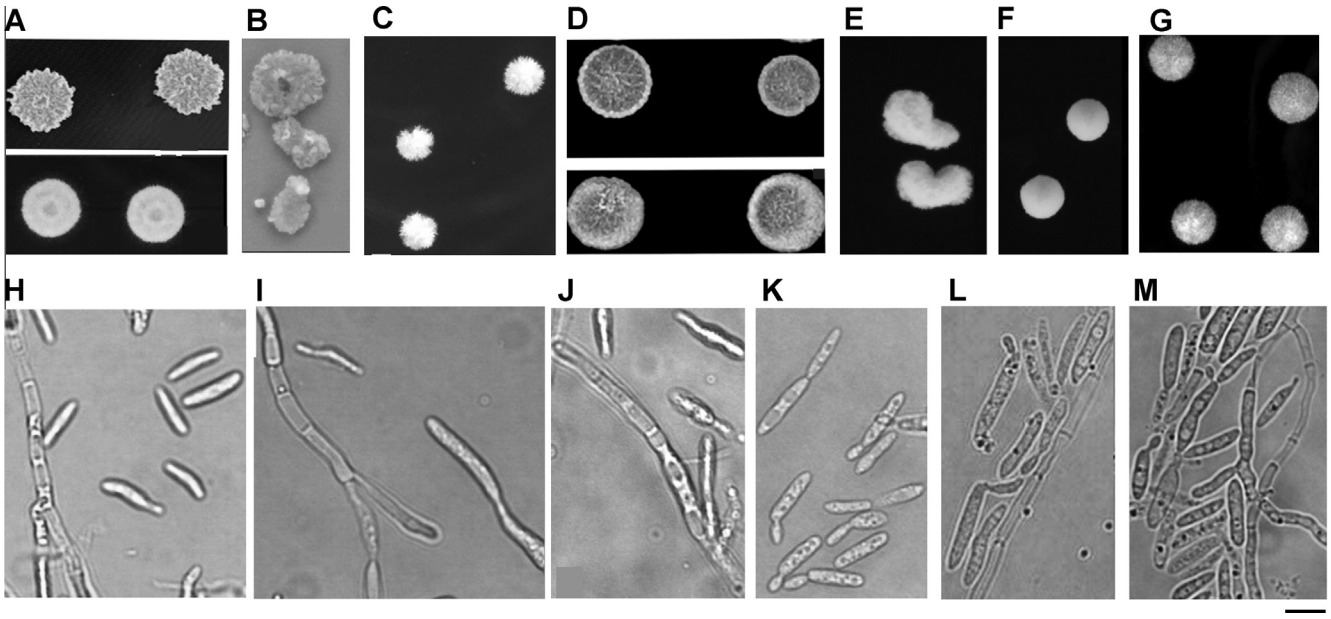

Fig. 5. Characterization of *MAT-1b*-expressing *S. scitamineum* and of *U. maydis* strains. Cells were grown in solid or liquid medium and analyzed by microscopy. (A) Colonies of wild-type *MAT-1* (Ss17) and *MAT-2* (Ss18) strains. Upper: non-mixed haploid strain; lower: mixed (mating) *S. scitamineum MAT-1* (Ss17) and *MAT-2* (Ss18). (B) *Ss*Δ*MAT-1b* mutant. (C) *S. scitamineum MAT-1* carrying *MAT-2* strain. (D) Colonies of wild-type *U. maydis* Umn9 and Umn10 strains. Upper: non-mating Umn9 or Umn10; lower: mixed (mating) Umn9 and Umn10 of *U. maydis* (E) solopathogenic *U. maydis*. (F) *U. maydis* Umn9 strain carrying ectopic *MAT-1*. (G) *U. maydis* Umn10 strain that expresses an ectopic *MAT-1*. (H) Microscopic image showing sporidia and hyphae of mixed *S. scitamineum MAT-1* (Ss17) and *MAT-2* (Ss18) strain. (I) Microscopic image showing sporidia and hyphae of *MAT-1b*-expressing Ss18 strain. (J, K) Microscopic images of hyphae and sporidia of the Umn9 and Umn10 strains of *U. maydis*. (L) Microscopic image of sporidia and hyphae of *MAT-1*-expressing *U. maydis* Umn9 strain. (M) Microscopic image of sporidia and hyphae of *MAT-1b*-expressing *U. maydis* Umn10 strain. Scale bar: 10 μm.

