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RESEARCH PAPER

Two transcriptional activators of *N*-acetylserotonin *O*-methyltransferase 2 and melatonin biosynthesis in cassava

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Abstract

Similar to the situation in animals, melatonin biosynthesis is regulated by four sequential enzymatic steps in plants. Although the melatonin synthesis genes have been identified in various plants, the upstream transcription factors of them remain unknown. In this study on cassava (*Manihot esculenta*), we found that MeWRKY79 and heat-shock transcription factor 20 (MeHsf20) targeted the W-box and the heat-stress elements (HSEs) in the promoter of *N*-acetylserotonin *O*-methyltransferase 2 (*MeASMT2*), respectively. The interaction between MeWRKY79, MeHsf20, and the MeASMT2 promoter was evidenced by the activation of promoter activity and chromatin immunoprecipitation (ChIP) in cassava protoplasts, and by an *in vitro* electrophoretic mobility shift assay (EMSA). The transcripts of *MeWRKY79*, *MeHsf20*, and *MeASMT2* were all regulated by a 22-amino acid flagellin peptide (flg22) and by *Xanthomonas axonopodis* pv *manihotis* (*Xam*). In common with the phenotype of *MeASMT2*, transient expression of *MeWRKY79* and *MeHsf20* in *Nicotiana benthamiana* leaves conferred improved disease resistance. Through virus-induced gene silencing (VIGS) in cassava, we found that *MeWRKY79*- and *MeHsf20*-silenced plants showed lower transcripts of *MeASMT2* and less accumulation of melatonin, which resulted in disease sensitivity that could be reversed by exogenous melatonin. Taken together, these results indicate that *MeASMT2* is a target of MeWRKY79 and MeHsf20 in plant disease resistance. This study identifies novel upstream transcription factors of melatonin synthesis genes in cassava, thus extending our knowledge of the complex modulation of melatonin synthesis in plant defense.

Key words: Cassava, disease resistance, heat-shock transcription factor 20, Hsf20, melatonin, *N*-acetylserotonin *O*-methyltransferase, WRKY79.

Introduction

As an important animal hormone, *N*-acetyl-5-methoxytryptamine (melatonin) was first discovered in the pineal gland of cows in 1958 (Lerner *et al.*, 1958). About four decades years later, melatonin was identified in plants in 1995 (Dubbels *et al.*, 1995; Hattori *et al.*, 1995). Because of the increased awareness of the importance of melatonin in human health and its potential utility in food, beverage, and agriculture industries, research related to melatonin in plants has significantly increased in recent years (Arnao and Hernández-Ruiz, 2014; Hardeland, 2015, 2016).

Although concentrations differ in various organs, at different developmental stages, and under different growth conditions, melatonin has been identified and quantified in almost all plant species using techniques including

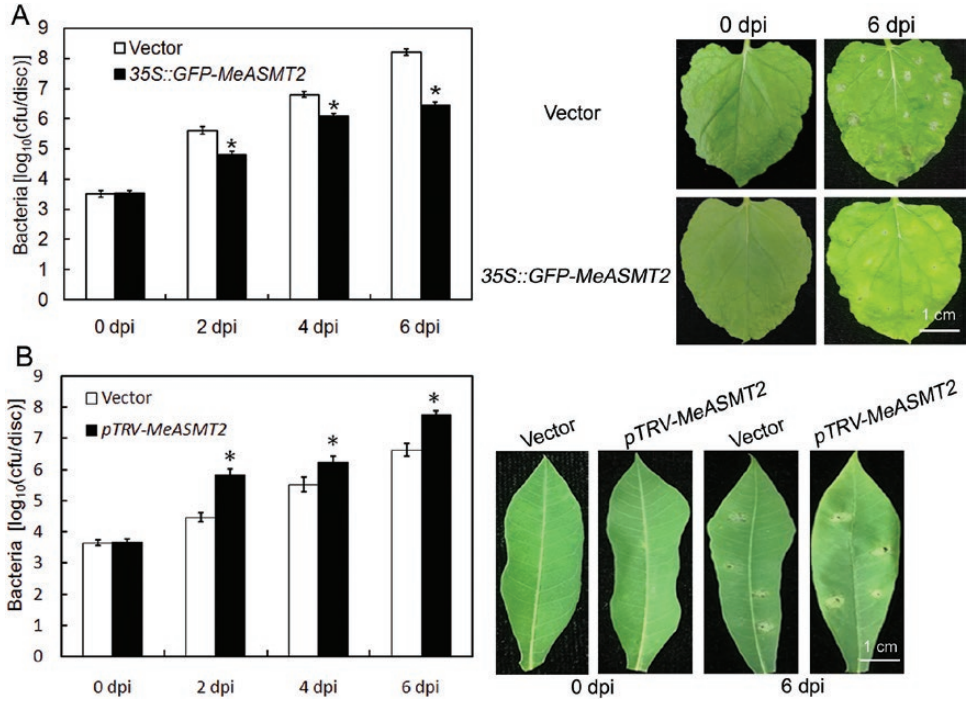

Fig. 1. *MeASMT2* confers improved disease resistance against cassava bacterial blight. (A) Transient expression of *MeASMT2* in *Nicotiana benthamiana* leaves conferred enhanced disease resistance against cassava bacterial blight. After transient expression for 2 d in the leaves, 10^8 colony-forming units (cfu) ml^{-1} of *Xam* were syringe infiltrated. (B) *MeASMT2*-silenced cassava plants produced by VIGS resulted in decreased disease resistance against *Xam*. The *Agrobacterium tumefaciens* GV3101 cell culture harbouring the pTRV2 or *MeASMT2*-pTRV2 together with pTRV1 were mixed with ratio of 1:1 and syringe-infiltrated into cassava leaves. At 14 d post-inoculation (dpi), the leaves were used for gene expression assay and 10^8 cfu ml^{-1} of *Xam* syringe-infiltration. At least three biological replicates were included in the data, with about 20 leaves in every biological repeat. All data were analysed for significant differences using ANOVA and Student's *t*-test: *, $P<0.05$. (This figure is available in colour at *JXB* online.)

