The Potassium Channel Gene *BmTWK7* Contributes to Cocoon-Spinning Behavior in *Bombyx mori*

Supplementary Methods

Validation of recombinant plasmids in BmN cells

To evaluate the genome-editing activity of the gRNAs, transient transfection was performed in BmN cells. BmN cells were cultured at 27 °C and transfected at 70–90% confluence. The recombinant gRNA expression plasmid and Nos-Cas9 expression plasmid were co-transfected into BmN cells using X-tremeGENE HP DNA Transfection Reagent (Roche, Penzberg, Germany). The medium was replaced with complete culture medium 6–8 h after transfection, and the cells were collected 48 h after transfection. Genomic DNA was extracted using a TaKaRa MiniBEST Genomic DNA Extraction Kit (TaKaRa, Dalian, China). Fragments encompassing the *BmTWK7* target sites were amplified by PCR, separated by 2% agarose gel electrophoresis, gel-purified, and ligated into the pMD-19T vector. The recombinant vectors were subsequently transformed into DH5α competent cells. Positive clones were selected and subjected to Sanger sequencing to identify nucleotide changes at the target sites.

Mutation detection in individuals using the T7E1 assay.

Mutations at the BmTWK7 target sites were identified using T7 endonuclease I (T7E1; ViewSolid Biotech, Beijing, China) digestion and Sanger sequencing. PCR fragments encompassing the *BmTWK7* target sites were amplified from the individuals to be tested and from WT individuals. Equal volumes of the two PCR products were mixed and subjected to denaturation and reannealing according to the manufacturer's instructions for T7E1: 95 °C for 5 min, followed by a decrease from 95 to 75 °C at 0.1 °C/cycle and then from 75 °C to approximately 16 °C at 0.1 °C/cycle. After reannealing, 0.5 μL of T7E1 was added, and the reaction was incubated at 37 °C for 30 min. Digestion products were analyzed by 2% agarose gel electrophoresis, and gel images were analyzed using Image Lab software (Bio-Rad, CA, USA). Sanger sequencing was subsequently performed using the cloning and sequencing procedure described above. Sequence alignment was used to determine the specific nucleotide changes at the target sites and the genotype of the *BmTWK7*-KO line.

Assessment of Body weight

When WT and *BmTWK7*-KO larvae reached the beginning of the fifth instar (5L0h), females and males were reared separately in different containers, with 20 larvae per container and three biological replicates for each strain. Thereafter, the total body weight of the larvae in each container was measured at the same time each day using an analytical balance. The average body weight per larva was calculated based on the actual number of larvae in each container.

Supplementary figures

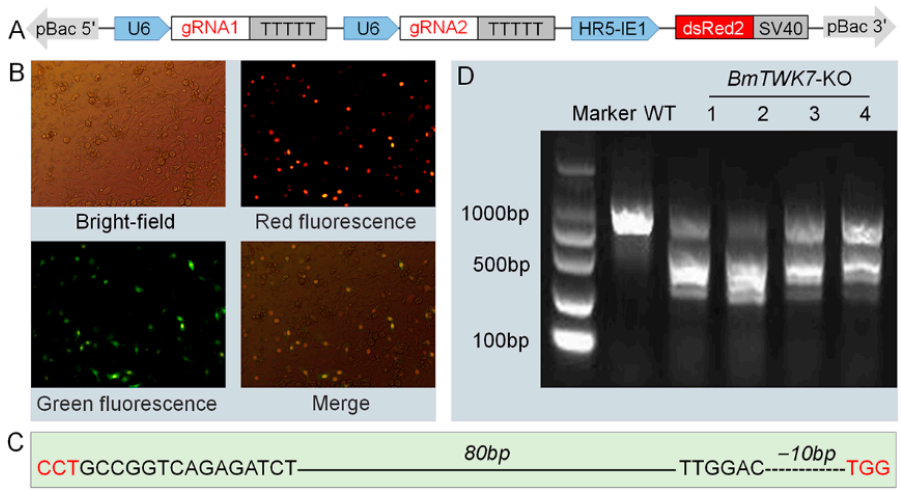


Figure S1. Validation of recombinant plasmids in BmN cells and mutation detection in individuals using the T7E1 assay. (**A**) Schematic representation of the recombinant plasmid constructs. (**B**) Fluorescence images of BmN cells co-transfected with the recombinant gRNA expression vector and the Nos-Cas9 expression vector. (**C**) Representative mutant sequences identified in co-transfected BmN cells. (**D**) T7E1 assay for mutation detection in F_1 progeny.

