The fascinating role of neuropeptide Bursicon and its receptor in shaping insect seasonal polyphenism

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Abstract

Seasonal polyphenism enables organisms to adapt to environmental challenges by increasing phenotypic diversity. *Cacopsylla chinensis* exhibits remarkable seasonal polyphenism, specifically in the form of summer-form and winter-form, which have distinct morphological phenotypes. Previous research has shown that low temperature and the temperature receptor *CcTRPM* regulate the transition from summer-form to winter-form in *C. chinensis* by impacting cuticle content and thickness. However, the underling neuroendocrine regulatory mechanism remains largely unknown. Bursicon, also known as the tanning hormone, is responsible for the hardening and darkening of the insect cuticle. In this study, we report for the first time on the novel function of Bursicon and its receptor in the transition from summer-form to winter-form in *C. chinensis*. Firstly, we identified *CcBurs-α* and *CcBurs-β* as two typical subunits of Bursicon in *C. chinensis*, which were regulated by low temperature (10°C) and *CcTRPM*. Subsequently, *CcBurs-α* and *CcBurs-β* formed a heterodimer that mediated the transition from summer-form to winter-form by influencing the cuticle chitin contents and cuticle thickness. Furthermore, we demonstrated that *CcBurs-R* acts as the Bursicon receptor and plays a critical role in the up-stream signaling of the chitin biosyntheis pathway, regulating the transition from summer-form to winter-form. Finally, we discovered that miR-6012 directly targets *CcBurs-R*, contributing to the regulation of Bursicon signaling in the seasonal polyphenism of *C. chinensis*. In summary, these findings reveal the novel function of neuroendocrine regulatory mechanism underlying seasonal polyphenism and provide critical insights into insect Bursicon and its receptor.

Keywords: *Cacopsylla chinensis*; seasonal polyphenism; Neuropeptide; Bursicon, *CcBurs-R*; miR-6012

Introduction

Polyphenism is a fascinating phenomenon of phenotypic plasticity, where a single genome produces multiple distinct phenotypes in response to environmental cues (Simpson and Sword, 2011). In recent years, polyphenism has garnered increasing attention and has become a focal point of research in cutting-edge fields, such as ecology, evolutionary biology, epigenetics, and entomology. Nature presents us with numerous remarkable examples of polyphenism. For instance, we observe seasonal polyphenism in psylla (Butt and Stuart, 1986) and butterflies (Emily et al., 2014; Baudach and Vilcinskas, 2021), sexual and wing polyphenism in aphids and planthoppers (Xu et al., 2015; Shang et al., 2020), caste polyphenism in ants and honeybees (Kucharski et al., 2008; Bonasio et al., 2012), sex determination in reptiles and fish regulated by temperature and social factors (Janzen and Phillips, 2006; Liu et al., 2017), and environmentally induced polyphenism in plants (Gratani, 2014). Undoubtedly, polyphenism plays a major contributor to the population dynamics of insects worldwide (Noor et al., 2008). Numerous studies have reported that insect polyphenism is influenced by a range of external environment factors, such as temperature, population density, photoperiod, and dietary nutrition (Simpson and Sword, 2011; Ma et al., 2011; An et al., 2012;). Additionally, internal neuro-hormones, including insulin, dopamine, and ecdysone, have been found to play crucial roles in insect polyphenism (Ma et al., 2011; Uehara et al., 2011; Xu et al., 2015; Vellichirammal et al., 2017). However, the specific molecular mechanism underling polyphenism still require further clarification.

Cacopsylla chinensis (Yang & Li) is a pear psylla belonging to Hemiptera order, which causes severe damage to trees and fruits in the major pear production areas across East Asian countries, including China and Japan (Hildebrand et al., 2010; Wei et al., 2020). This phloem-sucking psylla inflicts harm on young shoots and leaves in both adult and nymph stages, leading to stunted and withered pear trees (Ge et al., 2019). Furthermore, *C. chinensis* secretes a substantial amount of honeydew and acts as a vector for plant pathogenic microorganisms, such as the phytoplasma of pear

decline disease and *Erwinia amovora* (Hildebrand et al., 2010). Importantly, this pest demonstrates strong adaptability to its environment and exhibits seasonal polyphenism, manifesting as summer-form (SF) and winter-form (WF), which display significant differences in morphological characteristics throughout the seasons (Ge et al., 2019; Zhang et al., 2023). The summer-form has a lighter body color and causes more severe damage, while the winter-form, in contrast, has a brown to dark brown body color, a larger body size, and stronger resistance (Ge et al., 2019; Tougeron et al., 2021). In a previous study, Zhang et al. demonstrated that a low temperature of 10°C and the temperature receptor *CcTRPM* regulate the transition from summer-form to winter-form in *C. chinensis* by affecting cuticle thickness and chitin content (Zhang et al., 2023). Up to now, no insect hormones or neuropeptides underling this seasonal polyphenism in *C. chinensis* have been identified.

Bursicon, also known as the tanning hormone, was initially discovered in the 1960s through neck-ligated assays. It serves a highly conserved function in insects by inducing the clerotization and melanization of the new cuticle in larvae and facilitating wing expansion in adults (Dewey et al., 2004). Bursicon is a heterodimer neuropeptide composed of two subunits, Bursicon-α and Bursicon-β, which exert their effects through the leucine-rich repeats-containing G- protein-coupled receptor, also known as the Bursicon receptor (Luo et al., 2005). In *Drosophila*, flies with mutated Bursicon receptor, such as the *rk* gene, or deficient in one of Bursicon subunits, exhibit improper tanning and altered body shape (Luan et al., 2006). Similarly, in the model insect *Tribolium castaneum*, RNA interference experiments have demonstrated that the Bursicon receptor (*Tcrk*) is not only required for cuticle tanning, but also crucial for the development and expansion of integumentary structures (Bai and Palli, 2010). Interestingly, it has been reported that Bursicon homodimers can activate the NF-kB transcription factor *Relish*, leading to the induction of innate immune and stress genes during molting (An et al., 2012). In cold environments, insects generally taken longer to complete their development than those of the same species raised in warmer conditions (Nyamaukondiwa et al., 2011). Consequently, insects exposed to

cold conditions exhibit larger body size and darker cuticular melanization than those reared in warmer environments (Shearer et al., 2016). Given this background, Bursicon and its receptor are expected to play a significant role in the seasonal polyphenism of *C. chinensis*.

MicroRNAs (miRNAs), which are approximately 23 nucleotides in length and belong to a class of small noncoding RNAs, play a crucial role in the regulation of posttranscriptional gene expression (Lucas and Raikhel, 2013). Increasing studies have shown that miRNAs are important in insect polyphenism, such as miR-31, miR-9, and miR-252, as well as hormone signaling, for examples, miR-133 in dopamine synthesis (Yang et al., 2014; Zhang et al., 2020; Shang et al., 2020; Zhang et al., 2023). However, there have been no reports on miRNAs targeting Bursicon and its receptor. Therefore, studying the molecular mechanism of miRNA regulation of the Bursicon receptor at the post-transcriptional level would be highly innovation. In this study, we conducted bioinformatics analysis, qRT-PCR, and Western blot to identify two Bursicon subunits (*CcBurs-α* and *CcBurs-β*) and their association with low temperature of 10°C. We then employed RNAi, cuticle staining, and transmission electron microscopy to study the effects of *CcBurs-α* and *CcBurs-β* on cuticle content, cuticle thickness, and the transition percent from summer-form to winter-form in *C. chinensis*. Furthermore, we identified *CcBurs-R* as the Bursicon receptor and investigated its role in the transition from summer-form to winter-form. Finally, through *in vivo* and *in vitro* assays, we discovered that miR-6012 targets *CcBurs-R* and is involved in the seasonal polyphenism. These efforts not only shed light on the novel function of Bursicon and its receptor in mediating the transition from summer-form to winter-form in *C. chinensis*, but also enhance our understanding of the neuroendocrine basis of insect seasonal polyphenism.

Results

Molecular identification of *CcBurs-α* and *CcBurs-β* in *C. chinensis*

Sequence analysis showed that the open reading frame (ORF) of *CcBurs-α*

(GenBank: OR488624) was 480 bp long, encoding a predicted polypeptide of 159 amino acids. The polypeptide had a molecular weight of 17.45 kDa and a theoretical isoelectric point (*pI*) of 6.13. The complete ORF of *CcBurs-β* (GenBank: OR488625) was 405 bp, encoding a polypeptide of 134 amino acids residues. The predicated molecular weight of *CcBurs-β* was 15.21 kDa and a theoretical *pI* of 5.24. Amino acid sequence alignment analysis revealed that CcBurs-α and CcBurs-β shared high amino acid identity with homologs from other selected insect species (Figure 1A and 1B). Both subunits contained eleven conserved cysteine residues, marked with red stars. Phylogenetic analysis (Figure S1A and S1B) indicated that *CcBurs-α* was most closely related to the *DcBurs-α* homologue (*Diaphorina citri*, XP_008468249.2), while *CcBurs-β* was most closely related to *DcBurs-β* (*D. citri*, AWT50591.1) among the selected species. The potential tertiary protein structure and molecular docking of *CcBurs-α* and *CcBurs-β* were constructed using the Phyre[2] server and PyMOL-v1.3r1 software (Figure 1C). To investigate the identities of homodimer and heterodimer of *CcBurs-α* and *CcBurs-β*, SDS-PAGE with reduced and non-reduced gels was used. When expressed as individual subunits, they formed α+α and β+β homodimers, as the molecular size of α or β doubled in the non-reduced gel compared to the reduced gel (Figure 1D). When co-expressed, most α and β subunits formed the *CcBurs-α+β* heterodimer (Figure 1D).