radioimmunoassays, gas chromatography/mass spectrometry (GC/MS), and enzyme-linked immunosorbent assays (ELISA) (Arnao and Hernández-Ruiz, 2014; Hardeland, 2015, 2016). To date, melatonin has been discovered in, for example, fruits (apple, banana, cucumber, sweet cherry, tomato) (Okazaki and Ezura, 2009; Posmyk *et al.*, 2009; Okazaki *et al.*, 2010; Wang *et al.*, 2012; Zhao *et al.*, 2013), plant-derived beverages (coffee, tea) (Hardeland, 2016), model plants (*Arabidopsis*, tobacco) (Shi and Chan, 2014; Shi *et al.*, 2015*a*, 2015*b*, 2015*c*, 2015*d*, 2016*a*, 2016*b*), and crops (rice, corn) (Byeon *et al.*, 2012). As a result of its wide distribution, the functions of melatonin in animal and plants have largely been determined. Comprehensive studies have found that melatonin is widely involved in plant development and stress responses, including seed germination (Tiryaki and Keles, 2012), root formation (Pelagio-Flores *et al.*, 2012; Zhang *et al.*, 2013, 2014), floral transition (Shi *et al.*, 2016*b*), leaf and fruit ripening and senescence (Wang *et al.*, 2012, 2013*a*, 2013*b*; Shi *et al.*, 2015c; Sun *et al.*, 2015), post-harvest physiological deterioration (Hu *et al.*, 2016; Ma *et al.*, 2016), circadian rhythms (Shi *et al.*, 2016*a*), yield, innate immunity (Yin *et al.*, 2013; Lee *et al.*, 2014, 2015, 2016; Shi *et al.*, 2015a; Zhao *et al.*, 2015), and responses to cold (Posmyk *et al.*, 2009; Bajwa *et al.*, 2014; Shi and Chan, 2014), heat (Shi *et al.*, 2015*d*), salt (Liang *et al.*, 2015), drought (Shi *et al.*, 2015*b*), oxidative (Shi *et al.*, 2015e; Zhang *et al.*, 2015) and cadmium stress (Byeon *et al.*, 2015).

The melatonin biosynthetic and metabolic pathways have largely been revealed in plants (Tan *et al.*, 2014). In common with animals, melatonin biosynthesis in plants begins from tryptophan (Kang *et al.*, 2007, 2010, 2011, 2013; Park *et al.*, 2013; Byeon and Back, 2014; Zuo *et al.*, 2014). At least four sequential enzymatic steps are responsible for melatonin biosynthesis in plants, including tryptophan decarboxylase (TDC), tryptamine 5-hydroxylase (T5H), serotonin *N*-acetyltransferase (SNAT), and *N*-aceylserotonin methyltransferase (ASMT) (Tan *et al.*, 2016; Wei *et al.*, 2016*b*). Thereafter, melatonin 2-hydroxylase (M2H) is responsible for melatonin metabolism, through the conversion of melatonin to 2-hydroxymelatonin (Byeon and Back, 2015). By constructing transgenic plants with overexpression or knockdown of these genes, the effects of endogenous melatonin levels as well as their related phenotype have been shown in various species (Kang *et al.*, 2007, 2010, 2011, 2013; Park *et al.*, 2013; Byeon and Back, 2014, 2015; Zuo *et al.*, 2014).

It is notable that melatonin is a universal molecule in plants, with endogenous levels from almost undetectable to high concentrations (Arnao and Hernández-Ruiz, 2014; Hardeland, 2015, 2016). It is maintained at a relative low level under normal conditions, but it can be greatly and rapidly induced in response to unfavorable conditions, such as cold, heat, salt, drought, oxidative and nutrient stress, and bacterial infection (Shi *et al.*, 2015*a*, 2015*b*, 2015*c*, 2015*d*, 2015*e*; Zhang *et al.*, 2015). This may be a defense strategy of plants, with the capacity to modulate melatonin production helping their ability to survive and to thrive (Arnao and Hernández-Ruiz, 2015;

