Calmodulin-mediated suppression of 2-ketoisovalerate reductase in *Beauveria bassiana* beauvericin biosynthetic pathway

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Summary

Ketoisovalerate reductase (KIVR, E.C. 1.2.7.7) mediates the specific reduction of 2-ketoisovalerate (2-Kiv) to D-hydroxyisovalerate (D-Hiv), a precursor for beauvericin biosynthesis. Beauvericin, a famous mycotoxin produced by many fungi, is a cyclooligomer depsipeptide, which has insecticidal, antimicrobial, antiviral and cytotoxic activities. In this report, we demonstrated that *Beauveria bassiana* 2-ketoisovalerate reductase (BbKIVR) acts as a typical KIVR enzyme in the entomopathogenic fungus *B. bassiana.* In addition, we found that BbKIVR interacts with calmodulin (CaM) *in vitro* and *in vivo*. The functional role of CaM-binding to BbKIVR was to negatively regulate the BbKIVR activity in *B. bassiana*. Environmental stimuli such as light and salt stress suppressed BbKIVR activity in *B. bassiana*. Interestingly, this negative effect of BbKIVR activity by light and salt stress was recovered by CaM inhibitors, suggesting that the inhibitory mechanism is mediated through stimulation of CaM activity. Therefore, this work suggests that BbKIVR plays an important role in the beauvericin biosynthetic pathway mediated by environmental stimuli such as light and salt stress via the CaM signaling pathway.

Introduction

Beauveria bassiana is a fungus that grows naturally in soils and acts as a parasite on various arthropod species, causing white muscardine disease. Thus, it belongs to the entomopathogenic fungi (Shah and Pell, 2003). At present, it is being used as a biological insecticide to control a number of pests (Shah and Pell, 2003). Fungi are exposed to severe changes in various environmental conditions. Environmental signal such as light represents information enabling fungi to react to the different ambient conditions between day and night (Adams and Yu, 1998; Adams *et al.*, 1998; Belozerskaya *et al.*, 2012). Besides perception of the light, integration of light signal with other environmental stimuli mediates light-induced regulation of multiple signaling pathways and metabolic pathways (Adams and Yu, 1998;

Adams *et al.*, 1998; Belozerskaya *et al.*, 2012). Especially, light is known to inhibit sexual reproduction and secondary metabolism in some fungi (Adams and Yu, 1998; Adams *et al.*, 1998; Belozerskaya *et al.*, 2012).

Fungi produce a large number of secondary metabolites that play roles in multiple cellular events during growth and development (Adams and Yu, 1998; Yu and Keller, 2005; Rai *et al.*, 2009). These metabolites play crucial roles for useful applications in agricultural, pharmaceutical and industrial fields (Adams and Yu, 1998; Yu and Keller, 2005; Rai *et al.*, 2009). Beauvericin, which has multiple cellular effects, is a cyclooligomer depsipeptide produced by many fungi (Hamill *et al.*, 1969; Gupta *et al.*, 1991; Fukuda *et al.*, 2004; Jow *et al.*, 2004; Lin *et al.*, 2004; Chen *et al.*, 2006; Zhan *et al.*, 2007). Nonribosomal peptides, including beauvericin, belong to an extensive family of biologically active natural products. Their structural diversity is mainly caused by the features of nonribosomal peptide synthetase (NRPS) enzymatic machinery (Kopp and Marahiel, 2007). The NRPS is a multifunctional enzyme that catalyzes the iterative synthesis of oligopeptide monomer units through the recursive oligomerization of the monomers via a nonribosomal, thiol-templated mechanism (Kopp and Marahiel, 2007). Hydroxyisovalerate (Hiv) is a common 2-hydroxycarboxylic acid ingredient of depsipeptides including beauvericin. In fungi, D-Hiv is formed by the highly specific reduction reaction of 2-ketoisovalerate (2-Kiv) by 2-ketoisovalerate reductase (KIVR) (Lee *et al.*, 1992; Zhang *et al.*, 2012). Beauvericin production is inhibited in a *KIVR* knockout *B. bassiana*, suggesting a critical role of *KIVR* in the beauvericin biosynthesis (Xu *et al.*, 2009).

