Expression Profiles and Response to Exogenous Hormones of β-Fructofuranosidase Gene *BmSuc1* in Silkworms (*Bombyx mori*)

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Abstract [**Objectives**] In order to clarify the regulatory effects of insect hormones on the expression of *BmSuc1* and provide a reference for further analysis of the function and expression regulation mechanism of *BmSuc1*, this study explored the expression profiles of *BmSuc1* in different tissues and periods of silkworm larvae and the expression changes of *BmSuc1* after treatment with exogenous hormones. [**Methods**] By using the real-time fluorescence quantitative PCR technique, the expression characteristics of *BmSuc1* were detected in different periods, different tissues and after treatment with exogenous hormones during the development of silkworm larvae. The expression of *BmSuc1* and 20E receptor gene *USP* was detected after RNA interference with double-stranded RNA (dsRNA) of USP. [**Results**] The relative expression of *BmSuc1* gene in the midgut was the highest, followed by the silk glands, epidermis and hemolymph. However, there was much lower or almost no expression in other tissues. In addition, the *BmSuc1* expression profile exhibited a pulse-like pattern in silkworm larvae. The expression level of *BmSuc1* was higher at each instar stage before molting, late fifth instar before cocooning and prepupal stage. Silkworm larvae at day 2 of the fifth instar were treated with 20-hydroxyecdysone (20E) and juvenile hormone (JH). It was found that the expression of *BmSuc1* was extremely significantly higher at 12 and 18 h after 20E treatment than the control group injected with 0.1% dimethyl sulfoxide (DMSO) ($P<0.01$, the same below). But there were no significant difference in *BmSuc1* expression between the JH treatment and the control group during the measurement time range ($P>0.05$). The dsRNA of USP was synthesized *in vitro* and injected into silkworm larvae at day 3 of the fifth instar. It was showed that the USP relative expression was extremely significantly down-regulated at 24 and 36 h after injection, which indicated that dsRNA interference was successful. RNAi of USP would block 20E signal transduction, and the expression of *BmSuc1* was inhibited and significantly down-regulated at 24 and 36 h after injection of dsRNA of USP ($P<0.05$). [**Conclusions**] The *BmSuc1* expression peaks appeared in the molting of silkworm larvae and the metamorphosis of larvae to pupae, which suggests that *BmSuc1* may be involved in the metamorphic development process of silkworms. Treatment with exogenous ecdysone 20E can activate the expression of *BmSuc1*, but blocking the 20E signal transduction pathway may suppress expression of *BmSuc1*. It indicates that *BmSuc1* as a downstream target gene in the 20E signal transduction pathway is directly or indirectly regulated by 20E signals.

Key words *Bombyx mori*; β-Fructofuranosidase gene; Hormones; Expression characteristics

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Silkworms (*Bombyx mori*) are the only kind of Lepidoptera model insect determined by the International Invertebrate Association, as well as the biological foundation supporting the silk industry, with extremely high economic value[1-2]. β-Fructofuranosidase (β-FFase) is a sucrose hydrolase, which is ubiquitous in plants and microorganisms. The view that there is no β-FFase in animals has long existed, which affects people's scientific judgment[3-5]. The *BmSuc1* gene is the first animal-type β-FFase gene cloned and identified, and its enzyme activity is not affected by the inhibition of 1-deoxynojirimycin (DNJ), a mulberry leaf alkaloid[6]. The *BmSuc1* gene is highly expressed in the midgut, anterior and central silk glands of silkworm larvae, and encodes β-FFase, which may play an important physiological role in the resistance of silkworms to the toxicity of mulberry leaf alkaloids[5]. Therefore, clarifying the transcription and expression characteristics of gene *BmSuc1* and its response to hormones can provide reference for revealing the physiological function of gene *BmSuc1* in silkworm body and clarifying the molecular regulation mechanism of insect hormones on gene β-FFase. Daimon *et al.*[7] took the lead in cloning gene *BmSuc1* encoding β-FFase in silkworm genome, and obtained the fusion protein *BmSuc1* through *in-vitro* recombination, and confirmed that the fusion protein *BmSuc1* has sucrose hydrolase activity, and its activity is not affected by alkaloids such as mulberry DNJ. Since then, scholars have cloned the β-FFase homologous genes from the genomes of *Samia cynthia* ricini and *Antherea pernyi*[8]. Among them, there are two β-FFase homologous genes (*ScSuc1* and *ScSuc2*) in *S. cynthia* ricini and three genes (*ApSuc1a*, *ApSuc1b* and *ApSuc1c*) in *A. pernyi*, but these genes are hardly transcribed in the larvae of non-mulberry-eating insects *S. cynthia* ricini and *A. pernyi*, and it has been found that the sucrose hydrolase in *S. cynthia* ricini and *A. pernyi* is mainly α-glucosidase, which has no β-Ffase activity[8]. Li *et al.*[9] cloned five β-FFase homologous gene fragments (*DpSuc1a*, *DpSuc1b*, *DpSuc2a*, *DpSuc2b* and *DpSuc2c*) from *Diaphania pyloalis* (Walker), of which *DpSuc1a* and *DpSuc2c* genes were highly expressed in the foregut, midgut, silk glands and fat