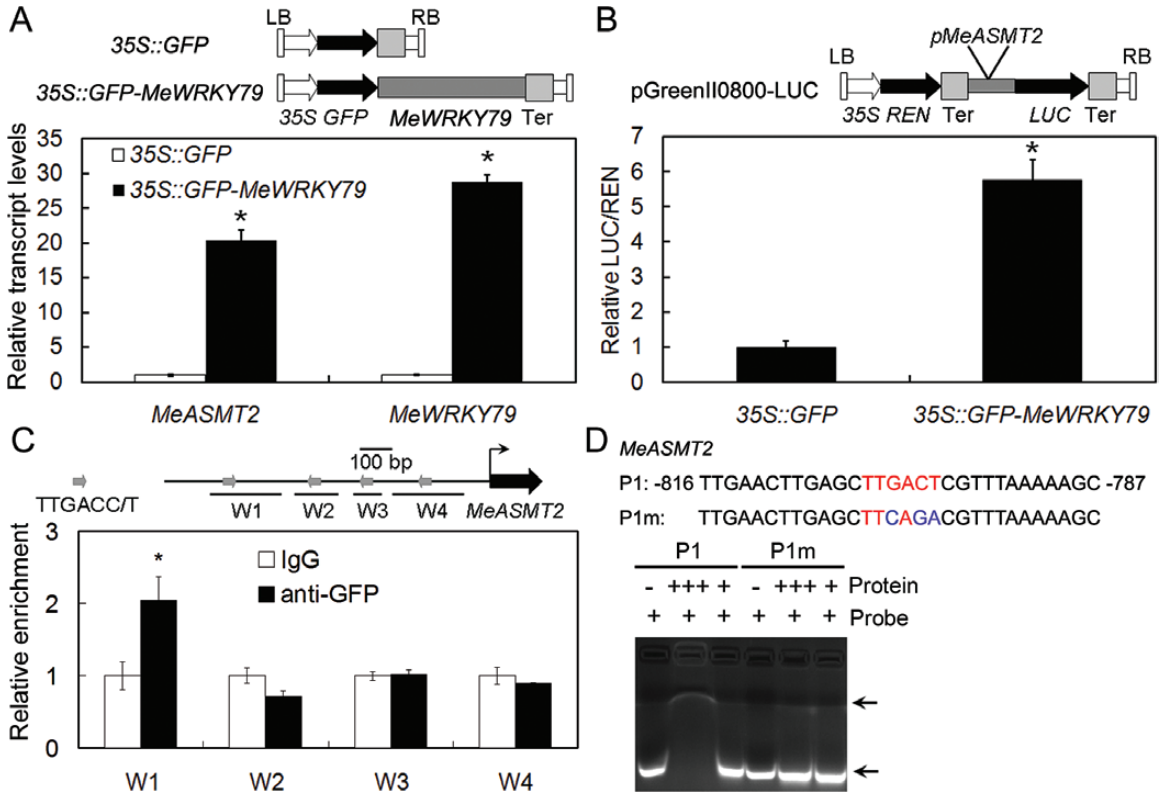

Fig. 2. MeWRKY79 is a transcriptional activator of *MeASMT2* in cassava. (A) Transcript levels of *MeASMT2* in cassava leaf protoplasts with transient expression of pEGAD or *MeWRKY79*-pEGAD. (B) The relative LUC activity of the *MeASMT2* promoter in cassava leaf protoplasts with transient expression of pEGAD or *MeWRKY79*-pEGAD. (C) Enrichment of the *MeASMT2* promoter fragment in ChIP-PCR assays from cassava leaf protoplasts. The regions of the four W-boxes were successively: –622~–839; –468~–602; –348~–435; and –76~–297. (D) Binding of the probe containing the W-box of the *MeASMT2* promoter to MeWRKY79 in EMSA. The probe sequences of the wild-type and the mutation are listed. The wild-type probe sequence of P1 contains 30 bp of the *MeASMT2* promoter with the W-box (TTGACT), and the mutation probe sequence of P1m changes the W-box to TTCAGA. All data were analysed for significant differences using ANOVA and Student's *t*-test: *, $P<0.05$. (This figure is available in colour at *JXB* online.)

Reiter *et al.*, 2015). Hence it is very important to investigate the complex regulation of endogenous melatonin in plants and the underlying mechanisms, for example the translation and post-translational regulation of melatonin synthesis enzymes, and the upstream transcription factors of these rate-limiting enzymes or isoenzymes (Ganguly *et al.*, 2001, 2005).

In a previous study (Wei *et al.*, 2016*a*), we identified three *N*-acetylserotonin *O*-methyltransferase genes (*MeASMT*s) in cassava (*Manihot esculenta*), an important tropical crop (Wang *et al.*, 2014). Overexpression of each gene was found to increase endogenous melatonin in *Nicotiana benthamiana* leaves. In this current study, the upstream transcription factors of MeASMT2 were first identified in cassava, and *in vivo*, *in vitro*, and genetic studies determined that *MeASMT2* is a target of MeWRKY79 and heat-shock transcription factor 20 (MeHsf20) in disease resistance. This is the first study that identifies novel upstream transcription factors of a melatonin synthesis gene in plants, and it helps to extend our knowledge of complex modulation of melatonin synthesis as well as plant melatonin signaling.

Materials and methods

Plant materials and growth conditions

Cassava (*Manihot esculenta*) variety South China 124 (SC124) and *Nicotiana benthamiana* were used in this study. The plants were grown in soil with Hoagland's solution in a controlled-environment greenhouse with 12/12 h light/ dark at 28/25 °C and irradiance of about 120–150 µmol quanta m^{-2} s^{-1}.

RNA isolation and quantitative real-time PCR

Total RNA in plant leaves was extracted using RNAprep Pure Plant Kit (Tiangen, DP441, Beijing, China) together with RNase-free DNase (NEB, M0303S, USA), according to the manufacturers' protocols. First-strand cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit and further diluted and quantified using NANODROP 2000 (both Thermo Scientific, Waltham, MA, USA). Quantitative real-time PCR was then performed in a LightCycler® 96 Real-Time PCR System (Roche, Basel, Switzerland) using the diluted cDNA and TransStart Tip Green qPCR SuperMix (TransGen Biotech, AQ141, Beijing, China), and was further analysed using the comparative $2^{-\Delta\Delta CT}$ method. The primers are listed in Supplementary Table S1 at *JXB* online.

Vector construction and transient expression in Nicotiana benthamiana *leaves*

For the transient expression, the coding sequences of *MeWRKY79* and heat-shock transcription factor 20 (*MeHsf20*) were amplified by PCR and cloned into the pEGAD vector by restriction enzyme digestion and ligase (Cutler *et al.*, 2004). For the luciferase (LUC) reporter construct, the promoter sequence of *MeASMT2* was amplified by PCR and cloned into the pGreenII0800-LUC vector. For the protein expression and induction, the coding sequences of *MeWRKY79* and *MeHsf20* were amplified by PCR and cloned into the pET28a vector. After verification by DNA sequencing for