The temporal expression profile revealed that both *CcBurs-α* and *CcBurs-β* were ubiquitous in all developmental stages, with lower expression in eggs and nymphs and higher expression in adults of both summer-form and winter-form (Figure S2A -S2D). The mRNA levels of both *CcBurs-α* and *CcBurs-β* were higher in each developmental stage of the winter-form than in the summer-form, suggesting a potentially more important role in the winter-form. Spatially, *CcBurs-α* and *CcBurs-β* were detected in all investigated nymph tissues and were expressed most prominently in the head (Figure S2E and S2F). In addition to the midgut, both *CcBurs-α* and *CcBurs-β* showed higher expression in other selected tissues of the winter-form compared to the summer-form, especially in the head and cuticle. Results from temperature treatment

exhibited that the mRNA expression of *CcBurs-α* and *CcBurs-β* significantly increased after 10°C treatment for 3, 6, and 10 days compared to 25°C treatment (Figure 1E and 1F). Meanwhile, qRT-PCR results indicated that the transcription levels of both *CcBurs-α* and *CcBurs-β* were noticeably down-regulated after successful knockdown of the temperature receptor *CcTRPM* by RNAi at 3, 6, and 10 days (Figure 1G-1H, S3). These data suggest that *CcBurs-α* and *CcBurs-β* are regulated by a low temperature of 10°C and *CcTRPM*, and may serve as down-stream signals involved in the seasonal polyphenism of *C. chinensis*.

CcBurs-α and _CcBurs-β_ were essential for the transition from summer-form to winter-form

To investigate the role of *CcBurs-α* and *CcBurs-β* in the transition from summer-form to winter-form of *C. chinensis*, newly hatched 1st instar nymphs of summer-form were fed with dsCcBurs-α, dsCcBurs-β, or dsEGFP. qRT-PCR results exhibited that feeding dsCcBurs-α or dsCcBurs-β extremely reduced the expression of the target gene under 10°C condition. The RNAi efficiencies of *CcBurs-α* and *CcBurs-β* were approximately 66-78% and 69-79% at 3, 6, and 10 days compared to dsEGFP feeding (Figure 2A and 2B).

After successful knockdown of *CcBurs-α*, *CcBurs-β*, or both, the UV absorbance of total pigment extraction at a wavelength of 300 nm in dsCcBurs-α-treated (0.18), dsCcBurs-β-treated (0.19), and dsCcBurs-α+β-treated (0.07) nymphs was dramatically lower than that in dsEGFP-treated nymphs (0.85) under 10°C condition (Figure 2C). This finding indicates that *CcBurs-α* and *CcBurs-β* play a prominent role in cuticle pigment formation in the winter-form in *C. chinensis*. Moreover, both the results of cuticle chitin content determination and cuticle ultrastructure observation indicated that knockdown of *CcBurs-α*, *CcBurs-β*, or both markedly reduced the cuticle chitin content (about 0.33, 0.32, 0.14) and cuticle thicknesses (about 1.44, 1.53, 0.73 μm) compared with dsEGFP-treated nymphs (1.00, 3.39 μm) under 10°C condition, respectively (Figure 2D-2G). Interestingly, the results of pigmentation

absorbance and cuticule thickness after *CcBurs-α* or *CcBurs-β* knockdown were similar to those after *CcTRPM* knockdown (Table S2). Additionally, dsCcBurs-α feeding (25.48%), dsCcBurs-β feeding (26.03%), or both feeding (11.84%) obviously decreased the transition percent from summer-form to winter-form compared to dsEGFP feeding (84.02%) (Figure 2H-2I). Together, these data suggest that the two subunits of Bursicon, *CcBurs-α* and *CcBurs-β*, are essential for the transition from summer-form to winter-form of *C. chinensis* by affecting cuticle contents and thickness.

CcBurs-R* was identified as the Bursicon receptor in *C. chinensis

To study the role of neuropeptide Bursicon in seasonal polyphenism, we identified a leucine-rich repeat-containing G protein-coupled receptor and named it as Bursicon receptor *CcBurs-R* (GenBank: OR488626). The open reading frame of *CcBurs-R* is 3498 bp long and encodes a 1165-amino acid protein with a predicted molecular weight of 118.61 kDa, a theoretical *pI* of 8.64, and seven predicted transmembrane domains. Multiple alignment analysis showed a high degree of conservation in the transmembrane domains of *CcBurs-R* with *Burs-R* sequences from other four selected insect species (Figure 3A). Phylogenetic tree analysis indicated that *CcBurs-R* is most closely related to the *DcBurs-R* homologue (*D. citri*, KAI5703609.1) in evolutionary relationship, and both are important Hemiptera pest of fruit trees (Figure 3B). The potential tertiary protein structure of *CcBurs-R* and its molecular docking with *CcBurs-α* and *CcBurs-β* were constructed using the online server Phyre2 and modified with PyMOL-v1.3r1 software (Figure 3C).

Development expression pattern indicated that *CcBurs-R* had relatively lower levels of expression in eggs and nymphs, but extremely higher levels in adult stages (Figure S4A-B). The mRNA level of *CcBurs-R* was higher in each developmental stage of winter-form than summer-form, suggesting its important role in the transition from summer-form to winter-form. In terms of tissue-specific expression, *CcBurs-R* was found to be present in all determined tissues, with relatively higher expression in

five tissues (head, cuticle, midgut, wings, and foot) of winter-form than summer-form (Figure S4C). To confirm that *CcBurs-R* is the Bursicon receptor of *C. chinensis*, we determined the effect of *CcBurs-α* or *CcBurs-β* knockdown on its mRNA expression. qRT-PCR results showed that RNAi-mediated knockdown of *CcBurs-α* or *CcBurs-β* significantly decreased *CcBurs-R* expression after dsRNA feeding at 3, 6, and 10 days compared to the dsEGFP group under 10°C condition (Figure 3D-3E). Moreover, the heterodimer protein of *CcBurs-α+β* fully rescued the effect of RNAi-mediated knockdown on *CcBurs-R* expression, while α+α or β+β homodimers did not (Figure 3F). Additional results demonstrated that 10°C treatment markedly increased *CcBurs-R* expression compared to 25°C treatment, and *CcTRPM* knockdown obviously decreased the mRNA level of *CcBurs-R* compared to the dsEGFP treatment (Figure 3G-3H). Therefore, these findings indicate that *CcBurs-R* is the Bursicon receptor of *C. chinensis* and is regulated by a low temperature of 10°C and *CcTRPM*.

***CcBurs-R* regulated the transition from summer-form to winter-form**

The function of *CcBurs-R* in seasonal polyphenism was further investigated using RNAi technology. qRT-PCR results revealed that RNAi efficiency was 66-82% after dsCcBurs-R feeding for 3, 6, and 10 days compared to the dsEGFP treatments (Figure 4A). As shown in Figure 4B-4F, the total pigment extraction at a wavelength of 300 nm (0.14 *VS* 0.85), cuticle chitin content (0.31 *VS* 1.00), and cuticle thicknesses (1.34 μm *VS* 3.39 μm) were all significantly decreased in dsCcBurs-R-treated nymphs compared to the dsEGFP control. Expectedly, the results of pigmentation absorbance and cuticule thickness after *CcBurs-R* knockdown were similar to those of *CcTRPM*, *CcBurs-α*, or *CcBurs-β* knockdown (Table S2). In addition, RNAi-mediated down-regulation of *CcBurs-R* expression markedly affected the transition percent from summer-form to winter-form compared to dsEGFP feeding (26.70% *VS* 83.79%), while the heterodimer protein of *CcBurs-α+β* could fully rescue the effect of *CcBurs-R* knockdown on the transition percent (Figure 4G-4H). Therefore, our results suggest that *CcBurs-R* mediates the transition from summer-form to winter-form by directly affecting cuticle contents and thickness.

Since *CcTre1* and *CcCHS1*, two rate-limiting enzyme genes in the chitin biosyntheis pathway, have been demonstrated to be involved down-stream in this transition of *C. chinensis*, we next investigated the relationship between Bursicon signal and these two genes. The results showed that the mRNA levels of *CcTre1* and *CcCHS1* were obviously decreased in dsCcBurs-α, dsCcBurs-β, or dsCcBurs-R feeding nymphs on the 6th day compared to the control (Figure 4I-4J). This data indicates that *CcBurs-R* functions up-stream of the chitin biosyntheis pathway and is involved in the transition from summer-form to winter-form in *C. chinensis*.

miR-6012 directly targeted *CcBurs-R* by inhibiting its expression

To determine if miRNAs are involved in the regulation of Bursicon hormone in the seasonal polyphenism of *C. chinensis*, we amplified the 3'UTR of *CcBurs-R* and predicted relevant miRNAs. Four miRNAs, including miR-6012, miR-375, miR-2796, and miR-1175, were predicted to have binding sites in the 3'UTR of *CcBurs-R* by two software programs, miRanda and Targetscan (Figure 5A). To confirm the target relationship, *in vitro* dual-luciferase reporter assays were performed. After introducing the 3'UTR full sequence of *CcBurs-R* into the pmirGLO vector, the relative luciferase activity was significantly reduced compare to the negative control in the present of agomir-6012, while there was no change with the other three miRNAs (Figure 5B). Next, *in vivo* RNA immunoprecipitation results showed that the expression levels of *CcBurs-R* and miR-6012 increased approximately 15-fold and 23 fold, respectively, in the Ago-1 antibody-mediated RNA complex of agomir-6012 fed nymphs compared to the IgG control (Figure 5C and S5A-B). FISH results indicated that *CcBurs-R* and miR-6012 had opposite expression trends during the developmental stages and were co-expressed in the 3rd instar nymphs (Figure 5D). qRT-PCR results also revealed that low temperature prompted the expression of *CcBurs-R*, while miR-6012 had an inhibitory effect (Figure 5E-5F). These data suggest that miR-6012 directly targets *CcBurs-R* by inhibiting its expression.

miR-6012 mediated the seasonal polyphenism of *C. chinensis* by targeting

CcBurs-R

To decipher the function of miR-6012 in regulating seasonal polyphenism, we increased its abundance by feeding agomir-6012 to the 1st instar nymphs. qRT-PCR results indicated that the expression levels of miR-6012 were markedly higher at 3, 6, and 10 days after agomir-6012 feeding compared to the agomir-NC control (Figure 6A). Furthermore, the results showed that agomir-6012 treatments significantly affected pigmentation absorbance, cuticle chitin content, cuticle thicknesses, the transition percent from summer-form to winter-form, and morphological phenotype compared to the negative control of agomir-NC feeding (Figure 6B-6H). Additionally, agomir-6012 feeding also inhibited the mRNA expression of *CcTre1* and *CcCHS1* (Figure 6I). Together, these results display that miR-6012 plays an important role in the transition from summer-form to winter-form in *C. chinensis*.