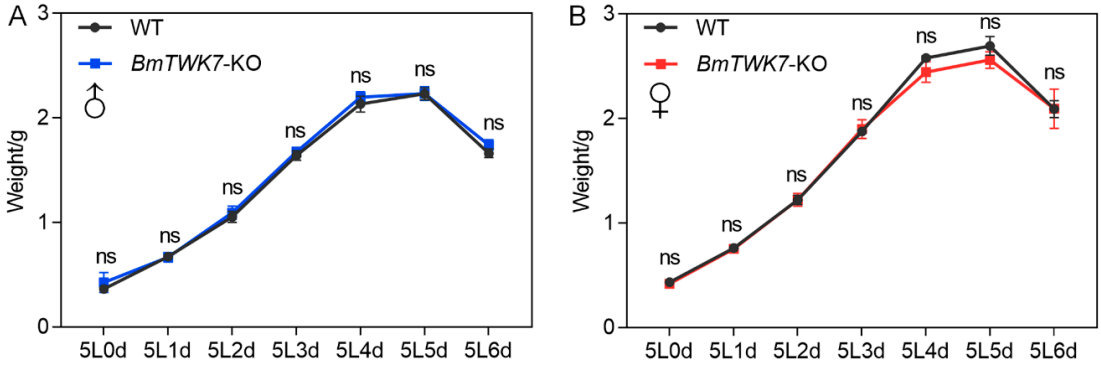


Figure S2. Effects of *BmTWK7* deficiency on body weight gain in male and female fifth-instar larvae. (**A**) Changes in body weight of WT and *BmTWK7*-KO male larvae throughout the fifth instar. (**B**) Changes in body weight of WT and *BmTWK7*-KO female larvae throughout the fifth instar.

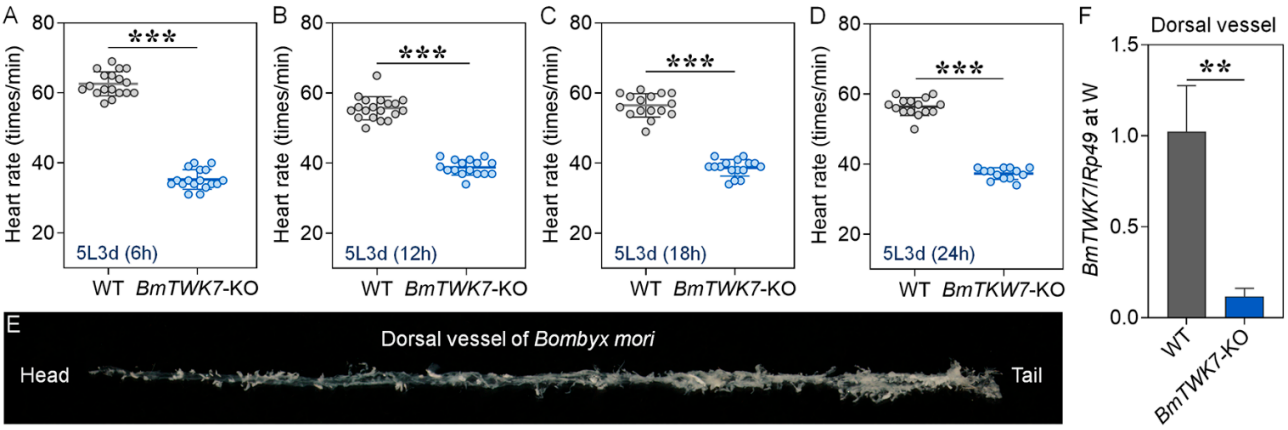


Figure S3. Effects of *BmTWK7* deficiency on heart rate and *BmTWK7* expression in the dorsal vessel of silkworm larvae, with representative dorsal vessel dissection. (**A–D**) Heart rates of WT and *BmTWK7*-KO silkworms at 5L3d (6 h), 5L3d (12 h), 5L3d (18 h) and 5L3d (24 h), measured by direct visual counting. (E) Dissection image showing the silkworm dorsal vessel, with the anterior (Head) and posterior (Tail) directions indicated. (**F**) Relative expression level of *BmTWK7* in the dorsal vessel of WT and *BmTWK7*-KO silkworms at W. Data in (A–D, F) were analyzed using Student's t-test. Data are presented as the mean ± SD; **, $p < 0.01$; ***, $p < 0.001$.