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body, while genes *DpSuc1b*, *DpSuc2a* and *DpSuc2b* were almost expressed in all tissues of *D. pyloalis*; and β-FFase in the midgut of *D. pyloalis* has the activity of decomposing sucrose, and its activity is not affected by alkaloid DNJ. *D. pyloalis* is a kind of mulberry pest that hosts mulberry leaves. Although closely related to silkworms, their bodies harbor β-FFase homologous gene with β-FFase activity, indicating that β-FFase has mportant biological functions in the sugar metabolism pathway of mulberry-eating insects, helping them to hydrolyze sucrose normally and avoid the toxic pathway of mulberry leaf alkaloids[10]. The molting and metamorphosis development of insects is realized by inducing or inhibiting the expression of a large number of genes in a certain time and space order under the synergistic regulation of molting hormone (MH) and juvenile hormone (JH)[11-12]. JH plays an important role in maintaining the morphology and characters of larvae and promoting the maturation of gonads, while the molting, metamorphosis and reproduction of insects are strictly regulated by MH[13-14]. MH is one of the most critical internal factors to control insect metamorphosis, and any gene directly or indirectly regulated by MH may participate in insect molting and metamorphosis. Gene *BmSuc1* is the first animal-type β-FFase gene cloned and identified, but there are few reports on its transcription and expression characteristics in the whole life cycle of silkworms and the regulation of insect hormones on *BmSuc1* gene expression. With silkworms, a kind of Lepidoptera model insect, as the research object, the regulation effects of insect hormones on gene *BmSuc1* were clarified through the expression characteristics of gene *BmSuc1* in different tissues and different periods of silkworm larvae detected by real-time fluorescence quantitative PCR and the expression change laws after treatment with insect hormones [20-hydroxyecdysone (20E) and JH], providing reference for in-depth analysis on the function and expression regulation mechanism of gene *BmSuc1*.

Materials and Methods

Experimental materials

The tested silkworm strain was Xianghui, which was provided by the Silkworm Resources Laboratory of Sericultural and Agricultural Research Institute of Yunnan Academy of Agricultural Sciences. The materials for the tissue expression profile was obtained from the head, malpighian tubule, hemolymph, epidermis, midgut, trachea, fatbody, silk gland, testis and ovary of silkworms at day 3 of the fifth instar. The materials for the stage expression profile were whole silkworms at various stages from the first instar to pupation. Ecdysone 20E (Y0002054) and juvenile hormone JH analog (33375) were purchased from Sigma Company. The rapid extraction kits of high-purity total RNA (RP1202) were purchased from BioTeke Corporation. Reverse transcription kits (RR047A) and real-time fluorescence quantitative PCR kits TB Green Premix EXTaq (RR 42LR) were purchased from TaKaRa. RiboMAX Large Scale System-T7 *in-vitro* transcription kits (P1300) were purchased from Promega.

Exogenous hormone treatment

According to the method of Zhang *et al.*[16], silkworms developing well at day 2 of the fifth instar were selected, and 2 μl of 20E (1.0 μg/μl) or JH (0.5 μg/μl) was injected into the second stomata of each silkworm with a micro syringe (10 μl). Samples were taken at 6, 12, 18 and 24 h after injection, respectively, and stored at −80 ℃. A control group was set by injecting the same amount of 0.1% dimethyl sulfoxide (DMSO). Four replicates were set for each treatment and three replicates for each sampling.