Discussion

Polyphenism is a conserved adaptive mechanism in species ranging from insects to mammalian, and evidence is mounting that it also extends to many nematode and fish species (Stockton et al., 2018; Yang and Pospisilik, 2019). Seasonal polyphenism can provide overwintering species with better adaptability to extreme climates through beneficial shifts in morphology, physiology, or behavior (Simpson and Sword, 2011). Physiological studies have shown that the neuroendocrine hormone system communicates environmental signals to facilitate downstream morphology and physiology transformation (Zera and Denno, 1997; Overgaard and MacMillan, 2017). Having a good model is extremely important for answering specific scientific questions (Bhardwaj et al., 2020). To clarify the role of neuropeptide Bursicon in the seasonal polyphenism of *C. chinensis*, we identified two Bursicon subunits, *CcBurs-α* and *CcBurs-β*, in this study. The SDS-PAGE results of non-reduced and reduced gels showed that *CcBurs-α* and *CcBurs-β* can form both homodimers (α+α or β+β) and a heterodimer (α+β) (Figure 1D). Temporal expression patterns showed that *CcBurs-α* and *CcBurs-β* have very similar gradually increasing expression trends and higher

expression in winter-form than summer-form (Figure S1C-1D), indicating that Bursicon may play a significant role in winter-form. This result is consistent with the report on gypsy moths, where transcript levels of *Ldbursicon* in adult stages were higher than in larvae (Zhang et al., 2022b). The transcript levels of both subunits were higher in the head and cuticle of winter-form compare to summer-form, implying a potential role of Bursicon in seasonal polyphenism of *C. chinensis* (Figure S1E-1F) (Luan et al., 2006).

As the transition of *C. chinensis* from summer-form to winter-form is regulated by a low temperature of 10°C and *CcTRPM*, we next determined the effect of 10°C treatment and *CcTRPM* RNAi on the expression of *CcBurs-α* and *CcBurs-β*. As expected, 10°C treatment significantly increased the expression of *CcBurs-α* and *CcBurs-β*, while *CcTRPM* RNAi markedly decreased their mRNA levels (Figure 1E-1H). This is the first report on the relationship between the neuropeptide Bursicon and low temperature. Further results from RNAi-mediated knockdown of *CcBurs-α*, *CcBurs-β*, or both showed that Bursicon prominently regulates the transition from summer-form to winter-form in *C. chinensis* by affecting cuticle pigment content, cuticle chitin content, and cuticle thickness (Figure 2C-2I). In many insects, such as *Drosophila* and *T. castaneum*, Bursicon is believed to be the main hormone responsible for cuticle tanning (Luo et al., 2005; Bai et al., 2010). However, knockdown of Bursicon subunits did not cause visible defects in cuticle sclerotisation or pigmentation of *Bombyx mori* and *Lymantria dispar* adults (Huang et al., 2007; Zhang et al., 2022b). These researches indicate that Bursicon may not be necessary for cuticle tanning in all insects. Although the reactions involved in cuticle tanning are well-known, further studies are needed to understand how Bursicon mediates the seasonal polyphenism of *C. chinensis*.

To further elucidate the role of the Bursicon signal in seasonal polyphenism, we identified the Bursicon receptor of *CcBurs-R* in *C. chinensis*. Temporal and spatial expression patterns of *CcBurs-R* were very similar to those of *CcBurs-α* and *CcBurs-β*, and it also had higher expression in winter-form than summer-form (Figure S4). By

comparing its expression profiles with those in other insects, we can conclude that the spatio-temporal expression of Bursicon receptor is related to the specificity of insect species. A recent study indicated that silencing of *Burs-α*, *Burs-β*, or its receptor significantly affected the reproduction of *T. castaneum* (Bai et al., 2010). Knockdown of *CcBurs-α*, *CcBurs-β*, or both obviously decreased the expression of *CcBurs-R*, while feeding the heterodimer protein of α+β fully rescued *CcBurs-R* expression after knockdown of *CcBurs-α* and *CcBurs-β* together, which further confirmed the relationship between subunits and the receptor (Figure 3D-3F). 10°C treatment clearly improved the expression of *CcBurs-R*, but *CcTRPM* RNAi sharply reduced its mRNA level (Figure 3G-3H). Notably, elimination of *CcBurs-R* in *C. chinensis* obviously affected cuticle pigment content, cuticle chitin content, and cuticle thickness, leading to the failure of the transition from summer-form to winter-form (Figure 4B-4H). Feeding the α+β heterodimer protein fully rescued the defect in the transition percent and morphological phenotype after *CcBurs-R* knockdown (Figure 4G-4H). Therefore, these findings strongly support our hypothesis that Bursicon and its receptor are essential for the transition from summer-form to winter-form in *C. chinensis*. Actually, seasonal polyphenism is a complex process that may be regulated by multiple cascade reaction. Further studies are needed to clarify the regulatory mechanism of Bursicon and its receptor in mediating the seasonal polyphenism of *C. chinensis*.

In animals, miRNAs are essential for tissue development and behavioral evolution (Lucas and Raikhel, 2013). Previous studies have reported that many miRNAs function upstream of the neurohormone signaling pathway in insect polyphenism (Suderman et al., 2006). For example, miR-133 controls behavioral aggregation by targeting the dopamine synthesis gene in Locusts (Yang et al., 2014), and miR-9b targets insulin receptor to mediate dimorphism and wing development in aphids (Shang et al., 2020). In this study, we identified miR-6012 as a regulator of *CcBurs-R* in the Bursicon hormone signaling pathway for the first time. We found that miR-6012 was inhibited by a low temperature of 10 °C and targeted *CcBurs-R* by binding to its 3'UTR. When nymphs were treated with agomir-6012, they exhibited

lower cuticle pigment content, reduced cuticle chitin content, and thinner cuticle thickness compared to the agomir-NC control under 10 °C condition. In addition, agomir-6012 treatment markedly decreased the transition percent from summer-form to winter-form and affected the morphological phenotype compared to the control. The significantly decreased in *CcTre1* and *CcCHS1* expression after agomir-6012 treatment suggested that miR-6012 also functions as the up-stream regulator of chitin biosynthesis signaling.

In conclusion, our study uncovered a novel role of Bursicon and its receptor in regulating the seasonal polyphenism of *C. chinensis*, in addition to their known functions in cuticle-hardening of larvae and wing expansion of adults. In Figure 7, we proposed a molecular working model to describe this novel mechanism. Under 10 °C condition, Bursicon signaling pathway is first activated in the head of *C. chinensis* by low temperature and *CcTRPM*. Then, *CcBurs-α* and *CcBurs-β* form a heterodimeric neuropeptide that acts on its receptor *CcBurs-R* to mediate the transition from summer-form to winter-form by affecting cuticle pigment content, cuticle chitin content, and cuticle thickness. Moreover, miR-6012 targets *CcBurs-R* to modulate the function of Bursicon signaling pathway in this seasonal polyphenism. As a result, the 1st instar nymphs of summer-form develop into 3rd instar nymphs of winter-form to better adapt to low-temperature adversity. Future research will focus on: (1) studying the combined effect of Bursicon with other neuro-hormones on the seasonal polyphenism of *C. chinensis*, (2) identifying the down-stream signaling of Bursicon in mediating this phenomenon through multi-omics and RNAi approaches.

Materials and Methods

Insect rearing

C. chinensis populations of summer-form and winter-form were collected in June and December 2018, respectively, from pear orchards in Daxing, Beijing, China. The nymphs and adults of summer-form were reared on host plants in a greenhouse under condition of 25 ± 1°C, a photoperiod of 12L:12D, and a relative humidity of 65 ± 5%

(Zhang et al., 2023). Meanwhile, the nymphs and adults of winter-form were reared at 10 ± 1°C with a photoperiod of 12L:12D and a relative humidity of 25 ± 5% in an artificial incubator. Unless otherwise specified, the photoperiod of all subsequent treatments was 12L: 12D. Korla fragrant pear seedlings, 2-3 years old with a height of 50-80 cm, were used as host plants and received conventional water and fertilizer management.

Gene identification and sequence analysis

From the transcriptome database of *C. chinensis*, we obtained the predicted sequences of Bursicon subunits and its receptor. After sequencing validation, we named them *CcBurs-α* (GenBank accession number: OR488624), *CcBurs-β* (GenBank accession number: OR488625), and *CcBurs-R* (GenBank accession number: OR488626). The physicochemical properties of *CcBurs-α*, *CcBurs-β*, and *CcBurs-R* were analyzed using the online bioinformatics ProtParam tool (http://web.expasy.org/protparam/). The putative transmembrane domains of *CcBurs-R* were identified using the online software SMART (Simple Modular Architecture Research Tool). The tertiary protein structures of *CcBurs-α*, *CcBurs-β*, and *CcBurs-R* were predicted using the online server Phyre[2] (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and modified with PyMOL-v1.3r1 software. Homologous protein sequences from different insect species were searched using BLASTP in the NCBI database. Multiple alignments of the amino acid sequences for *CcBurs-α*, *CcBurs-β*, and *CcBurs-R* with other homologs were performed using DNAman software. Phylogenetic analysis was carried out based on the neighbor-joining (NJ) method in MEGA10.1.8 software.