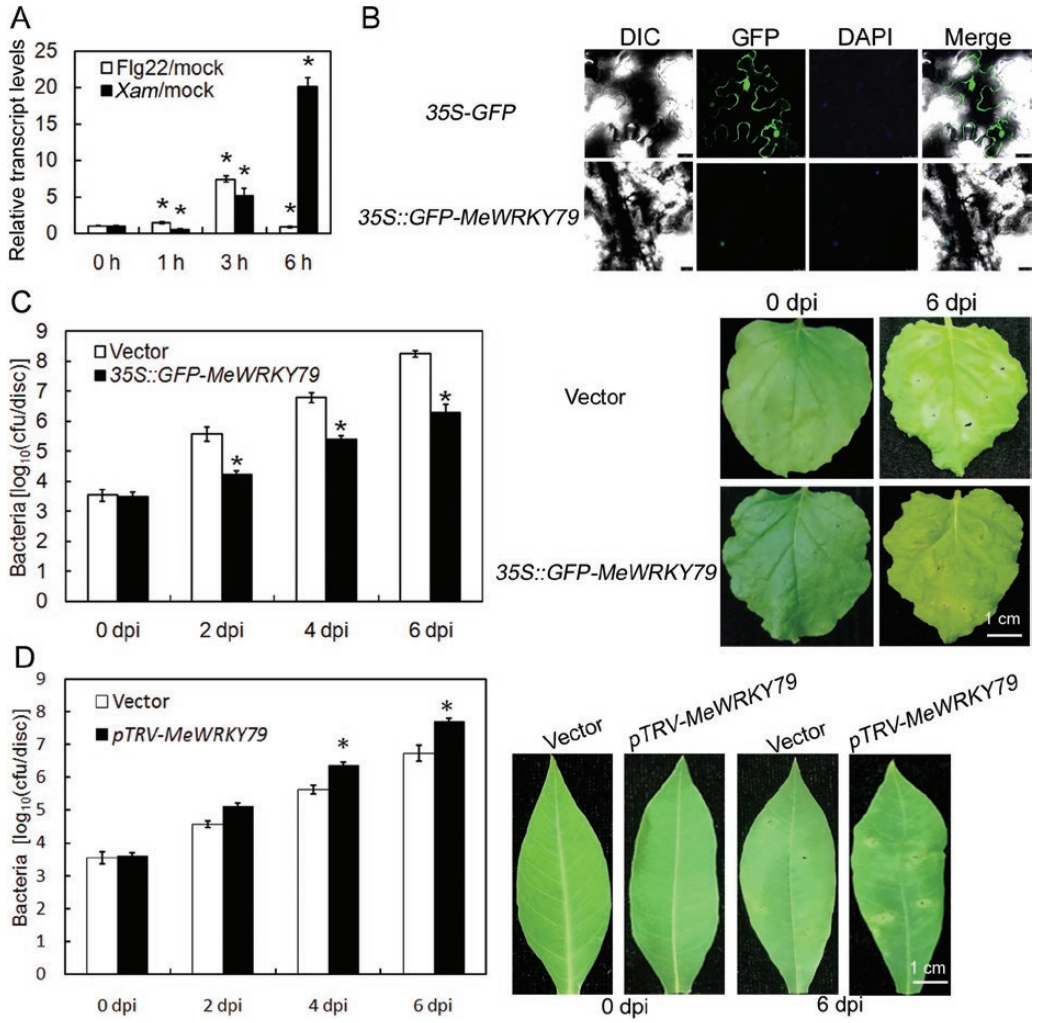

Fig. 3. *MeWRKY79* confers improved disease resistance against cassava bacterial blight. (A) The expression patterns of *MeWRKY79* in response to treatment with flg22 and *Xam*. For the quantitative real-time PCR assay, leaves of 30-d-old cassava (SC124 variety) were treated with water (mock), 10 μM flg22, or 10^8 cfu ml^{-1} of *Xam* for 0, 1, 3, and 6 h before for sampling. (B) Subcellular localization of MeWRKY79 in *Nicotiana benthamiana* leaves through transient expression. GFP-fused MeWRKY79 and 1 μg ml^{-1} DAPI-stained cell nuclei were detected using a confocal laser-scanning microscope. Scale bar = 25 μm. (C) Transient expression of *MeWRKY79* in *N. benthamiana* leaves conferred enhanced disease resistance against cassava bacterial blight. After transient expression for 2 d in the leaves, 10^8 cfu ml^{-1} of *Xam* was syringe-infiltrated. (D) *MeWRKY79*-silenced cassava plants resulted in decreased disease resistance against *Xam*. The *Agrobacterium tumefaciens* GV3101 cell culture harbouring the pTRV2 or *MeWRKY79*-pTRV2 together with pTRV1 were mixed with ratio of 1:1 and syringe-infiltrated into cassava leaves. At 14 dpi, the leaves were used for gene expression assay and syringe-infiltration of 10^8 cfu ml^{-1} of *Xam*. At least three biological replications were included in the data, with about 20 leaves in every biological repeat. All data were analysed for significant differences using ANOVA and Student's *t*-tests: *, $P<0.05$. (This figure is available in colour at *JXB* online.)

confirmation, the recombinant plasmids were used for further analysis. The primers are listed in Supplementary Table S2.

For the transient expression, the *Agrobacterium tumefaciens* GV3101 cell culture harbouring the *35S::green fluorescent protein* (GFP), *35S::GFP-MeWRKY79*, *35S::GFP-MeHsf20*, or *35S::GFP-MeASMT2* together with P19 were infiltrated into *N. benthamiana* leaves, as described by Sparkes *et al.* (2006). At 2 d post-infiltration (dpi), green fluorescence in the transformed *N. benthamiana* leaves was visualized using a confocal laser-scanning microscope (TCS SP8, Leica, Heidelberg, Germany).

Transient expression assay of dual LUC reporter gene and chromatin immunoprecipitation (ChIP)

Transient expression assays in cassava protoplasts were performed to investigate the interaction between MeWRKY79 or MeHsf20 and the MeASMT2 promoter as described in Yoo *et al.* (2007). For the LUC assay, the plasmid of pEGAD or MeWRKY79-pEGAD or MeHsf20-pEGAD was used as the effector, and *35::REN-pMeASMT2::LUC* (pMeASMT2-pGreenII0800-LUC) was used as the reporter. The *Renilla* luciferase and firefly luciferase in the transformed protoplasts were assayed using the Dual Luciferase Reporter Gene Assay Kit (Beyotime, RG027, Haimen City, Jiangsu Province, China), according to the manufacturer's protocol.

For the ChIP assay, pEGAD or MeWTKY3-pEGAD or MeHsf20-pEGAD was transformed into cassava protoplasts, and then the protoplasts were harvested for chromatin pellet extraction and following nuclease digestion as described by O'Neill and Turner (2003). The anti-GFP antibody (AG281, Beyotime, China) was used to immunoprecipitate the MeWRKY– or MeHsf20–DNA complex, and the DNA fragment enrichment was analysed by quantitative real-time PCR using the specific primers listed in Supplementary Table S2.