Total RNA extraction and cDNA preparation

According to the operation instructions of rapid extraction kits for high-purity total RNA, total RNA was extracted from silkworms. The integrity of the total RNA was detected by 1.0% agarose gel electrophoresis, and its concentration and purity were detected by SimpliNano ultra-low volume nucleic acid and protein analyzer. Next, reverse transcription kits were used to reversely transcribe RNA according to their instructions into cDNA for real-time fluorescence quantitative PCR detection.

Real-time fluorescence quantitative PCR

With silkworm *BmSuc1* gene (GenBank accession number: NM_001126249.1) published by NCBI as the target gene and ribosomal protein gene Rp49 (GenBank accession number: NM_001098282) as the internal reference gene, primers for real-time fluorescent quantitative PCR (Table 1) were designed by Primer Premier 5.0, and the synthesis was entrusted to Sangon Biotech (Shanghai) Co., Ltd. A 20 μl of real-time fluorescence quantitative PCR reaction system was prepared according to instructions of TB Green® Premix Ex Taq™ [containing TB Green Premix Ex Taq (2X) 10 μl, PCR forward primer (10 μM) 0.5 μl, PCR reverse primer (10 μM) 0.5 μl, cDNA template 1.5 μl, sterile water 7.5 μl]. The amplification program was started with pre-denaturation at 95 ℃ for 5 min, followed by 40 cycles of 95 ℃ for 10 s and 60 ℃ for 30 s. According to the data recorded by the CFX-96 real-time fluorescence quantitative PCR instrument, the relative expression level of the target gene was calculated using the $2^{-\triangle\triangle}$Ct method. Statistical analysis and mapping of experimental data were conducted using Excel 2016 and SPSS 21.0.

Table 1 Primers used in quantitative real-time PCR

Gene name	Primer sequence
BmSuc1	F: 5'-AATCCAGTCCTCTCCTACGTGC-3'
	R: 5'-TCCGGTCTGATACGTGTTCTTG-3'
Rp49	F: 5'-CAGGCGGTTCAAGGGTCAATAC-3'
	R: 5'-TGCTGGGCTCTTTCCACGA-3'

dsRNA interference test

RNA interference (RNAi) is a phenomenon that specifically degrades target gene mRNA through double-stranded RNA (dsRNA), leading to post-transcriptional gene silencing. On-line prediction of potential siRNA sites in the CDS sequence of silkworm 20E receptor gene (*USP*) was made by using siDirect v2.0 (http://sidirect2.rnai.jp/). Fragments with concentrated siRNA sites and good specificity were selected as dsRNA interference fragments, and silkworm red fluorescent protein gene RFP was

used as a control. Primer 5.0 was employed to design RNA interference primers, and a T7 linker sequence (blackened bases in Table 2) was added to the 5 end of each primer. According to instructions of RiboMAX Large Scale System-T7 kits, dsRNA fragments of genes *USP* and *RFP* were transcribed and synthesized *in vitro*. On day 3 of fifth-instar silkworm larvae, dsRNA was injected by a glass capillary needle according to the dosage of 30 μg per silkworm. Samples were taken at 24, 36, 48 and 60 h after injection, and the interference effect of dsRNA and the expression changes of gene *BmSuc1* were detected by real-time fluorescence quantitative PCR.

Table 2 RNAi primer sequences

Gene name	Primer sequence
USP	F: 5'-TAATACGACTCACTATAGGGAGACCCTAACCATCCCTTGA-3'
	R: 5'-TAATACGACTCACTATAGGGAGATGAATCCGCAACTAACG-3'
RFP	F: 5'-TAATACGACTCACTATAGGGAGACTTCAAGGTGCGCATGGAG-3'
	R: 5'-TAATACGACTCACTATAGGGAGATGTGGATCTCGCCCTTCAG-3'

Results and Analysis

Expression characteristics of *BmSuc1* in different tissues and periods

Real-time fluorescence quantitative PCR was adopted to detect the expression of gene *BmSuc1* in 10 tissues from silkworm larvae at day 3 of the fifth instar. The results (Fig. 1) showed that the relative expression of gene *BmSuc1*was highest in the midgut, followed by the silk gland, epidermis and hemolymph. It was expressed in other tissues at a very-low relative expression level or almost not expressed. The expression profile of gene *BmSuc1* at various stages from the first instar to pupation is shown in Fig. 2. *BmSuc1* exhibited a pulse-like expression pattern, and the relative expression level of *BmSuc1* was relatively low in silkworms from each instar of the first to fourth instar. As silkworms began to consume mulberry leaves, the relative expression level of gene *BmSuc1* gradually increased, reaching the highest level before molting (marked by a red arrow), and then began to decrease. The relative expression level was the lowest at the end of molting, and then entered the next cycle. Moreover, there was a peak of expression in both late fifth instar (before cocooning) and the prepupal stage (marked by a red arrow).