Calmodulin (CaM) is one of the most conserved Ca^{2+}-binding proteins in eukaryotes. The CaM-binding domain of target proteins is composed of 16–35 contiguous amino acids with positively charged amphipathic characteristics that form an alpha-helix (Cyert, 2001; O'Day, 2003; Reddy *et al.*, 2011). CaM interacts with a variety of target proteins and controls their functions. The CaM-targeted proteins play important roles in the regulation of multiple

cellular functions (Cyert, 2001; O'Day, 2003; Reddy *et al.*, 2011). Even though a number of CaM-binding proteins are currently known in yeast, animals and plants, little is known about CaM-targeted proteins in filamentous fungi (Kim *et al.*, 2015). Therefore, it is required to identify and characterize the CaM-binding proteins for extending our understanding in the CaM-mediated signaling pathways of filamentous fungi.

Here, we report that a KIVR from *B. bassiana* (BbKIVR) binds to CaM *in vitro* and *in vivo*, and CaM suppresses BbKIVR activity via the beauvericin biosynthetic pathway. In addition, we show that environmental changes such as light and salt stress affect BbKIVR activity through CaM signaling pathway.

Results and discussion

Identification of a putative CaM-binding motif in BbKIVR

B. bassiana genome contained only a *KIVR* gene (*BbKIVR*), and its predicted molecular mass was 51.9 kDa and its *p*I was 8.96, respectively. BbKIVR contained a typical C-terminal ketopantoate reductase domain (Fig. 1A). In addition, it had the conserved GXGXXG nucleotide-binding signature in the N-terminal region (Fig. 1A). Interestingly, our computational analyses (calmodulin target database and helical wheel projection) predicted that BbKIVR has a putative CaM-binding motif in the middle region (Fig. 1A). Generally, CaM-binding motifs are stretches of 16-35 residues, formed by basic amphipathic α-helix (Cyert, 2001; O'Day, 2003; Reddy *et al.*, 2011). Helical wheel projection predicted that the putative CaM-binding domain of BbKIVR is likely restricted to amino acids 167-184 that form a typical basic amphipathic α-helix (Fig. 1B). This motif appears to be fit a 1-12 motif (FILVW)xxxxxxxxxx(FILVW) (Rhoads and Friedberg, 1997). Multiple sequence alignment showed that BbKIVR has sequence identity to the KIVR domains of fungal KIVRs (Fig. 1C). In addition, the putative CaM-binding domains of KIVRs were highly conserved in fungi, indicating that CaM-binding to KIVRs might be a common feature in fungi (Fig. 1C).

Biochemical characterization of recombinant His-BbKIVR

The KIVR is required for the formation of beauvericin through the NRPS biosynthetic pathway (Fig. 2A). It catalyzes the NADPH-specific reduction of 2-Kiv to D-Hiv (Lee *et al.*, 1992; Zhang *et al.*, 2012). To test the *in vitro* KIVR activity of BbKIVR, we cloned full-length *BbKIVR* cDNA into a pET100 expression vector, and recombinant His-BbKIVR was then purified by a His-Select purification system (Sigma-Aldrich) in *Escherichia coli* BL21(DE3) cells. The substrate specificity of the His-BbKIVR was performed using the following substrates: 2-Kiv, pyruvate, 2-ketobutyrate and 2-ketoglutarate. His-BbKIVR exhibited the highest activity to 2-Kiv as substrate, while the homologous compounds pyruvate, 2-ketobutyrate and 2-ketoglutarate were shown to be poor substrates for the His-BbKIVR activity (Fig. 2B). The optimal activity of His-BbKIVR was obtained at pH 7.5 and 35 °C (Fig. 2B). Therefore, our results suggest that BbKIVR acts as a typical KIVR enzyme.