Electrophoretic mobility shift assay (EMSA)

The recombinant protein of MeWRKY79- or MeHsf20-pET28a was expressed and induced in *Escherichia coli* strain BL21 (DE3). After sequence synthesis, the responding probes were vortexed and

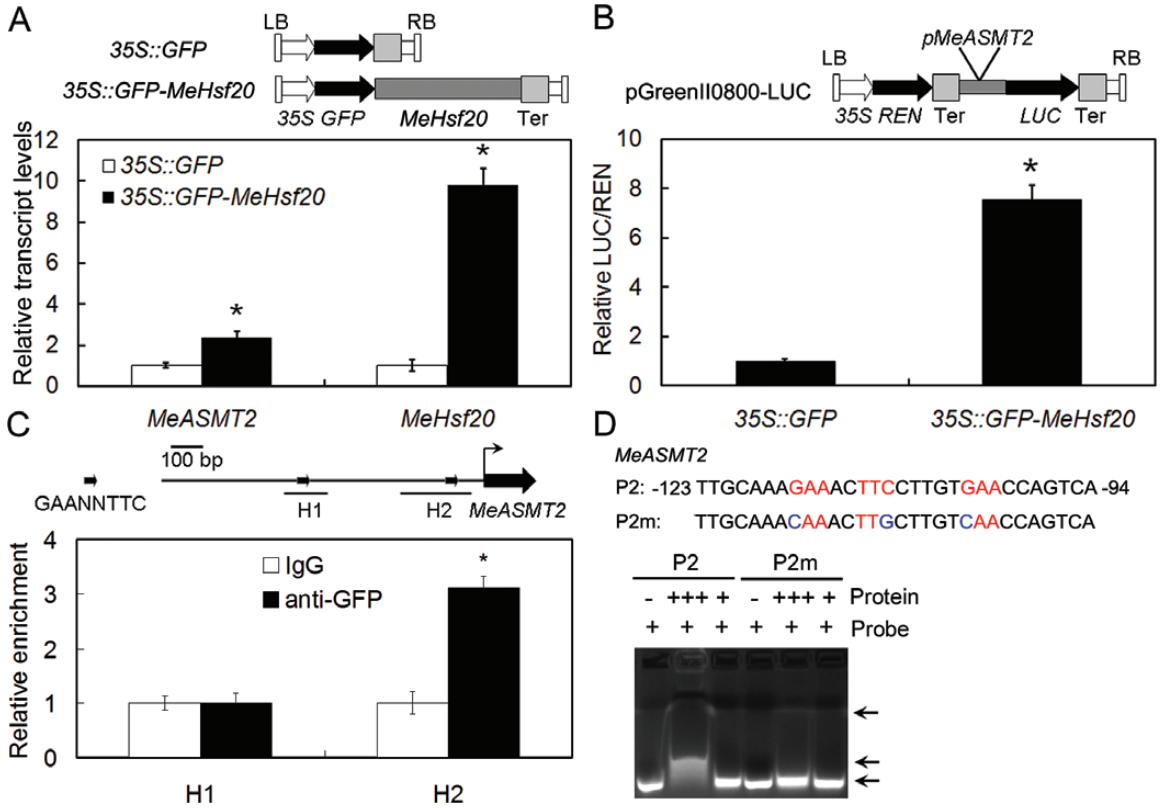

Fig. 4. MeHsf20 is a transcriptional activator of *MeASMT2* in cassava. (A) Transcript levels of *MeASMT2* in cassava leaf protoplasts with transient expression of pEGAD or *MeHsf20*-pEGAD. (B) The relative LUC activity of the *MeASMT2* promoter in cassava leaf protoplasts with transient expression of pEGAD or *MeHsf20*-pEGAD. (C) Enrichment of the *MeASMT2* promoter fragment in the ChIP-PCR assay from cassava leaf protoplasts. The regions of the heat-stress elements H1 and H2 were –468~–602 and –76~–297, respectively. (D) Binding of the probe containing HSEs of the *MeASMT2* promoter to MeHsf20 in EMSA. The probe sequences of the wild-type and the mutation are listed. The wild-type probe sequence of P2 contains 30 bp of the *MeASMT2* promoter with HSEs (GAAACTTCCTTGTGAA), and the mutation probe sequence of P2m changes the HSEs to CAAACTTGCTTGTCAA. All data were analysed for significant differences using ANOVA and Student's *t*-tests: *, $P<0.05$. (This figure is available in colour at *JXB* online.)

incubated with 10 µg protein at 25 °C for 2 h. Then the complexes of recombinant protein of MeWRKY79- or MeHsf20-pET28a and the probes were analysed in 2% (w/v) garose gel as described by Ream *et al.* (2016), followed by visualization using a GelDoc imaging system (BIO-RAD, Hercules, California, USA).

Virus-induced gene silencing (VIGS) in cassava

The pTRV1 and pTRV2 vectors (Liu *et al.*, 2002) were used for VIGS in cassava. The partial coding sequences of *MeWRKY79*, *MeHsf20*, and *MeASMT2* were amplified by PCR and cloned into the pTRV2 vector by restriction enzyme digestion and ligase. The primers used are listed in Supplementary Table S2. The *Agrobacterium tumefaciens* GV3101 cell culture harbouring the pTRV2, *MeWRKY79*-pTRV2, *MeHsf20*-pTRV2, or *MeASMT2*-pTRV2 together with pTRV1 were mixed with ratio of 1:1 and syringe-infiltrated into cassava leaves as described by Liu *et al.* (2002). At 14 dpi, the infiltrated cassava leaves were used for gene expression, melatonin, and disease-resistance assays.

Quantification of plant melatonin level

Plant melatonin was extracted and quantified using a plant melatonin enzyme-linked immunosorbent assay (ELISA) Kit (Jianglai Biotechnology, Shanghai, China), as described by Wei *et al.* (2016*a*).

Statistical analysis

At least three biological replicates were included in the data, and all data were analysed using ANOVA and Student's *t*-test for determination of significant differences.

Accession numbers

The accession numbers of genes are as follows: *MeASMT2* (Me17G050500), *MeWRKY79* (KT827654), *MeHsf20* (Me08G051600).

Results

MeASMT2 *positively regulates disease resistance against cassava bacterial blight*

Through transient expression in *N. benthamiana* leaves, we found that overexpression of *MeASMT2* conferred enhanced disease resistance against cassava bacterial blight, which results from infection by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) (Fig. 1A). Through use of VIGS in cassava, we found that *MeASMT2*-silenced plants exhibited decreased disease resistance against *Xam* (Fig. 1B). These results indicate that *MeASMT2* positively regulates disease resistance against cassava bacterial blight.