Bursicon protein expression and determination

To express the Bursicon proteins in HEK293T cells, we first inserted the ORF sequences of *CcBurs-α* or *CcBurs-β* into the modified vector pcDNA3.1-his-P2A-mCherry to construct the recombinant vectors of pcDNA3.1-CcBurs-α-his-P2A-mCherry and pcDNA3.1-CcBurs-β-his-P2A-mCherry

using the *pEASY*-Basic Seamless Cloning and Assembly Kit (Cat# CU201, TransGen, Beijing, China) (Table S1). After confirming the sequences through sequencing and obtaining endotoxin-free plasmids, the recombinant vectors of *CcBurs-α* or *CcBurs-β* were transfected into HEK293T cells either individually or simultaneously following the protocol of *TransIntro*® EI Transfection Reagent (Cat# FT201, TransGen, Beijing, China). Control cells were transfected with the blank vector pcDNA3.1-his-P2A-mCherry (without *CcBurs-α* or *CcBurs-β* cDNA insert). After 6-10 h of transfection, the serum-free DMEM cell culture medium was replaced with fresh medium supplemented with 10% fetal bovine serum. After another 24 h of incubation, the medium was replaced again with serum-free DMEM. The medium was collected and centrifuged at 1000 × g for 10 min to remove cell debris after 48 h (An et al., 2012). The expressed Bursicon proteins were purified using Ni-NTA His·bind® resin (Cat# 70666, Merck, Germany). Then, western blotting was conducted to separate and identify these proteins using 15% SDS-PAGE (for reduced gel) and 12% SDS-PAGE (for non-reduced gel) with *ProteinFind*® Anti-His Mouse Monoclonal Antibody (Cat# HT501, TransGen, Beijing, China). Lastly, the protein bands were imaged using enhanced chemiluminescence with the Azure C600 multifunctional molecular imaging system (USA).

qRT-PCR for mRNA and miRNA

Samples for the temporal expression profile were collected at different developmental stages of summer-form and winter-form, including egg; nymphs of the 1st, 2nd, 3rd, 4th, and 5th instar; and adults of the 1st, 3rd, and 7th day. For the tissue expression pattern, six types of tissue (head, cuticle, midgut, fat body, wings, and foot) were dissected from both summer-form and winter-form of 5th instar nymphs. To examine the effect of different temperatures treatments on the expression of mRNAs and miRNAs, the newly hatched 1st instar nymphs of summer-form were treated at 25°C and 10°C, respectively. Samples were collected on the 3rd, 6th, and 10th day after different temperatures treatments. For the effect of *CcTRPM* knockdown on the transcription level of *CcBurs-α*, *CcBurs-β*, and *CcBurs-R* under 10°C conditions, the

newly hatched 1st instar nymphs of summer-form were fed with *CcTRPM* dsRNA, and the samples were collected on the 3rd, 6th, and 10th day after dsRNA feeding. Each sample was performed in three replications, with approximately 100 individuals for each replication of egg samples and at least 50 insects were included for each nymph or adult sample. All samples were immediately stored at -80°C for total RNA extraction.

Total RNAs were isolated from the above *C. chinensis* samples using TRNzol Universal (Cat# DP424, TIANGEN, Beijing, China) and miRcute miRNA isolation kit (Cat# DP501, TIANGEN, Beijing, China) for mRNA and miRNA, respectively, based on the manufacturer' protocol. The first-strand cDNA of mRNA or mature miRNA was synthesized from 500 ng or 1 μg of total RNAs using PrimeScript™ RT reagent kit with gDNA Eraser (Cat# RR047A, Takara, Kyoto, Japan) or miRcute Plus miRNA First-Strand cDNA Synthesis Kit (Cat# KR211, TIANGEN, Beijing, China) according to the instruction manual. The relative gene expression was quantified using TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat# RR820A, Takara, Kyoto, Japan) or miRcute Plus miRNA qPCR Detection Kit (Cat# FP411, TIANGEN, Beijing, China) in a total 20 μL reaction mixture on a CFX96 Connect™ Real-Time PCR System (Bio-Rad, Hercules, CA, USA). The conditions were as follows: denaturation for 3 min at 95°C, followed by 40 cycles at 95°C for 10s, and then 60°C for 30s. *Ccβ-actin* (GenBank accession number: OQ658571) or U6 snRNA was used as the internal reference gene for qRT-PCR in *C. chinensis* (Liu et al., 2020; Zhang et al., 2023). To check for specificity, melting curves were analyzed for each data point (Figure S2-S5). The $2^{-\Delta\Delta CT}$ method (CT means the cycle threshold) was used to quantify gene expression of qRT-PCR data, where ΔΔCT is equal to $\Delta CT_{treated\ sample}$ $-\Delta CT_{control}$ (Livak and Schmittgen, 2001).

dsRNA synthesis and RNAi experiments

The synthesis of double-stranded RNA (dsRNA) and the stem-leaf device for dsRNA feeding were conducted as previously described (Zhang et al., 2023). Briefly,

467 MEGAscript™ RNAi kit (AM1626, Ambion, California, USA) was used to
468 synthesize dsRNA in vitro using primers ligated with T7 RNA polymerase promoter
469 sequences at both ends (Table S1). The dsRNAs were further purified with the
470 phenol/chloroform method, air dried, dissolved in diethyl pyrocarbonate
471 (DEPC)-treated nuclease-free water, and stored at -80°C for later used. The purity and
472 concentration of dsRNA were measured using ultraviolet spectrophotometry and gel
473 electrophoresis.

474 For RNAi experiments, newly hatched 1st instar nymphs of summer-form were
475 fed with dsRNAs (500 ng/μL) targeting different genes and then divided into three
476 groups. (1) Samples were collected at 3, 6, and 10 d after dsRNAs feeding under 10°C
477 condition for the RNAi efficiency analysis and gene expression analysis by qRT-PCR.
478 (2) Nymph samples were collected at 12-15 d after dsRNA feeding for total cuticle
479 pigment analysis, comparison of cuticle ultrastructure, cuticle chitin staining with
480 WGA-FITC, and determination of cuticle chitin content under 10°C condition using
481 the following methods. (3) Morphological characteristics were observed every two
482 days, and the number of summer-form and winter-form individuals was counted until
483 the 3rd instar under 10°C condition, following the previous description (Zhang et al.,
484 2023).

485 **miRNA prediction and target validation with *CcBurs-R***

486 To study the post-translation function of *CcBurs-R*, the 3'UTR sequence of
487 *CcBurs-R* was amplified using the specific primers (Table S1) and the 3'-Full RACE
488 Core Set with PrimeScript RTase kit (Cat# 6106, Takara, Kyoto, Japan). Two software
489 programs, miRanda and Targetscan, were employed to predict miRNAs targeting
490 *CcBurs-R*, following previously described methods (Zhang et al., 2023). The
491 following methods were used to validate the target relationship between miRNAs and
492 *CcBurs-R*.

493 *In vitro* luciferase reporter gene assays: The full sequence of the 3'UTR or 3'UTR
494 sequence with binding sites removed from *CcBurs-R* was amplified and inserted

downstream of the luciferase gene in the pmirGLO vector (Promega, Wisconsin, USA) to construct recombinant plasmids. Agomir-6012 and antagomir-6012, chemically synthesized and modified RNA oligos with the same sequence or anti-sense oligonucleotides of miR-6012, were obtained from GenePharma (Shanghai, China). Agomir-NC and antagomir-NC, provided by the manufacture, were used as negative controls. Approximate 500 ng of the recombinant plasmid and 275 nM of agomir were co-transfected into HEK293T cells using the Calcium Phosphate Cell Transfection Kit (Cat# C0508, Beyotime, Nanjing, China). After 24 h of co-transfection, the activity of the luciferase enzymes was determined following the protocol of Dual-Luciferase Reporter Assay System (Cat# E1910, Promega, Wisconsin, USA).

In vivo RNA-binding protein immunoprecipitation assay (RIP): The RIP assay was performed using the Magna RIP Kit (Cat# 17–704, Merck, Millipore, Germany) (Zhang et al., 2023). Fifty nymphs were collected after feeding with agomir-6012 or agomir-NC for 24 h, and crushed with an auto homogenizer in ice-cold RIP lysis buffer. Magnetic beads were incubated with 5 μg of Ago-1 antibody (Merck, Millipore, Germany) or IgG antibody (Merck, Millipore, Germany) to form a magnetic bead-antibody complex. The target mRNAs were pulled down by the magnetic bead-antibody complex from the supernatants in the RIP lysates. The immunoprecipitated RNAs were released by digestion with protease K and quantification of *CcBurs-R* and miR-6012. Each experiment had six replicates.

Fluorescence in situ hybridization (FISH): The antisense nucleic acid probes for *CcBurs-R* (5'-GCGCUUGUGCUGCUUCUGCU-3') were labeled with FAM, and miR-6012 (5'-UGACCGACUAGAGUAGCGGCUU-3') was labeled with FITC (GenePharma, Shanghai, China). In short, nymph samples at different stages were immersed in Carnoy's fixative for 24-48 h at room temperature. After washing and decolorization, the samples were pre-hybridized three times using the hybridization buffer without the probes keep in dark. For co-localization, two fluorescent probes (1 μM) were combined to hybridize the samples for about 12 h in the dark. DAPI (1 μg/mL) was used to stain cell nuclei. The signals were observed and the images were

recorded using a Leica SP8 confocal microscopy (Weztlar, Germany). To exclude false positive, RNAi-treated samples or no-probe samples were used as negative controls.

Treatments of agomir-6012 and antagomir-6012

To examine the affect of miR-6012 on the mRNA expression of *CcBurs-R*, *CcTre1*, and *CcCHS1*, summer-form 1st instar nymphs were fed with agomir-6012 (1 μM) or antagomir-6012 (1 μM). The samples were first collected at 3, 6, and 10 d after feeding for agomir efficiency determination. Then, samples were collected at 6 days after treatment for total RNA extraction and qRT-PCR analysis. Agomir-NC and antagomir-NC were fed as negative control.

To explore the function of miR-6012 in seasonal polyphenism, summer-form 1st instar nymphs were fed with agomir-6012 (1 μM) or agomir-NC (1 μM). Subsequently, cuticle ultrastructure comparison, cuticle chitin staining with WGA-FITC, determination of cuticle chitin content, and observation of morphological characteristics were performed as described in the following methods.