BbKIVR interacts with CaM in vitro *and* in vivo

BbKIVR contains a putative CaM-binding motif at the middle region, suggesting BbKIVR might be a novel CaM-binding protein (Fig. 1B). First, we examined whether BbKIVR has the ability to bind CaM *in vitro*. Recombinant His-tagged BbKIVR was produced in *E. coli* BL21(DE3) cells, and then subjected to the CaM-Sepharose pull-down assay. The result revealed that His-BbKIVR binds to CaM in a Ca^{2+}-independent manner (Fig. 3A). Ca^{2+} signal can be decoded by receptor proteins such as CaM and alters a number of cell responses involved in metabolism and physiology (Cyert, 2001; O'Day, 2003; Reddy *et al.*, 2011). Ca^{2+}/CaM binds to target proteins and alters their functions by transducing the Ca^{2+} signal (Cyert, 2001; O'Day, 2003; Reddy *et al.*, 2011). Ca^{2+}-free apocalmodulin (ApoCaM) binds to other proteins and has other specific functions. ApoCaM plays crucial roles in the cell that apparently do not require the ability to bind Ca^{2+} at all, and these roles appear to be

essential for cell growth and development (Cyert, 2001; O'Day, 2003; Reddy *et al.*, 2011). Basically, ApoCaM differs from Ca^{2+}/CaM in its tertiary structure (Cyert, 2001; O'Day, 2003; Reddy *et al.*, 2011). Here, we describe that BbKIVR is a novel ApoCaM-binding protein in *B. bassiana*.

In vivo coimmunoprecipitation (CoIP) assay also revealed an interaction between BbKIVR and CaM in *B. bassiana* (Fig. 3B). We isolated 2 mg of protein extracts from *B. bassiana* mycelia. Since the *B. bassiana* genome contains only one *KIVR* gene (*BbKIVR*), a protein having KIVR activity was expected to be BbKIVR among *B. bassiana* extracts. Using a CaM antibody, we immunoprecipitated CaM-associated proteins from the protein extracts, and these proteins were then subjected to *in vitro* KIVR activity assay. As shown in Figure 3B, we were able to detect KIVR activity from the co-immunoprecipitated proteins. When the protein extracts were immunoprecipitated using an anti-His antibody as a negative control, we barely detected BbKIVR activity from the co-immunoprecipitated proteins (Fig. 3B). This suggests that BbKIVR interacts with CaM in *B. bassiana*. Therefore, our results suggest that BbKIVR plays a role in mediating a molecular crosstalk between CaM signaling and the beauvericin biosynthesis by directly interacting with CaM.

CaM inhibits the BbKIVR activity

To investigate a role of CaM-binding to BbKIVR, an *in vitro* KIVR activity assay using His-BbKIVR was performed under the presence of CaM. As shown in Figure 4A, His-BbKIVR showed significant CaM-dependent inhibition in the *in vitro* assay of KIVR activity, indicating that CaM has a negative role in the activity of His-BbKIVR *in vitro*.

To confirm the inhibitory effect of CaM on BbKIVR activity, we used CaM inhibitors W-5 (N-(6-Aminohexyl)-1-naphthalenesulfonamide), W-7 (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide) and W-12 (*N*-(4-Aminobutyl)-2-naphthalenesulfonamide) that bind hydrophobic residues of the CaM-binding pocket through Van der Waals interactions

(Hidaka *et al.*, 1981). The protein extracts from *B. bassiana* mycelia were subjected to an *in vitro* KIVR activity assay under W-5, W-7 or W-12. As shown in Figure 4B, CaM inhibitors increased the BbKIVR activity of *B. bassiana*. Therefore, our results suggest a negative role of CaM in regulation of the BbKIVR activity.

Ketopantoate reductase (PanE) catalyzes the NADPH-dependent reduction of ketopantoate to pantoate, an essential step for the biosynthesis of pantothenate (vitamin B_5) (Ciulli *et al.*, 2007). Since BbKIVR is a member of family ketonate reductases, BbKIVR might have a similar 3D structure with that of PanE. Crystal structure suggests that PanE forms a dimer having two monomers (Ciulli *et al.*, 2007). BbKIVR might also form a dimer. CaM is known to inhibit the formation of a multimeric protein assembly (Zhang *et al.*, 2009). The inhibitory effect of BbKIVR activity by CaM might be triggered by a physical disturbance of CaM in the formation of an appropriate dimer. However, further studies are required for proving this possibility.