MeWRKY79 directly regulates MeASMT2 *expression and confers disease resistance*

Because four W-box elements (TTGACC/T) were enriched in the *MeASMT2* promoter (see Supplementary Fig. S1),

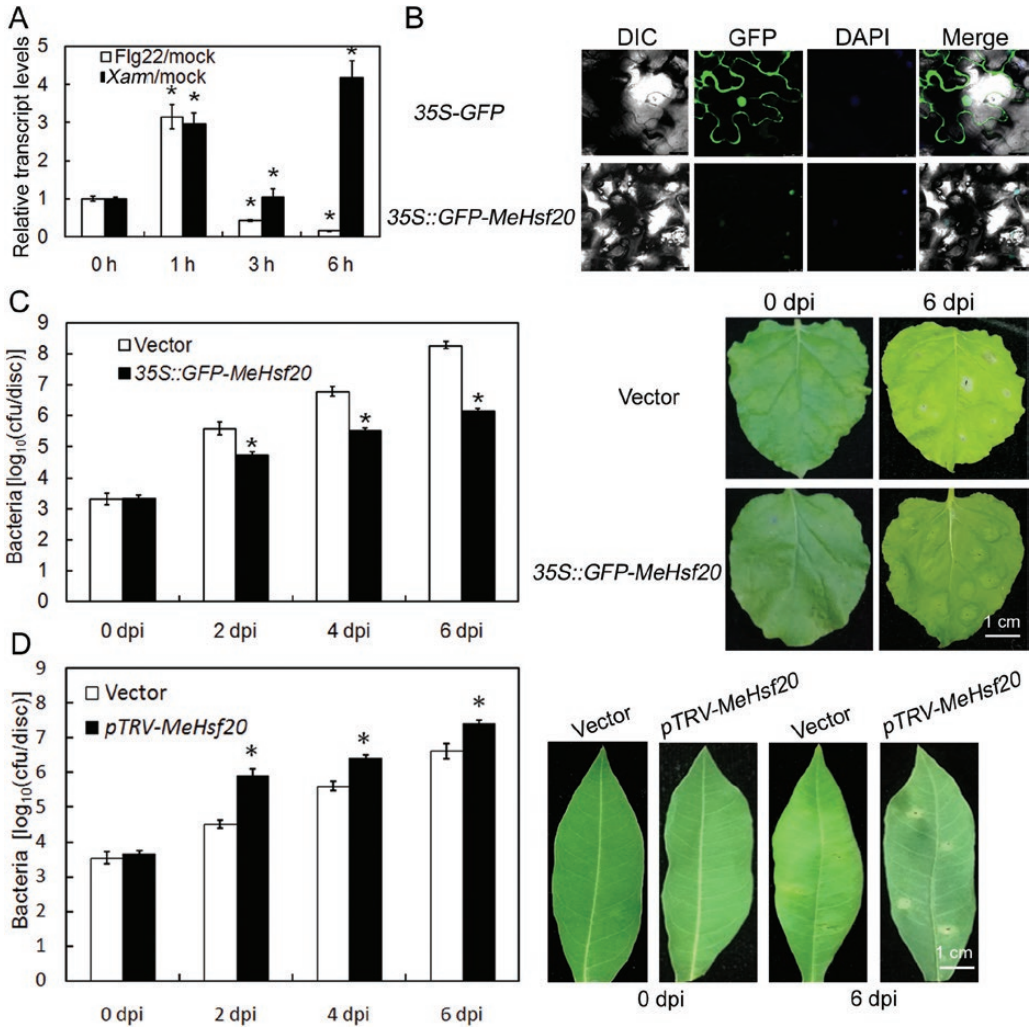

Fig. 5. *MeHsf20* confers improved disease resistance against cassava bacterial blight. (A) Expression patterns of *MeHsf20* in response to treatment with flg22 and *Xam*. For the quantitative real-time PCR assay, leaves of 30-d-old cassava (SC124 variety) were treated with water (mock), 10 μM flg22, or 10^8 cfu ml^{-1} of *Xam* for 0, 1, 3 and 6 h before sampling. (B) Subcellular localization of MeHsf20 in *Nicotiana benthamiana* leaves through transient expression. GFP-fused MeHsf20 and 1 μg ml^{-1} DAPI-stained cell nuclei were detected using a confocal laser-scanning microscope. Scale bar = 25 μm. (C) Transient expression of *MeHsf20* in *N. benthamiana* leaves conferred enhanced disease resistance against cassava bacterial blight. After transient expression for 2 d in the leaves, 10^8 cfu ml^{-11} of *Xam* was syringe-infiltrated. (D) *MeHsf20*-silenced cassava plants resulted in decreased disease resistance against *Xam*. The *Agrobacterium tumefaciens* GV3101 cell culture harbouring the pTRV2 or *MeHsf20*-pTRV2 together with pTRV1 were mixed with ratio of 1:1 and syringe-infiltrated into cassava leaves. At 14 dpi, the leaves were used for gene expression assay and syringe-infiltration of 10^8 cfu ml^{-1} of *Xam*. At least three biological replications were included in the data, with about 20 leaves in every biological repeat. All data were analysed for significant differences using ANOVA and Student's *t*-tests: *, $P<0.05$. (This figure is available in colour at *JXB* online.)

we identified the upstream regulators of *MeASMT2* in this study. Quantitative real-time PCR showed that *MeWRKY79* overexpression in cassava protoplasts increased the mRNA level of *MeASMT2* (Fig. 2A). A dual LUC reporter assay indicated that *MeWRKY79* overexpression in the protoplasts resulted in promoter activity of *MeASMT2* (Fig. 2B). To further determine which W-box was responsible for MeWRKY79 binding, four fragments including each W-box were chosen for ChIP analysis. ChIP-PCR found that the W1 fragment but not W2, W3, and W4 was enriched in MeWRKY79 immunoprecipitated fragments (Fig. 2C). Moreover, EMSA showed that MeWRKY79 directly binds to the P1 but not the mutated P1m that was included in the promoter of *MeASMT2* (Fig. 2D). These results indicate that MeWRKY79 positively regulates *MeASMT2* expression through directly activating its promoter.

Through quantitative real-time PCR, we found that the mRNA level of *MeWRKY79* was significantly regulated by treatments with a 22-amino acid flagellin peptide (flg22) and with *Xam* (Fig. 3A). Using transient expression of GFP-fused MeWRKY79, we found that MeWRKY79 was specifically located in the cell nucleus (Fig. 3B). Through transient expression in *N. benthamiana* leaves, we found that overexpression of *MeWRKY79* conferred enhanced disease resistance against cassava bacterial blight (Fig. 3C). VIGS in cassava showed that *MeWRKY79*-silenced plants exhibited decreased disease resistance against *Xam* (Fig. 3D). These results suggest that *MeWRKY79* positively regulates disease resistance against cassava bacterial blight.