Analysis of total cuticle pigment and cuticle chitin contents

To compare the difference in cuticle contents between summer-form and winter-form nymphs, the total cuticle pigment and cuticle chitin contents were determined. For the measurement of total cuticle pigment, the cuticle of dsRNA-treated nymphs were dissected and treated with acidified methanol (with 1% concentrated hydrochloric acid). The cuticle tissues were then pestled and placed in a thermostatic oscillator at 200 rpm for 24 h under 25°C condition. The total pigment extraction was obtained after filtering and centrifuging the supernatants through a 0.45 μm filter membrane. The UV absorbance of the total pigment extraction at different wavelengths was determined using a NanoDrop 2000 (Thermo Fisher Scientific, USA).

For the analysis of cuticle chitin content, WGA-FITC staining was conducted as

previously described (Xie et al., 2022; Zhang et al., 2023). Briefly, nymph samples were fixed with 4% paraformaldehyde and subjected to a gradient concentration dehydration with sucrose solution (10%, 20%, 30%). The dehydrated samples were then embedded in Tissue-Tek O.C.T. compound (Cat# 4583, SAKURA, Ningbo, China) after the pre-embedding stages at -25°C. Ultra-thin sections (approximately 70 nm thickness) of the embedded material were cut using a Leica freezing ultra-cut microtome (CM1850, Leica, Weztlar, Germany). The sections were stained with WGA-FITC (50 μg/mL) and DAPI (10 μg/mL) for 15 min, followed by rinsing three times with sterile PBS buffer. Fluorescence images were acquired using a Leica SP8 confocal microscopy (Weztlar, Germany). To further quantify the cuticle chitin content, a chitin Elisa kit (Cat# YS80663B, Yaji Biotechnology, Shanghai, China) was used according to the previously described method (Zhang et al., 2023).

Transmission electron microscopy assay

The TEM assay was performed as previously described (Ge et al., 2019; Zhang et al., 2022a; Zhang et al., 2023). In short, nymph samples without heads were fixed in 4% polyformaldehyde (PFA) for 48 h, followed by post-fixation in 1% osmium tetroxide for 1.5 h. The samples were then dehydrated in a standard ethanol/acetone series, infiltrated, and embedded in spurr medium. Subsequently, superthin sections (-70 nm) of the thorax were cut and stained with 5% uranyl acetate followed by Reynolds' lead citrate solution. Lastly, the sections were observed, photographed and measured using a HT7800 transmission electron microscope (Hitachi, Tokyo, Japan) operated at 120 kv. Six nymphs were used for each sample.

Statistical analysis

Figures preparation and statistical analysis were performed with GraphPad Prism 8.0 software and IBM SPSS Statistics 26.0, respectively. All data were shown as means ± SE (Standard Error of Mean) with different independent biological replications. Student's *t*-test was performed for pairwise comparisons to determine statistically significant differences between treatments and controls (*$P < 0.05$, **$P <$

0.01, and ***P < 0.001). One-way ANOVA followed by Tukey's HSD multiple comparison test was used for multiple comparisons in SPSS Statistics 26.0 (different letters denoted by $P < 0.05$).

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Author contributions statement

Songdou Zhang designed research; Songdou Zhang and Zhixian Zhang performed research; Jianying Li and Yilin Wang contributed new analytic tools; Songdou Zhang, Zhixian Zhang, Jianying Li, Yilin Wang, Zhen Li, and Xiaoxia Liu analyzed data; Songdou Zhang and Zhixian Zhang wrote the paper; Songdou Zhang and Xiaoxia Liu provided the fund.

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Conflict of interest statement

The authors declare that no competing financial interests.

Data availability statement

The published article includes all data generated or analyzed during this study. The full sequences of *CcBurs-α*, *CcBurs-β*, and *CcBurs-R* were submitted to GenBank database of NCBI (Accession number: OR488624, OR488625, and OR488626).

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Figure legends

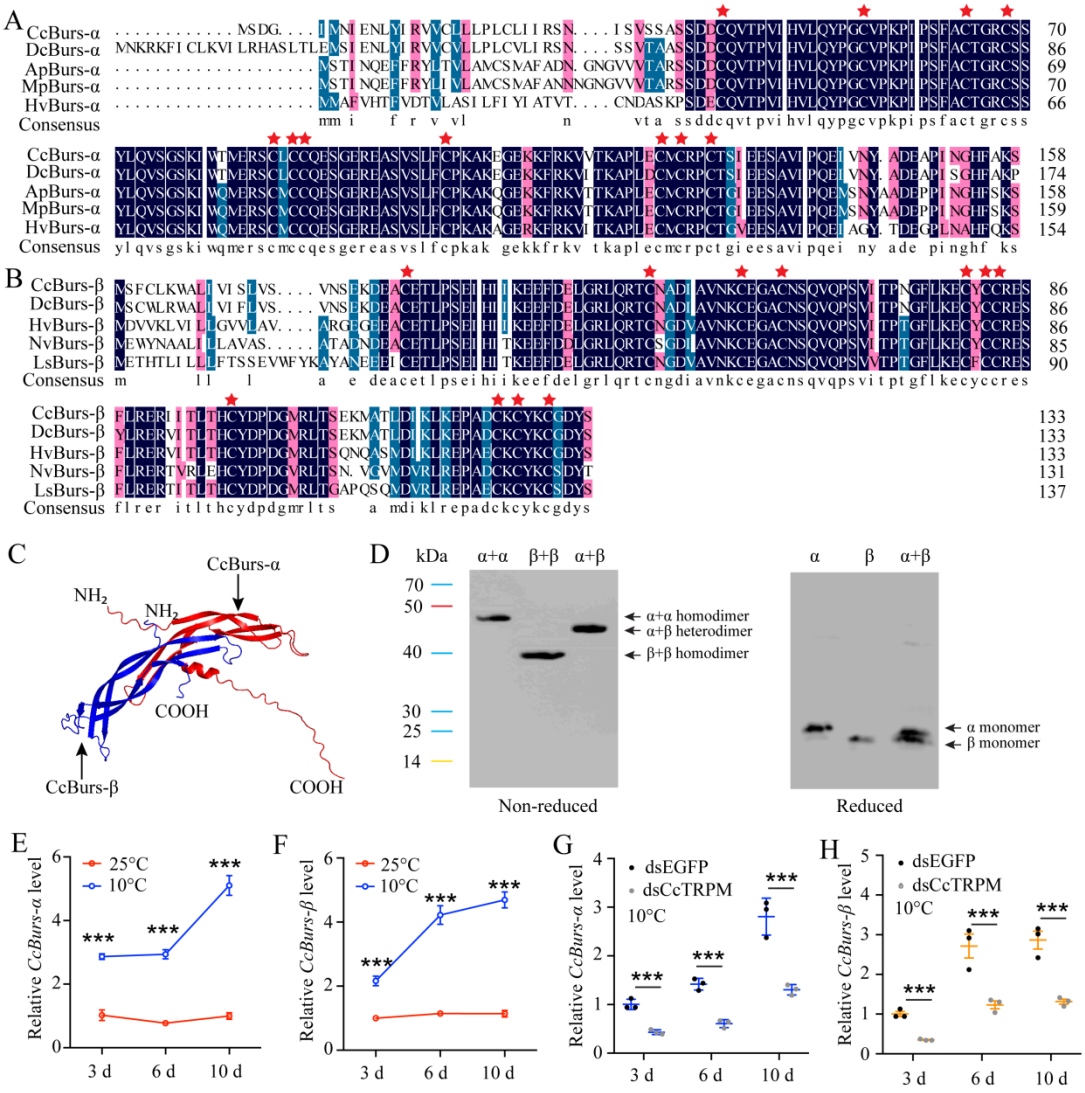


Figure 1. Molecular characteristic of *CcBurs-α* and *CcBurs-β* in *C. chinensis*.

A: Multiple alignments of the amino acid sequences of *CcBurs-α* with homologs from four other insect species. Black represents 100% identity, red represents 75% identity, and blue represents <75% identity. *CcBurs-α* (*C. chinensis*, OR488624), *DcBurs-α* (*Diaphorina citri*, XP_008468249.2), *ApBurs-α* (*Acyrthosiphon pisum*, XP_001946341.1), *MpBurs-α* (*Myzus persicae*, XP_022171710.1), *HvBurs-α* (*Homalodisca vitripennis*, XP_046670477.1). The corresponding GenBank accession numbers are as follows. **B: Multiple alignments of the amino acid sequences of *CcBurs-β* with homologs from four other insect species.** Black represents 100% identity, red represents 75% identity, and blue represents <75% identity. *CcBurs-β* (*C.*

chinensis, OR488625), *DcBurs-β* (*D. citri*, AWT50591.1), *HvBurs-β* (*H. vitripennis*, XP_046671521.1), *NvBurs-β* (*Nezara viridula*, AZC86173.1), *LsBurs-β* (*Laodelphax striatellus*, AXF48186.1). The corresponding GenBank accession numbers are as follows. **C: Predicted protein tertiary structures of *CcBurs-α* and *CcBurs-β*. D: Western blot analysis of Bursicon proteins using anti-His-Tag antibody with non-reduced and reduced SDS-PAGE.** The left numbers indicate the positions of pre-stained protein markers. Lanes of α, β, and α+β represent separate expression of CcBurs-α, CcBurs-β, or co-expressed of α+β. Monomers were not present under non-reduced conditions. **E-F: Relative mRNA expression of *CcBurs-α* and *CcBurs-β* after 25 °C or 10 °C treatments at 3, 6, and 10 d (n=3). G-H: Effect of temperature receptor *CcTRPM* knockdown on the mRNA expression of *CcBurs-α* and *CcBurs-β* at 3, 6, and 10 d under 10°C condition (n=3).** Data in 1E-1H are shown as the mean ± SE with three independent biological replications, with at least 50 nymphs for each biological replication. Statistically significant differences were determined using pair-wise Student's *t*-test in SPSS 26.0 software, and significance levels were denoted by *** ($p < 0.001$).