BbKIVR peptide binds to CaM and suppresses the inhibitory effect of CaM on BbKIVR activity

A synthetic BbKIVR peptide corresponding to residues 164-188 was synthesized, and its CaM-binding ability was tested using a gel mobility shift assay. The larger peptide-bound form of CaM should migrate with a lower mobility than free CaM under non-denaturing conditions. The result revealed that the peptide can form a complex with CaM (Fig. 5A). Heat shock protein 70 (HSP70) peptide was used as a positive control (Stevenson and Calderwood, 1990).

The BbKIVR peptide was also tested for influence on the inhibitory effect of CaM on BbKIVR activity. CaM-dependent inhibition of His-BbKIVR activity was monitored in the presence of the peptide *in vitro*. The peptide suppressed the inhibitory effect of CaM on His-BbKIVR activity *in vitro* (Fig. 5B). Therefore, our results revealed that the peptide

successfully competed with His-BbKIVR for CaM-binding, suggesting that the peptide has a CaM-binding feature.

CaM has a role in the regulation of BbKIVR activity through environmental changes

Light is known to inhibit secondary metabolism in some fungi (Adams and Yu, 1998; Adams *et al.*, 1998; Belozerskaya *et al.*, 2012). We examined whether CaM plays a role in the regulation of BbKIVR activity under environmental changes. As shown in Figure 6, light and salt stress suppressed BbKIVR activity in *B. bassiana* mycelia on agar culture compared to dark. Interestingly, this negative effect of BbKIVR activity was partially reversed by CaM inhibitor (W-5 or W-7) treatment under light and salt stress (Fig. 6). Therefore, the inhibitory mechanism of BbKIVR activity under light and salt stress is most likely mediated through CaM activation. Even though BbKIVR activity was not affected by oxidative stress, W-5 or W-7 slightly enhanced BbKIVR activity under oxidative stress (Fig. 6).

Adaptation to environmental changes is critical for the survival of all organisms. CaM plays critical roles in the regulation of cellular responses to environmental changes in eukaryotes (Cyert, 2001; O'Day, 2003; Reddy *et al.*, 2011). In this report, light and salt stress appear to affect the beauvericin biosynthetic pathway by suppressing BbKIVR activity through CaM signaling. The activity of BbKIVR might also be responsive to a variety of environmental stimuli, including nutrient depletion, UV irradiation, extreme temperatures, dryness, oxygen limitation, and other stress conditions in *B. bassiana*. Further studies are required for addressing this question. This work shows that BbKIVR activity is affected by light and salt stress in *B. bassiana*. Activated CaM under light and salt stress can suppress beauvericin biosynthesis through the inhibition of BbKIVR activity. On the contrary, dark appears to enhance BbKIVR activity by suppressing CaM activity.

Conclusions

Beauvericin is a fungal product with various kinds of bioactivities such as insecticidal, antimicrobial, antiviral and cytotoxic activities. These useful bioactivities make beauvericin a potential target for the applications in agricultural and pharmaceutical fields. In this report, we characterize a KIVR (BbKIVR), a critical enzyme for beauvericin biosynthesis in the entomopathogenic fungus *B. bassiana*. Mainly, we describe that CaM signaling is tightly involved in the molecular regulation of BbKIVR activity in response to environmental changes such as light and salt stress in *B. bassiana.* Therefore, this work represents novel findings in KIVR-mediated beauvericin biosynthetic pathway by which BbKIVR mediates a molecular crosstalk between the beauvericin biosynthesis and the CaM signaling in order to rapidly respond to light or salt stress in *B. bassiana*.

Experimental procedures

Fungal materials

The spore's suspensions of *B. bassiana* strain EFCC783 were inoculated onto sterilized cellophane (Bio-Rad) of SDAY agar plates, and incubated at 24 °C with 3,000 lux white light or without white light. Mycelia of the cellophane cultures were harvested and immediately used for further analysis.