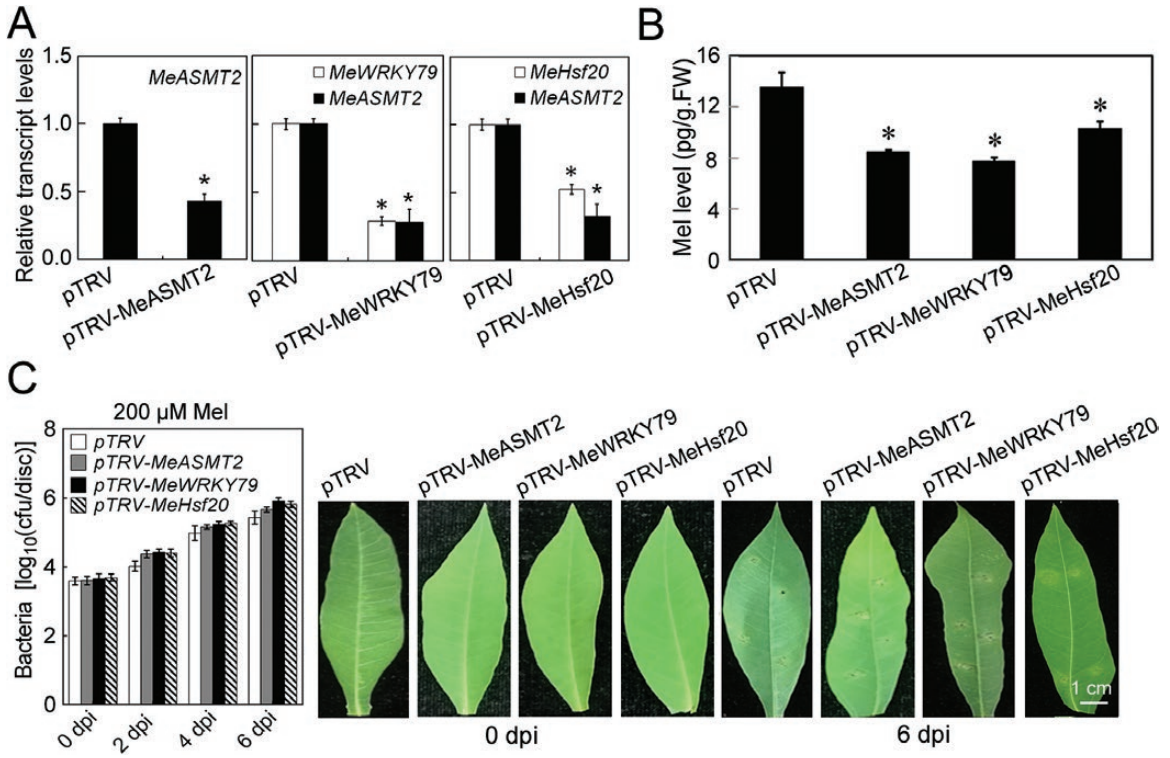

Fig. 6. *MeWRKY79* and *MeHsf20* regulate disease resistance against cassava bacterial blight through modulation of melatonin in cassava. (A) Transcript levels of *MeASMT2* in cassava leaves with transient expression of pTRV2, *MeASMT2*-pTRV2, *MeWRKY79*-pTRV2, or *MeHsf20*-pTRV2. (B) Endogenous melatonin levels of *MeASMT2*-, *MeWRKY79*-, and *MeHsf20*-silenced cassava plants. The *Agrobacterium tumefaciens* GV3101 cell culture harbouring the pTRV2 or *MeHsf20*-pTRV2 together with pTRV1 were mixed with ratio of 1:1 and syringe-infiltrated into cassava leaves. At 14 dpi, the leaves were used for gene expression and endogenous melatonin assay. (C) Exogenous melatonin restores disease resistance to the same level as controls in *MeASMT2*-, *MeWRKY79*-, and *MeHsf20*-silenced cassava plants. At 14 dpi of pTRV syringe-infiltration, the cassava leaves were sprayed with 200 μM melatonin, and 2 d later the leaves were used for 10^8 cfu ml^{-1} of *Xam* syringe-infiltration and further disease resistance assays. At least three biological replications were included in the data, with about 20 leaves in every biological repeat. All data were analysed for significant differences using ANOVA and Student's *t*-tests: *, $P<0.05$. (This figure is available in colour at *JXB* online.)

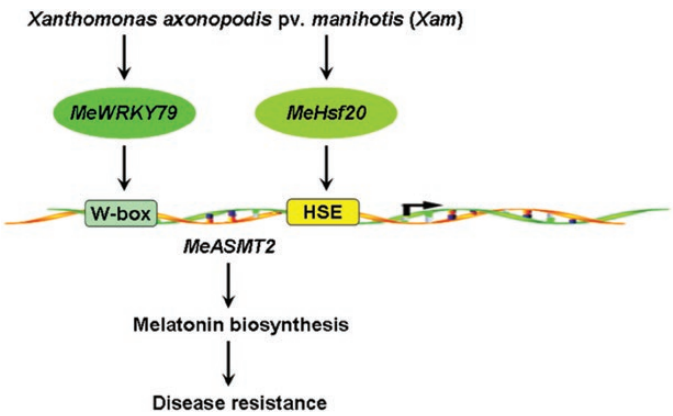

Fig. 7. A proposed model for a MeWRKY79- and MeHsf20-*MeASMT2* transcriptional module in disease resistance in cassava. (This figure is available in colour at *JXB* online.)