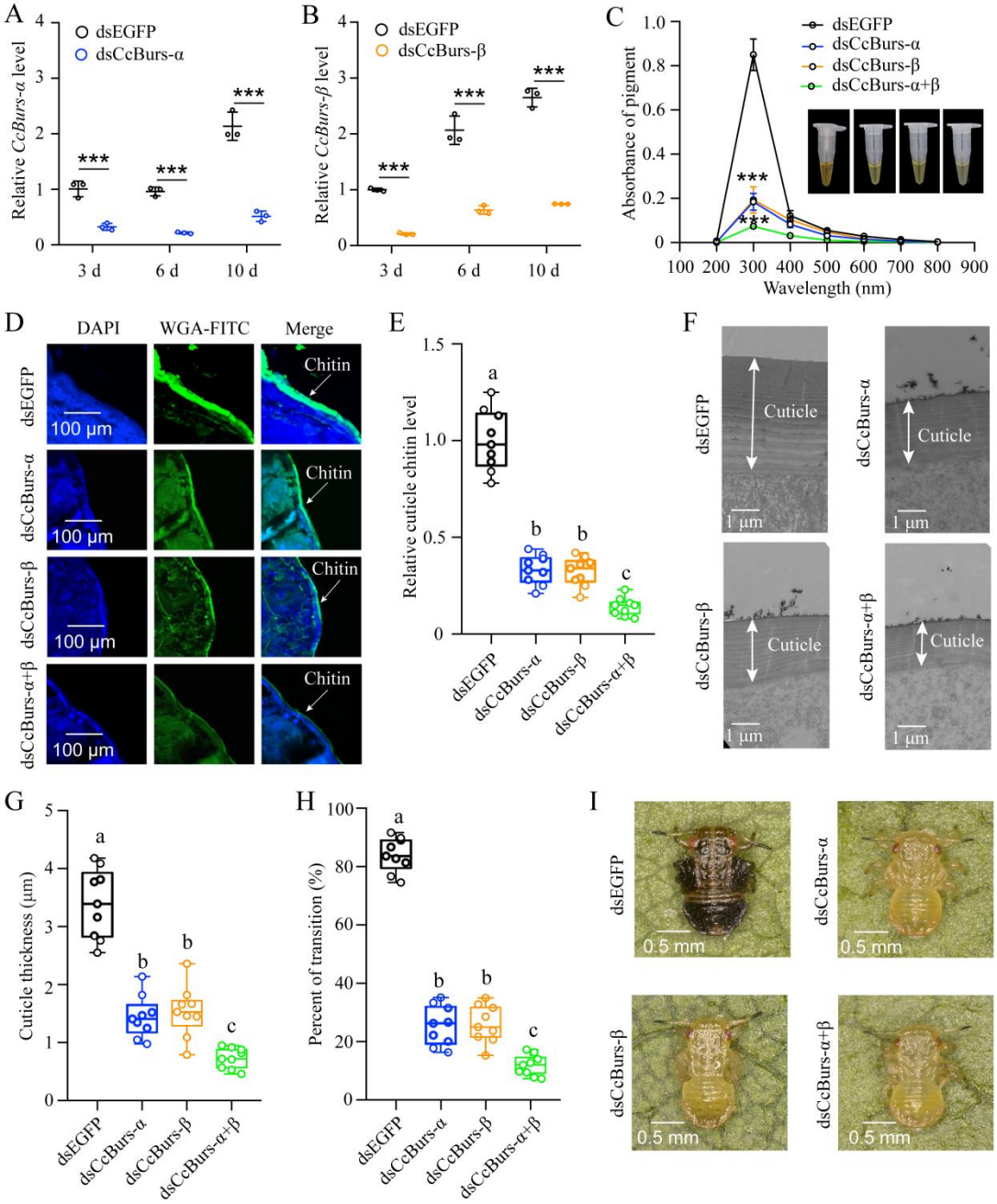


Figure 2. Neuropeptide Bursicon was essential for the transition from summer-form to winter-form in *C. chinensis*.

A-B: RNAi efficiency of *CcBurs-α* and *CcBurs-β* after dsRNA treatment at 3, 6, and 10 d by qRT-PCR under 10 °C condition (n=3). C-I: Effect of RNAi-mediated knockdown of *CcBurs-α* and *CcBurs-β* on the absorbance of total cuticle pigment, relative cuticle chitin content, cuticle thickness of the thorax, transition percent, and phenotype changes of 1st instar nymphs

compared to dsEGFP treatments (n=9). Data in 2A and 2B are shown as the mean ± SE with three independent biological replications, with at least 50 nymphs for each replication. Data in 2C, 2E, and 2G are presented as mean ± SE with three biological replications, with three technical replications for each biological replication. Data in 2H are presented as mean ± SE with nine biological replications. Statistically significant differences were determined using pair-wise Student's *t*-test, and significance levels were denoted by *** ($p < 0.001$). Different letters above the bars indicate statistically significant differences ($p < 0.05$), as determined by ANOVA followed by a Turkey's HSD multiple comparison test in SPSS 26.0 software.

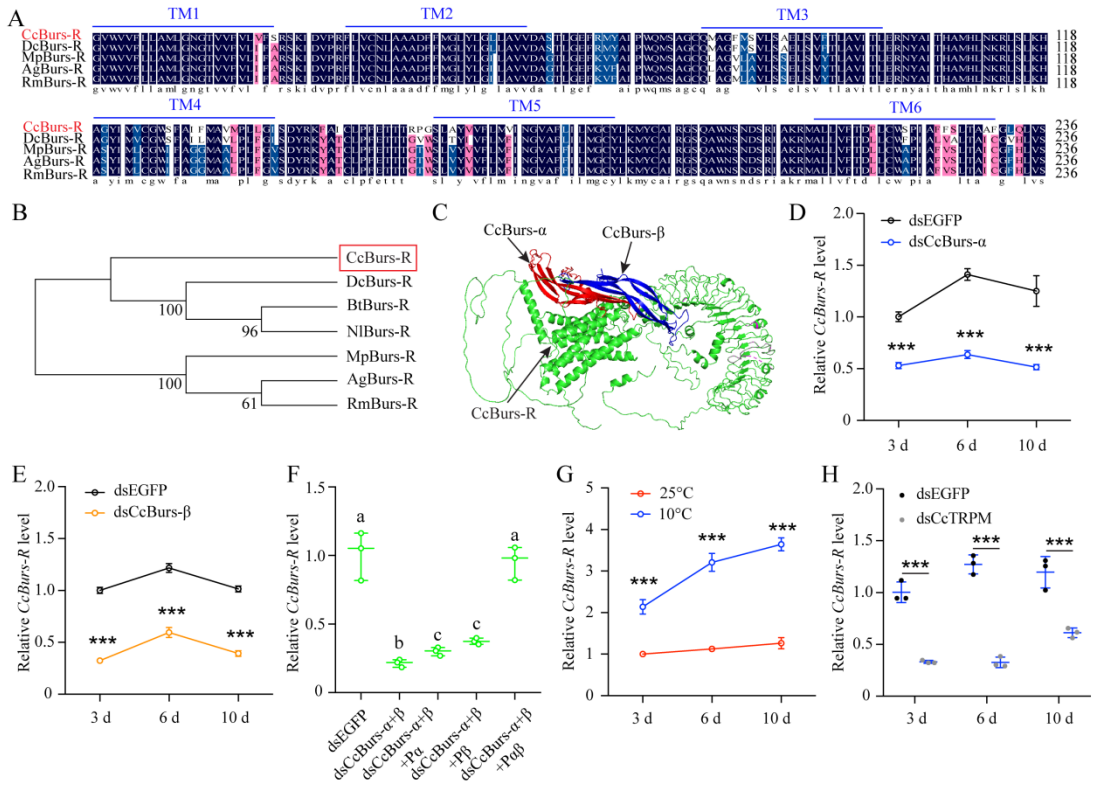


Figure 3. *CcBurs-R* was identified as the Bursicon receptor in *C. chinensis*.

A: Multiple alignments of the amino acid sequences of *CcBurs-R* transmembrane domain with homologs from four other insect species. The transmembrane domain from TM1 to TM6 is indicated by blue horizontal lines. *CcBurs-R* (*C. chinensis*,

OR488626), *DcBurs-R* (*D. citri*, KAI5703609.1), *MpBurs-R* (*M. persicae*, XP_022172830.1), *AgBurs-R* (*Aphis gossypii*, XP_027844917.2), *RmBurs-R* (*Rhopalosiphum maidis*, XP_026817427.1). The corresponding GenBank accession number as follows. **B: Phylogenetic tree analysis of *CcBurs-R* with its homologs in six other insect species.** *BtBurs-R* (*Bemisia tabaci*, XP_018898471.1), *NlBurs-R* (*N. lugens*, XP_022198758.2). **C: Predicted protein tertiary structure of *CcBurs-R* and its binding with *CcBurs-α* and *CcBurs-β*. D-E: Effect of *CcBurs-α* and *CcBurs-β* knockdown on the mRNA expression of *CcBurs-R* at 3, 6, and 10 d, respectively (n=3). F: CcBurs-α+β heterodimer protein could rescue the *CcBurs-R* expression after knockdown of *CcBurs-α* and *CcBurs-β* together. G: Relative mRNA expression of *CcBurs-R* after 25°C or 10°C treatments at 3, 6, and 10 d (n=3). H: Effect of temperature receptor *CcTRPM* knockdown on the mRNA expression of *CcBurs-R* at 3, 6, and 10 d (n=3). D**ata in 3D-3H are shown as the mean ± SE with three independent biological replications, with at least 50 nymphs for each replication. Statistically significant differences were determined using pair-wise Student's *t*-test in SPSS 26.0 software, and significance levels were denoted by *** ($p < 0.001$). Different letters above the bars indicated statistically significant differences ($p < 0.05$), as determined by ANOVA followed by a Turkey's HSD multiple comparison test in SPSS 26.0 software.