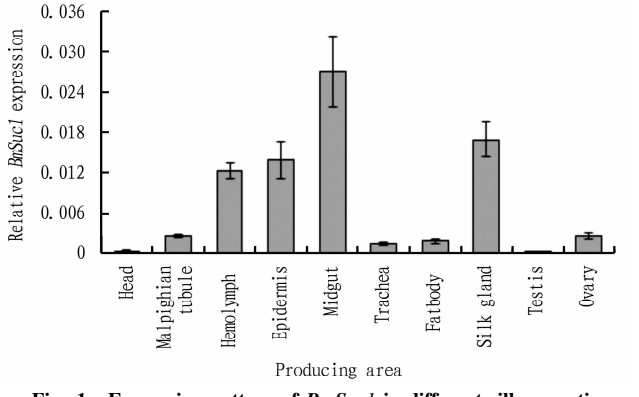
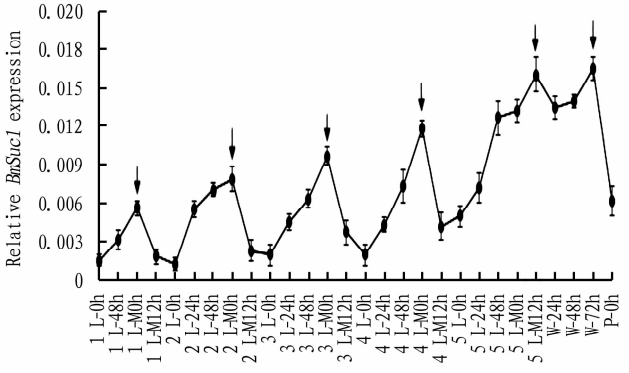


Fig. 1 Expression pattern of *BmSuc1* in different silkworm tissues

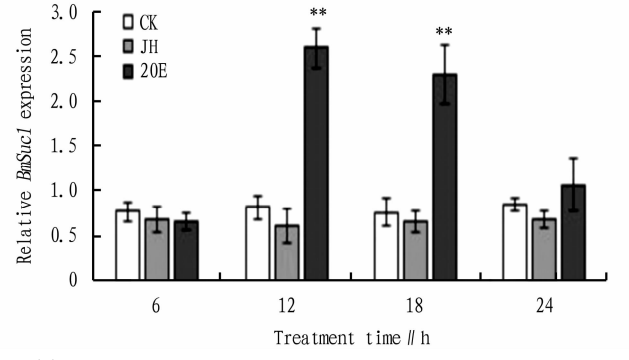


L: Larvae; M: molting; H: hour; W: wandering; P: pupa.

Fig. 2 Expression pattern of *BmSuc1* in silkworms at different developmental stages

Effects of exogenous hormones on the expression of *BmSuc1*

20E and JH synergistically regulate the molting and metamorphosis processes of insects, and after silkworms enter the stage before molting and metamorphosis, the content of 20E gradually increases, while the content of JH decreases[11]. According to the expression profile of *BmSuc1* in different periods (Fig. 2), it was speculated that gene *BmSuc1* was co-regulated by 20E and JH. Therefore, 20E and JH were selected to treat silkworms at day 2 of the fifth instar, and the expression changes of gene *BmSuc1* at different time points after treatment were detected. The results (Fig. 3) showed that at 12 and 18 h after 20E treatment, the relative expression level of gene *BmSuc1* was significantly higher than that of the control group ($P < 0.01$, the same below), but within the measured time range, there was no significant difference in *BmSuc1* expression between the JH treatment group and the control group ($P > 0.05$), indicating that gene *BmSuc1* was induced to express by 20E, but had no response to JH treatment.