Prediction of a CaM-binding motif on BbKIVR

A putative CaM-binding domain of BbKIVR was predicted using the helical wheel projection program (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi) and using the calmodulin target database (http://calcium.uhnres.utoronto.ca/ctdb/pub_pages/search/index.htm).

Purification of recombinant His-BbKIVR in E. coli

Total RNA was extracted from *B. bassiana* mycelia using the RNeasy mini kit (Qiagen) following the manufacturer's protocol. To remove the genomic DNA from the total RNA,

treatment with DNase I (Promega) was performed according to the manufacturer's instructions followed by chloroform extraction and precipitation. The cDNA was prepared from 1 μg of total RNA using the SuperScript III first-strand synthesis system (Life Technologies) according to the manufacturer's instructions. The cDNA encoding full-length *BbKIVR* was amplified by PCR with *BbKIVR*-specific primers. The *BbKIVR*-specific primers used were: forward primer, 5'- cacc atg cct tct ccg gag cat cca -3' and reverse primer, 5'- tta caa tag cct cct cgc cag -3'. The amplified PCR products were gel-purified (Bioneer) and inserted into a pET100 expression vector (Life Technologies). Recombinant His-BbKIVR was induced with 0.5 mM IPTG in *E. coli* strain BL21(DE3) cells (Life Technologies) and then His-BbKIVR was purified using a His-Select purification system (Sigma-Aldrich) as described by the manufacturer. The amount of protein was estimated by the Bradford's method using a protein assay kit (Bio-Rad). Purified His-BbKIVR was separated by 10 % SDS-PAGE and the gel was stained by Coomassie Brilliant Blue (Sigma-Aldrich).

In vitro *KIVR activity assay of the His-BbKIVR*

An assay measuring the KIVR activity of His-BbKIVR was performed as described previously with minor modifications (Zhang *et al.*, 2012). Briefly, the KIVR assay mixture contained 50 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 0.7 mM 2-Kiv, 0.29 mM NADPH, and 10 μg of His-BbKIVR. The reaction was initiated by the addition of substrate, and the decrease in absorbance at 340 nm was measured at 35 ºC for 30 min. A molar extinction coefficient of 6.22 cm^2/pmol NADPH was used for the calculation of enzyme activity. One unit was defined as the amount of enzyme caused the oxidation of 1 μmol of NADPH per min. To test the role of CaM in His-BbKIVR activity, 10 μg of His-BbKIVR was incubated with various concentrations of CaM (Sigma-Aldrich) for 20 min at room temperature before adding substrate.

In vitro *KIVR activity assay of protein extracts from* B. bassiana

B. bassiana mycelia were ground into fine powder in liquid nitrogen and homogenized in 2 ml ice-cold extraction buffer containing 25 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 150 mM NaCl, 1 % β-mercaptoethanol and protease inhibitor cocktail (Roche). After centrifugation at 8,000 rpm for 30 min at 4 °C, the supernatant was collected and the amount of protein extracts was estimated by the Bradford's method using a protein assay kit (Bio-Rad). KIVR activity assay was performed as described previously with minor modifications (Zhang *et al.*, 2012). Reaction mixtures (200 μg protein extracts, 50 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 0.7 mM 2-Kiv, 0.29 mM NADPH) were incubated for 30 min at 35 °C, and then KIVR activity was measured spectrophotometrically at OD_{340}. To test the role of CaM inhibitors (Sigma-Aldrich) on KIVR activity in protein extracts from *B. bassiana*, 200 μg of protein extracts were incubated with 250 μM of W-5, W-7 or W-12 for 20 min at room temperature before adding substrate.

CaM-Sepharose pull-down assay

Recombinant His-BbKIVR proteins were purified by CaM-Sepharose 4B beads (GE Healthcare) as described previously (Safadi *et al.*, 2000). Approximately 100 μl of pre-equilibrated CaM-Sepharose 4B beads were added to *E. coli* BL21(DE3) lysates, followed by incubation for 1 h at room temperature in a binding buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, and either 0.1 mM $CaCl_2$ or 5 mM EGTA. The beads were washed five times with the binding buffer. Proteins that bound to CaM were eluted by adding SDS-PAGE sample buffer and analyzed on 10 % SDS-PAGE. For Western blotting, an anti-His antibody was used with its respective horseradish peroxidase secondary antibody (Sigma-Aldrich). Enhanced chemiluminescent reagent (Bio-Rad) was used to detect the proteins.