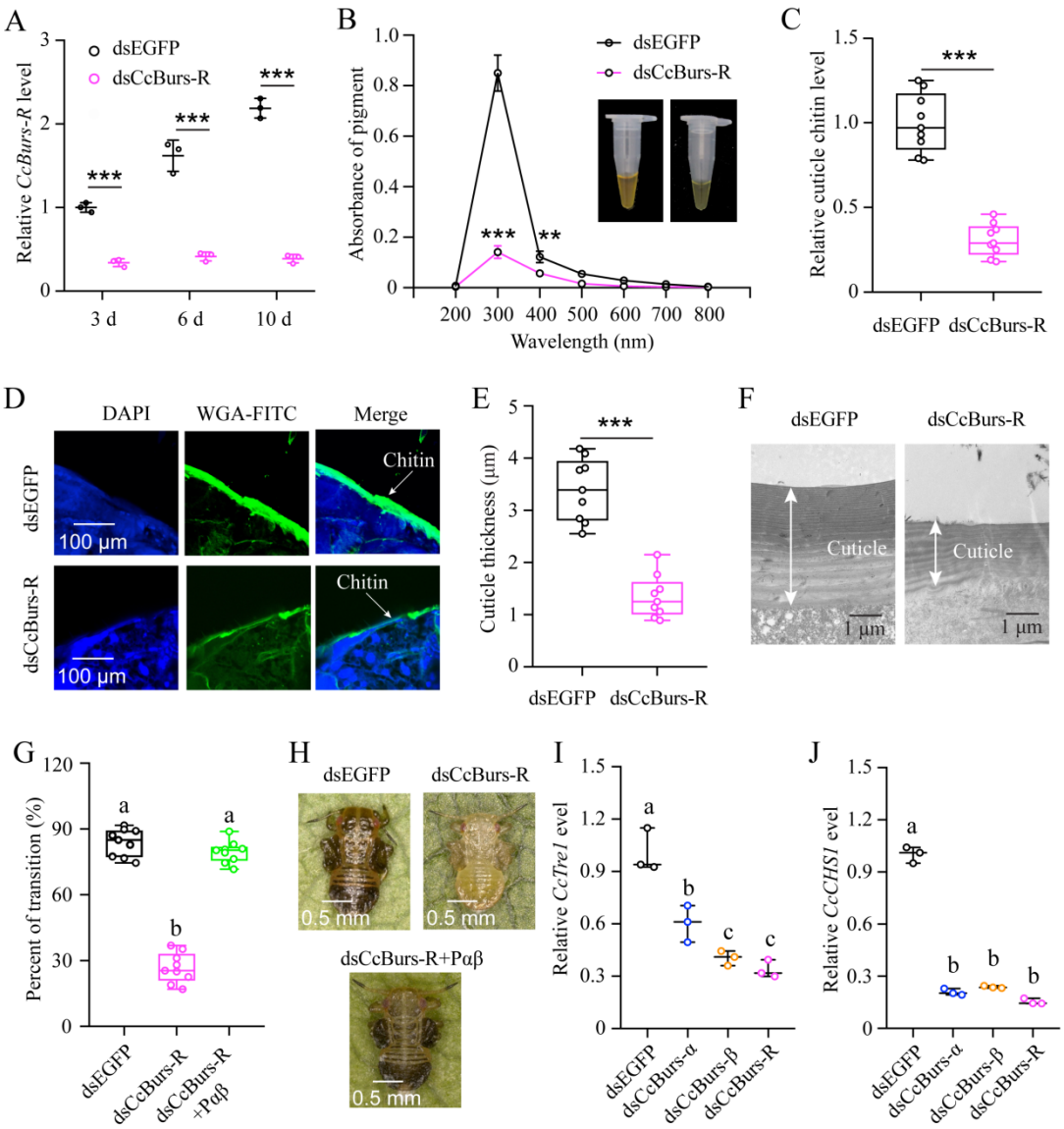
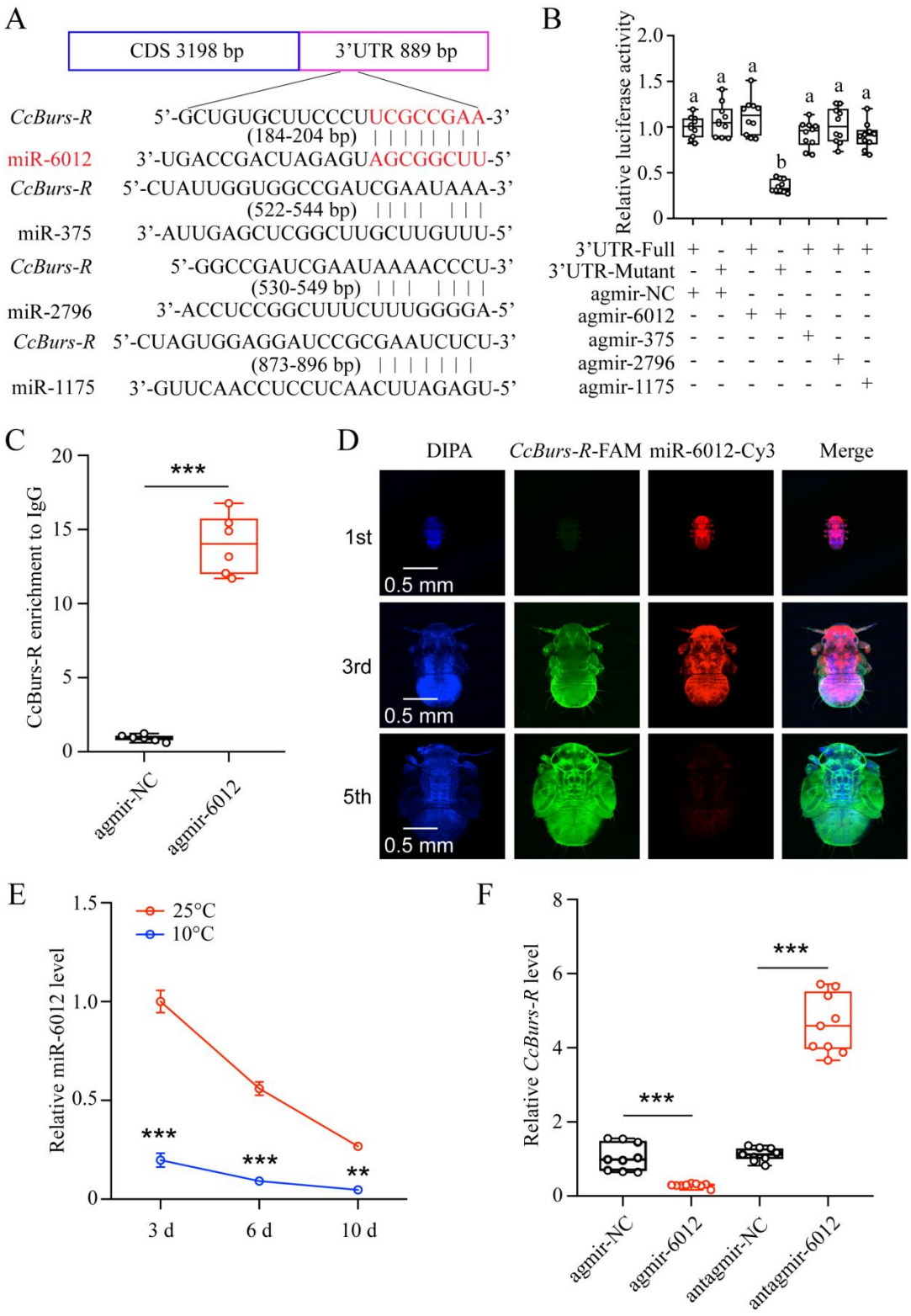


Figure 4. *CcBurs-R* directly mediated the transition from summer-form to winter-form in *C. chinensis*.

A: RNAi efficiency of *CcBurs-R* after dsRNA treatment at 3, 6, and 10 d by qRT-PCR under 10 °C condition (n=3). B-H: Effect of RNAi-mediated knockdown of *CcBurs-R* on the absorbance of total cuticle pigment, relative cuticle chitin content, cuticle thickness of the thorax, transition percent, and phenotype changes of 1st instar nymphs compared to dsEGFP treatments under 10 °C condition (n=9). I-J: Relative mRNA expression of *CcTre1* and *CcCHS1* after knockdown of *CcBurs-α*, *CcBurs-β*, and *CcBurs-R* at 10 d, separately (n=3). Data in 4A, 4I, and 4J are shown as the mean ± SE with three independent biological

replications, with at least 50 nymphs for each replication. Data in 4B, 4C, and 4E are presented as mean ± SE with three biological replications, with three technical replications for each biological replication. Data in 4G are presented as mean ± SE with nine biological replications. Statistically significant differences were determined using pair-wise Student's *t*-test, and significance levels were denoted by ** ($p < 0.01$) and *** ($p < 0.001$). Different letters above the bars indicate statistically significant differences ($p < 0.05$), as determined by ANOVA followed by a Turkey's HSD multiple comparison test in SPSS 26.0 software.



865

866 **Figure 5. miR-6012 directly targeted *CcBurs-R* to inhibit its expression.**

867 **A: Predicted binding sites of four miRNAs in the 3'UTR of *CcBurs-R*. B: *In vitro***
868 **confirmation of the target relationship between miR-6012 and *CcBurs-R* using**

dual luciferase reporter assays. C: *In vivo* validation of miR-6012 directly targeting *CcBurs-R* using RNA-binding protein immunoprecipitation (RIP) assay. D: Co-localization of miR-6012 and *CcBurs-R* in different development stages of *C. chinensis* using FISH. E: Effect of different temperature treatments on the expression of miR-6012 by qRT-PCR. F: Effect of miR-6012 agomir and antagomir treatments on the mRNA level of *CcBurs-R* at 6 d under 10 °C conditions. Data in 5B and 5F are presented as the mean ± SE with nine biological replicates. Results of 5C and 5E are indicated as the mean ± SE with six or three biological replicates. Statistically significant differences were determined using pair-wise Student's *t*-test, and significance levels were denoted by *** ($p < 0.001$). Different letters above the bars represent statistically significant differences ($p < 0.05$), as determined by ANOVA followed by a Turkey's HSD multiple comparison test in SPSS 26.0 software.