MeHsf20 directly regulates MeASMT2 *expression and confers disease resistance*

Because two heat-stress elements (HSEs, 5′-GAAnnTTC-3′) were enriched in the *MeASMT2* promoter (see Supplementary Fig. S1), we investigated the effect of MeHsf20 overexpression on the expression and promoter activity of *MeASMT2*. Quantitative real-time PCR showed that *MeHsf20* overexpression in cassava protoplasts increased the mRNA level of *MeASMT2* (Fig. 4A). A dual LUC reporter assay revealed that *MeHsf20* overexpression in the protoplasts resulted in promoter activity of *MeASMT2* (Fig. 4B). To further determine which HSE was responsible for MeHsf20 binding, two fragments including each HSE were chosen for ChIP analysis. ChIP-PCR showed that the H2 fragment but not H1 was enriched in MeHsf20 immunoprecipitated fragments (Fig. 4C). Moreover, EMSA showed that MeHsf20 directly bound to the P2 but not the mutated P2m that was included in the promoter of *MeASMT2* (Fig. 4D). These results indicate that MeHsf20 positively regulates *MeASMT2* expression through directly activating its promoter.

Through quantitative real-time PCR, we found that the mRNA level of *MeHsf20* was significantly regulated by flg22 and *Xam* treatments (Fig. 5A). Transient expression of GFP-fused MeHsf20 showed that MeHsf20 was specifically located in the cell nucleus (Fig. 5B). Transient expression in *N. benthamiana* leaves showed that overexpression of *MeHsf20* conferred enhanced disease resistance against cassava bacterial blight (Fig. 5C). Through VIGS in cassava, we found that *MeHsf20*-silenced plants exhibited decreased disease resistance against *Xam* (Fig. 5D). These results suggest that *MeHsf20* positively regulates disease resistance against cassava bacterial blight.

MeWRKY79 *and* MeHsf20 *regulate disease resistance through modulation of melatonin in cassava*

Consistent with the lower transcription of *MeASMT2* in *MeWRKY79*- and *MeHsf20*-silenced cassava plants (Fig. 6A), *MeWRKY79*- and *MeHsf20*-silenced plants accumulated less endogenous melatonin (Fig. 6B). Although *MeASMT2*-, *MeWRKY79*-, and *MeHsf20*-silenced plants exhibited decreased disease resistance against *Xam*, exogenous melatonin restored the resistance of these plants to the same level as the control (Fig. 6C). Thus, *MeWRKY79* and *MeHsf20* may regulate disease resistance through modulation of melatonin in cassava.

Discussion

In recent years, melatonin (*N*-acetyl-5-methoxytryptamine) has been shown to have wide involvement in plant development and stress responses. In both plants and animals, four sequential enzymes (TDC, T5H, SNAT, and ASMT/HIOMT) are responsible for melatonin biosynthesis (Tan *et al.*, 2016; Wei *et al.*, 2016*b*). Although the melatonin synthesis genes have been identified in various plants, their upstream transcription factors remain unknown. Recently, Cai *et al.* (2017) found that Hsf1a confers cadmium tolerance by activating caffeic acid *O*-methyltransferase 1 (*COMT1*) and inducing melatonin accumulation. Although COMT has a potent gene to alter melatonin levels as well as cadmium tolerance in tomato, the relationship between COMT and pathogen resistance was not considered in Cai *et al.* (2017). In our previous study (Wei *et al.*, 2016*a*), we identified seven melatonin biosynthesis genes in cassava (three *MeASMT*s, two *MeTDC*s, one *MeSNAT*, and one *MeT5H*), and overexpression of each gene increased endogenous melatonin in *Nicotiana benthamiana* leaves. In this current study, we found that MeWRKY79 and MeHsf20 target the W-box and HSEs in the promoter of *MeASMT2*, respectively, as evidenced by a transient expression assay of promoter activity and ChIP in cassava protoplasts, and *in vitro* EMSA. This study identified novel upstream transcription factors of melatonin synthesis genes in cassava, thus extending our knowledge of the complex modulation of melatonin synthesis. So far, no COMT has been identified and cloned in cassava: future identification and comparison of COMT with the seven melatonin biosynthesis genes may provide more clues to the mechanisms of melatonin synthesis and signaling in cassava.

The transcripts of *MeWRKY79*, *MeHsf20*, and *MeASMT2* were all regulated by flg22 and *Xam*. Through transient overexpression in *N. benthamiana* leaves and VIGS in cassava, we found that *MeWRKY79*, *MeHsf20*, and *MeASMT2* positively regulated disease resistance against cassava bacterial blight. When *MeWRKY79*, *MeHsf20*, or *MeASMT2* was transiently overexpressed in *N. benthamiana* leaves, the transcript levels of *Pathogen-related 1* (*PR1*), *PR2*, and *PR5* were largely activated in comparison to vector-transformed leaves (see Supplementary Fig. S2). In contrast, *MeWRKY79*-, *MeHsf20*-, and *MeASMT2*-silenced cassava plants showed significantly lower transcripts of *MePR*s than mock plants under both control and pathogen-infected conditions (Supplementary Fig. S2). Thus, the results for expression of defense genes in the overexpressed and silenced plants were consistent with the results for pathogen resistance. Notably, *MeWRKY79*- and *MeHsf20*-silenced cassava plants showed lower transcripts of *MeASMT2* and accumulated less endogenous melatonin, and the disease resistance of *MeWRKY79*- and *MeHsf20*-silenced plants could be restored by exogenous melatonin. Thus, *MeWRKY79*- and *MeHsf20*-regulated *MeASMT2* expression and melatonin accumulation are responsible for *MeWRKY79*- and *MeHsf20*-mediated disease resistance.

Taken together, this study demonstrated that MeWRKY79 and MeHsf20 confer disease resistance against cassava bacterial blight through regulation of *MeASMT2* and melatonin biosynthesis in cassava. Cassava bacterial blight induces the expression of *MeWRKY79* and *MeHsf20*, and the induced *MeWRKY79* and *MeHsf20* activate the expression of *MeASMT2* via binding to W-box and HSEs in the *MeASMT2* promoter, which in turn increases melatonin accumulation and confers improved disease resistance (Fig. 7).

Supplementary Data

Supplementary data are available at *JXB* online.

Fig. S1. The W-box and HSEs that were enriched in the promoter region of *MeASMT2*.

Fig. S2. *MeWRKY79*, *MeHsf20*, and *MeASMT2* regulate the transcript levels of defense-related genes.

Table S1. The primers used for quantitative real-time PCR.

Table S2. The primers used for vector construction.

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Author contributions

HS planned and designed the research; YW, GL, YB, and FX performed the experiments and conducted fieldwork; YW, CH, and HS analysed the data; and HS wrote and revised the manuscript.

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