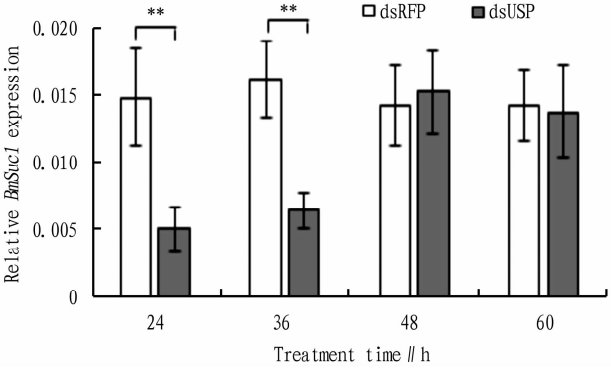
** $P < 0.01$ stands for an extremely significant difference from the control group. The same was applied in Fig. 4.

Fig. 3 Effects of 20E and JH treatment on the expression level of *BmSuc1* in silkworms

Effects of blocking 20E signals on the expression of *BmSuc1*

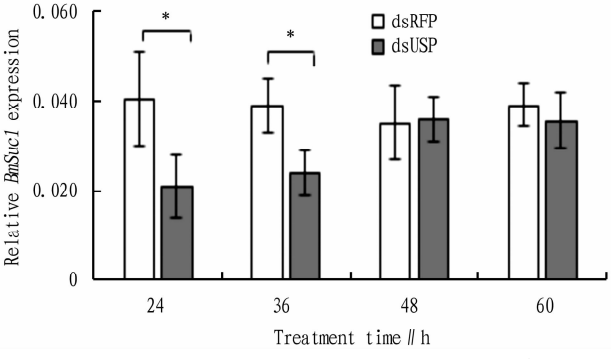
20E transmits signals through its receptor EcR/USP, which means that interfering with gene *EcR* or *USP* can effectively block 20E signal transduction. The dsRNA of gene *USP* synthesized by *in-vitro* transcription was injected into silkworm larvae on day 3 of the fifth instar, and real-time fluorescence quantitative PCR was adopted to detect the interference effect of gene *USP* and the

expression changes of gene *BmSuc1*. The results (Fig. 4) showed that the relative expression of gene *USP* was significantly down-regulated 24 and 36 h after injection of dsRNA containing gene *USP*, indicating successful dsRNA interference. From Fig. 5, it can be seen that using the RNAi technique to interfere with gene *USP* could block 20E signal transduction, resulting in an inhibition of *BmSuc1* expression. Its relative expression level was significantly down-regulated 24 and 36 h after injection of *USP*'s dsRNA ($P<0.05$). It was speculated that *BmSuc1*, as a downstream target gene in the 20E signal transduction pathway, was regulated by 20E signals.



dsRFP: dsRNA of genes injected with RFP; dsUSR: dsRNA of genes injected with USP. The same was applied in Fig. 5.

Fig. 4 Detection the interference effect on gene *USP*



* represented a significant difference from the control group ($P<0.05$).

Fig. 5 Effects of RNAi *USP* on the expression of *BmSuc1* in silkworm larvae

Discussion

With the completion of genome sequencing in more and more insects, it has been found that β-FFase homologous genes exist. Pauchet *et al.* [17] found transcripts encoding β-FFase in the midgut cDNA library of *Helicoverpa armigera*. Subsequently, two contigs (*MsSuc1* and *MsSuc2*) encoding β-FFase were found in the transcriptome sequencing database of *Manduca sexta*, which indicated that gene β-FFase was ubiquitous in genomes of insects in Lepidoptera[18]. Gene sequences encoding β-FFase were also found in the genomes of Coleoptera insects, such as *Dendroctonus ponderosae*, *Sphenophorus levis* and *Agrilus planipennis*[19-20]. Silkworms are a kind of model insect of Lepidoptera, whose *BmSuc1* gene is the first animal-type β-FFase encoding gene cloned and identified. However, the expression characteristics of *BmSuc1* in the whole life cycle and different tissues and organs of silkworms are not clear, and its transcriptional regulation mechanism has not been analyzed. In this study, the expression laws of *BmSuc1* in different tissues and periods of silkworm larvae were comprehensively and systematically analyzed. The results showed that *BmSuc1* was highly expressed not only in the midgut of silkworm larvae, but also in the epidermis and silk gland. Both molting and metamorphosis of insects involve the replacement of old and new epidermis, and the high expression of gene *BmSuc1* in the epidermis suggests that it may participate in the physiological process of molting and metamorphosis. Daimon *et al.* [7] detected the expression of *BmSuc1* in different tissues by semi-quantitative PCR. The results showed that gene *BmSuc1* was highly expressed in the midgut and silk gland, but it was not detected in the epidermal tissue. In this study, the results of real-time fluorescence quantitative PCR are different from those of Daimon *et al.*, which may be caused by the differences in test methods. Semi-quantitative PCR is based on the brightness of agarose gel electrophoresis bands, resulting many influencing factors, while the sensitivity and accuracy of real-time fluorescence quantitative PCR are higher than those of semi-quantitative PCR.