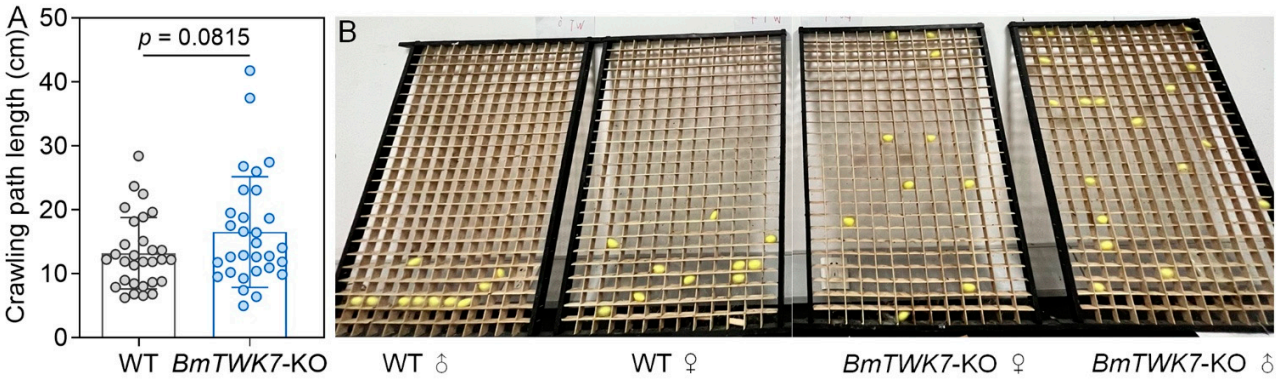


Figure S4. Quantification of crawling path length and cocooning height. (**A**) Quantification of crawling path length in WT and *BmTWK7*-KO larvae. (**B**) Original photographs used to determine the cocoon-spinning positions of WT and *BmTWK7*-KO silkworms. (**C**, **D**) Quantification of the height of the cocoon-spinning position in male and female WT and *BmTWK7*-KO silkworms, respectively.

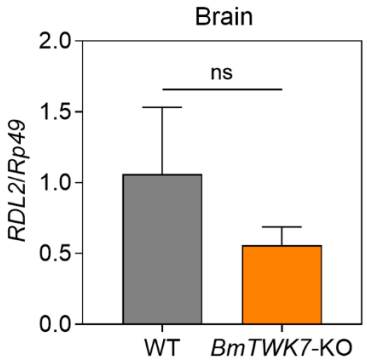


Figure S5. Relative expression level of *RDL2* in the brains of WT and *BmTWK7*-KO silkworms.

Supplementary Tables

Table S1. RT-qPCR and PCR primers sequence.

Using	Genes (*Bm*)		Primers Sequences
RT-qPCR	*Rp49*	F	GCATCAATCGGATCGCTATG
		R	GGACCTTACGGAATCCATTTG
	TWK7	F	GTTGGGTTTTTGTTTGTGTAT
		R	TGTATTTCGGTCTCTTTGGTA
	Crz	F	CGGCACGAACAGCCTATCA
		R	AACGAGACGGTCACAAGCG
	Fa	F	GCTCGGAGGAAAGTCATAAGG
		R	TCCACTCTGCCCGTCGTA
	CCAP	F	GGAGCAGTCTGGGAACG
		R	CGAACGAGGAGATGGTGA

	NPF	F	TCATCGGATTATTGGTGGT
		R	AGCCTCAACGAAGACGG
	GAD	F	TACCAAGTCAAAACCGGGCA
		R	ATCAAGATGAAGACCGGGGC
	GAT	F	TCTTCAATCTCGTGCAGTGG
		R	GTGACACGCCACAGGTACAC
	VGAT	F	GGATGGAATTGCTCCAAGAA
		R	TTTTAGCTCCTGGCTTTCCA
	GRD	F	TTATCCTTGGCAGCTACGCC
		R	CGGTGTGCCTTTTGAGGTTG
	RDL1	F	TCCAGAATGCCCTCCAG
		R	AAAACGGACTTCAGATGGTCT
	RDL2	F	TACCACCTAGCCGATCTTCG
		R	TTGTCCTCCTGCTTCTTCGT
	RDL3	F	TTCGCTACAAGGTCCGAGAT
		R	GTTCATCCTGCTCCTGCTG
	GABAT	F	CAAGACCGGAAAGGTTTTGA
		R	TCGGCCACGAACACTATGTA
	Ssadh	F	TGAATTTGGCATGGTTGCTA
		R	CCTTCACGCCCTATACCAGA
PCR	*BmTWK7*-gRNA1	F	TATCGTGCTCTACAAGTGGTAAAGAGATCTCTGACCGGCGTTTTAGAG CTAGAAATAGCAAGTTAAAATAA
		R	TAGATATCAAGCTGCTAGAAAAAAAAGCACCGACTCGGTGCCACTTT TTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTC
	BmTWK7-gRNA2	F	TATCGTGCTCTACAAGTGGATGATTGGACCTTCTACGAGTTTTAGAGCT AGAAATAGCAAGTTAAAATAA
		R	CTTATCGATACCGTCGAAAAAAAAAGCACCGACTCGGTGCCACTTTTT CAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCT
	BmTWK7-YZ	F	TTACACTGGCACAGTCACACC
		R	GACGACATTGCTAACACGAAC