Fig. 6. Pathogenicity assays with *U. maydis* solopathogenic mutants and *MAT-1b*-complemented *U. maydis* strains. Maize seedlings were inoculated with fungal cells of *U. maydis* solopathogenic or *MAT-1*-expressing strains, through injection. 1: Infection with a mixture of compatible wild-type *U. maydis* strains Umn9 and Umn10 resulted in tumor formation (arrow) within 7 days, and served as a positive control. 2: Solopathogenic strain (Um10b-carrying Umn9 strain). Arrow denotes tumor formation on maize stem. 3: *MAT-1b*-expressing *U. maydis* Umn9 strain. 4: *MAT-1b*-carrying *U. maydis* Umn10 strain, resulting in tumor formation on maize stems (arrow). 5: Injection with sterile water served as a negative control.

growth when introduced into opposite mating type strain, *MAT-2* (Ss18), which is similar to what has been reported in *S. reilianum* (Schirawski et al., 2005) and *U. hordei* (Bakkeren and Kronstad, 1993). However, full pathogenicity of *S. scitamineum* still depends on mating, which is slightly different from *U. maydis*, where the haploid strain CL13 containing an active pair of homeodomain proteins (bW2 and bE1) is pathogenic on maize but fails to grow filamentous mycelia because it only contains a single *a* allele (*a1*) (Bölker et al., 1995). Therefore, we hypothesize that functional bE/bW heterodimer could bypass the mating in *S. scitamineum* to trigger filamentous growth, but the pheromone response pathway is still required for pathogenic development during the dikaryotic phase. Alternatively, the position of ectopic *b*-locus inserted into the haploid of the opposite mating type may affect its full function, as the chimeric alleles of *b*-locus occupied the original chromosomal position in solopathogenic *U. maydis* (Bölker et al., 1995). However, in our study the *MAT-1 b*-locus was inserted into the *MAT-2* strain by random insertion. We also showed that the *b* locus from *S. scitamineum* could functionally replace *U. maydis b* locus in terms of triggering filamentation and tumors formation in maize, which is comparable to the solopathogenic strain. It has been reported that the *b* locus of *U. hordei* could also induce filamentous growth of *U. maydis* haploid strain with compatible allele of the *b* locus, but could not fully restore pathogenicity. Conversely, *b* locus of *U. maydis* could functionally trigger to yield mycelial colonies (Bakkeren and Kronstad, 1993). Our results confirm that the *S. scitamineum b* locus is functionally conserved with that from *U. maydis*, although we failed to restore filamentous differentiation in *S. scitamineum* by introducing the *U. maydis b* locus (data not shown).

Comparison of amino acid sequences of b proteins of *MAT-1* and *MAT-2* strains in *S. scitamineum* reveals that the homeodomain of bW and bE proteins is further divided into two subdomains, a conserved C-terminal region and a variable N-terminal motif. It has been reported that the variable domains serve functions in dimerization and/or for recognition of self and non-self in *U. maydis* (Bölker et al., 1995). The C-terminal domains of the bE and bW proteins of *S. scitamineum* showed significant identity to that of *S. reilianum,* whereas the N-terminal regions of the two bE or bW alleles showed marginal similarity to that of *S. reilianum*. Analysis of amino acid sequence of bE and bW proteins was also performed in *U. hordei*, and it was speculated that such alteration in amino acid sequence of *U. hordei b* locus occurred during evolution to

ensure allelic specificity (Bakkeren and Kronstad, 1993). Interestingly, when the incomplete *b* locus, encoding a truncated bE with the last 70 amino acids missed from the C-terminus, was introduced into the opposite mating type in *U. hordei*, resultant transformants could also initiate mycelia growth without mating (Bakkeren and Kronstad, 1993). Previous studies indicate that the C-terminus of the bE1 or bE2 polypeptides in *U. maydis* is not absolutely necessary for inducing mating or filamentous growth. This conclusion is based on the observations that single *bE* allele of *U. maydis* integrated in different mating type strain could induce mycelial growth and weak pathogenicity, and the *U. hordei bW* gene could functionally replace the *b* locus of *U. maydis* in terms of triggering filamentous growth, and was sufficient to trigger filamentous growth in *U. hordei* (Bakkeren and Kronstad, 1993).

In summary, our study identified a functional homolog of a mating type gene, *b* locus, in *S. scitamineum*, and confirmed that it serves as a major regulator of mating and pathogenicity in *S. scitamineum*. It remains unclear how the bE/bW heterodimer triggers such important developmental processes. The specific target genes downstream of the bE/bW transcription factor are also unknown and will be of interest in our future investigations.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.11.005.

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