In silkworms, the RNAi efficiency of gene *USP* is higher than that of gene *EcR*[21]. In this study, dsRNA of gene *USP* in silkworm larvae was synthesized by transcription *in vitro*, and could down-regulate the expression of gene *USP* after being injected into silkworms on day 3 of the fifth instar, thus blocking 20E signal transduction. Meanwhile, it was found that the expression of gene *BmSuc1* was inhibited, suggesting that *BmSuc1*, as a downstream target gene in the 20E signal transduction pathway, was directly or indirectly regulated by 20E signals. The 20E signal transduction pathway of insects is basically clear. In specific, with the help of molecular chaperone complexes, 20E first binds to heterodimer receptor EcR-USP, and then to 20E ecdysone receptor response element (EcRE) on the regulatory sequence of target gene, so as to recruit co-activators and start 20E primary response genes (transcription factor encoding genes E74, E75, E93 and Br-C) under the action of RNA polymerase[22-23]. 20E signals are induced by transcription factors encoded by the primary response genes and amplified in cascade, thus realizing the regulation of insect growth, molting and metamorphosis and immune defense[15].

There have been many reports on the regulation of silkworm gene expression by 20E. Zhao *et al.* [24] fed mulberry leaves soaked with 20E to silkworms, in which the transcription levels of glutathione-S-transferase genes (*BmGSTs1* and *BmGSTs2*) increased significantly in the intestine, fat body and malpighian tubule tissues, indicating that these two genes participated in the metabolism of ecdysone in silkworms. Yang *et al.* [25] showed that adding exogenous 20E significantly up-regulated the expression of silkworm vitellogenin gene (*Vg*). Yang *et al.* [26] found that 20E could regulate the expression of pyridoxine-5'-phosphate oxidase gene (*PNPO*) and pyridoxal kinase gene (*PLK*) in the silk gland of fifth-instar silkworm larvae, while JH had no effect on the expression of genes *PNPO* and *PLK*. Mai *et al.* [27] studied the expression and regulation mechanism of the antibacterial peptide

gene (*Lebocin*) in the midgut of silkworm larvae and found that 20E could activate *Lebocin* expression by up-regulating transcription factor genes (*BmBR-CZ4* and *BmEts*). Moreover, 20E could regulate the chitinase gene (*BmCHT5*) and participate in the molting and metamorphosis of silkworms through BrC-Z4 cis-acting elements[28]. 20E achieves transcriptional regulation of target genes by acting on EcRE or mediating transcription factor response genes such as E74, E75, E93 and Br-C[29]. The results of the exogenous hormone injection test in this study indicated that the expression of gene *BmSuc1* was regulated by 20E, but the specific regulatory mechanism is not yet clear, and the presence of EcRE or related transcription factor binding sites in the promoter sequence of *BmSuc1* remains to be further explored.

Conclusions

Gene *BmSuc1* is mainly highly expressed during molting and metamorphosis in silkworms, suggesting that it may be involved in the metamorphosis development process of silkworms. Treatment with exogenous ecdysone 20E can induce the transcription of *BmSuc1*, while blocking the 20E signal transduction pathway may suppress expression of *BmSuc1*. It indicates that *BmSuc1* as a downstream target gene in the 20E signal transduction pathway is directly or indirectly regulated by 20E signals.

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(Continued on page 70)

rapidly, and multi-day tours slowly recover; health and wellness tourism will be favored by more tourists; and domestic tourism is gradually recovering, and it is difficult for inbound tourism to recover in a short period of time.

(3) Based on the development prediction and existing problems of the tourism industry in Shandong Province in the post-epidemic era, following optimization paths were proposed: strengthening the construction of supporting infrastructure and enhancing service awareness, strengthening safety management and eliminating tourists' panic, vigorously developing health tourism and innovating characteristic tourism products, reasonably using the smart tourism system, and actively developing online tourism.

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