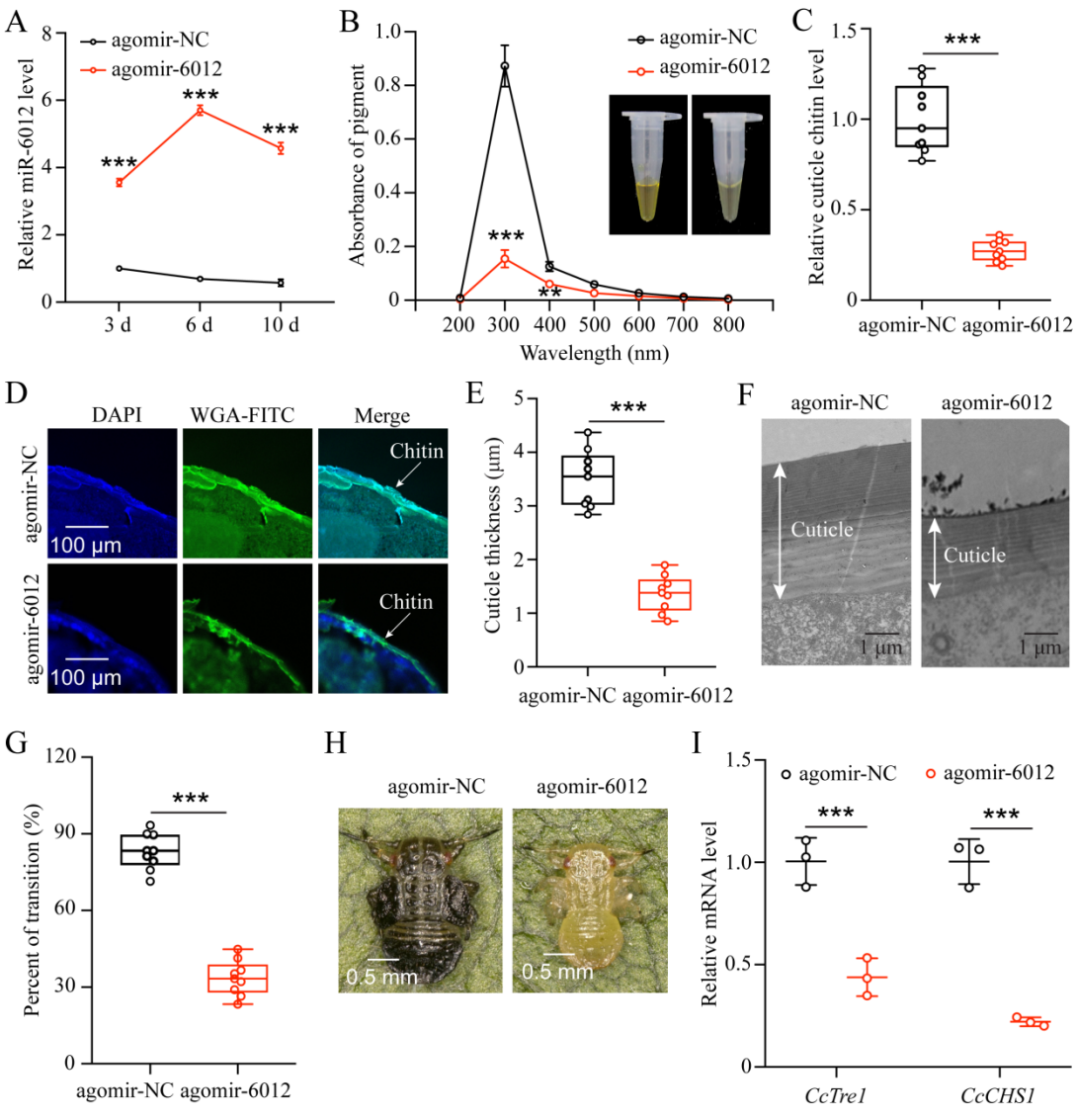


Figure 6. miR-6012 targeted *CcBurs-R* to mediate the seasonal polyphenism in *C. chinensis*.

A: Expression of miR-6012 after agomir-6012 treatment at 3, 6, and 10 d by qRT-PCR under 10 °C condition (n=3). B-H: Effect of agomir-6012 treatment on the absorbance of total cuticle pigment, relative cuticle chitin content, cuticle thickness of the thorax, transition percent, and phenotype changes of 1st instar nymphs compared to agomir-NC treatments under 10 °C condition (n=9). I: Relative mRNA expression of *CcTre1* and *CcCHS1* after agomir-6012 treatment at 6 d, separately (n=3). Data in 6A and 6I are shown as the mean ± SE with three independent biological replications, with at least 50 nymphs for each replication. Data

in 6C and 6E are presented as mean ± SE with three biological replications of three technical replications for each biological replication. Data in 6B and 6G are presented as mean ± SE with nine biological replications. Statistically significant differences were determined using pair-wise Student's *t*-test, and significance levels were denoted by ** ($p < 0.01$) and *** ($p < 0.001$).

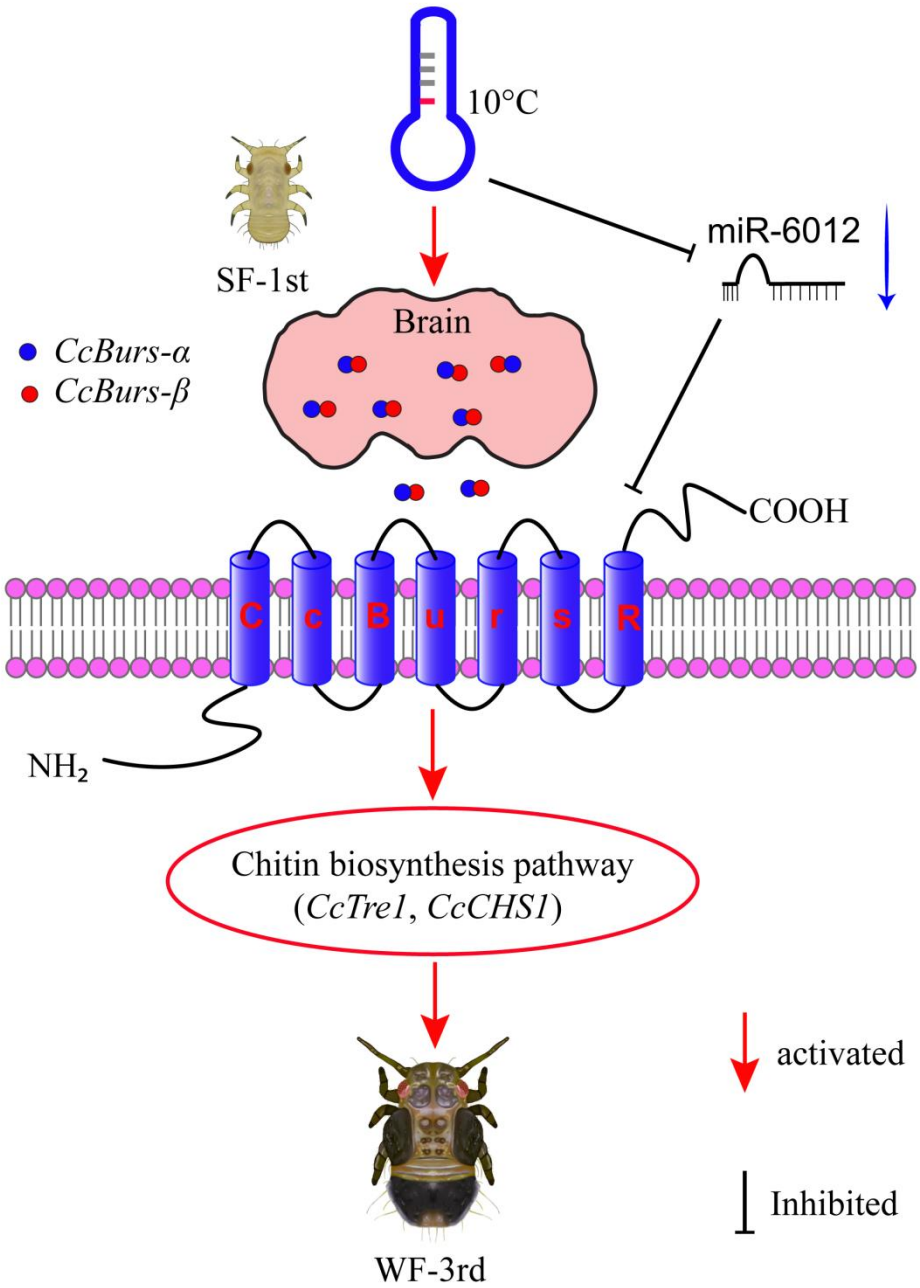


Figure 7. Schematic model of the novel functions of Bursicon signaling in the

seasonal polyphenism of *C. chinensis* in response to low temperature.

Under 10°C condition, low temperature significantly upregulated the expression of Bursicon signaling pathway. *CcBurs-α* and *CcBurs-β* then formed a heterodimeric neuropeptide to activate their receptor *CcBurs-R*, which mediated the transition from summer-form to winter-form in *C. chinensis* by acting on the chitin biosynthesis pathway. Furthermore, low temperature inhibited the expression of miR-6012, relieving its inhibitory effects on *CcBurs-R*. miR-6012 directly targeted *CcBurs-R*, contributing to the novel function of Bursicon signaling in seasonal polyphenism. Finally, the 1st instar nymphs of summer-form developed into 3rd instar nymphs of winter-form in *C. chinensis*.