In vivo *coimmunoprecipitation (CoIP) assay*

B. bassiana mycelia were ground to a fine powder in liquid nitrogen and homogenized in 2 ml ice-cold extraction buffer containing 25 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 150 mM NaCl, 1 % β-mercaptoethanol and protease inhibitor cocktail (Roche). After centrifugation at 8,000 rpm for 30 min at 4 °C, the supernatant was collected and the amount of protein extracts was estimated by the Bradford's method using a protein assay kit (Bio-Rad). Protein extracts (2 mg) from *B. bassiana* mycelia were immunoprecipitated with an anti-CaM antibody (Sigma-Aldrich) and protein A agarose (Millipore). After washing several times, co-immunoprecipitated proteins were subjected to an *in vitro* KIVR activity assay as described previously with minor modifications (Zhang *et al.*, 2012).

Peptide binding to CaM

The synthetic peptides were prepared by GL Biochem (http://www.glschina.com). Samples containing 303 pmol of CaM (Sigma-Aldrich) and the synthetic peptide (purity: > 90 %) in 100 mM Tris-HCl (pH 7.5) and 0.1 mM $CaCl_2$ in a total volume of 20 μl were incubated for 1 h at room temperature as described previously (Yang and Poovaiah, 2000). The mixtures were electrophoresed in 10 % nondenaturing PAGE containing 0.1 mM $CaCl_2$. The gels were stained with Coomassie Brilliant Blue.

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Figure legends

Fig. 1. BbKIVR has a putative CaM-binding motif.

A. Deduced amino acid sequences of BbKIVR were shown.

B. Helical wheel projection predicted a putative CaM-binding domain at the middle region that represents a typical basic amphipathic α-helix.

C. Sequence alignment was carried out with CLUSTALW program and BoxShade server (http://www.ch.embnet.org/software/Box_form.html). The putative CaM-binding domains

were boxed. *Fusarium proliferatum* (FpKIVR/KivRFp, Zhang *et al.*, 2012) and *Torrubiella hemipterigena* (CEJ80095).

Fig. 2. Biochemical features of recombinant His-BbKIVR.

A. Overall mechanism of beauvericin biosynthesis.

B. Substrate specificity of His-BbKIVR and the effects of pH and temperature on His-BbKIVR activity. The numeric values for 100 % activity were 310.5 U/mg protein (substrate), 320.5 U/mg protein (temperature), and 315.5 U/mg protein (pH).

Fig. 3. BbKIVR interacts with CaM *in vitro* and *in vivo*.

A. *In vitro* CaM-Sepharose pull-down assay. Recombinant His-tagged BbKIVR was incubated with CaM-Sepharose beads. Eluted His-BbKIVR proteins were detected using Western blotting with an anti-His antibody.

B. BbKIVR interacts with CaM *in vivo*. Protein extracts (2 mg) from *B. bassiana* mycelia were isolated and immunoprecipitated by using a CaM antibody. Co-immunoprecipitated proteins were subjected to *in vitro* KIVR activity assay. His antibody was used as a negative control. Error bars represent SD from three biological replicates ($p < 0.01$, Student's *t* test). The numeric value for 100 % activity was 3.6 U/mg protein.

Fig. 4. CaM suppresses BbKIVR activity.

A. CaM negatively regulates His-BbKIVR activity *in vitro*. His-BbKIVR (10 μg) was incubated with various concentrations of CaM for 20 min at room temperature before adding substrate, and *in vitro* KIVR activity was measured. Error bars represent SD from three technical replicates ($p < 0.01$, Student's *t* test). The numeric value for 100 % activity was 320.5 U/mg protein.

B. CaM inhibitors stimulate BbKIVR activity in *B. bassiana*. Protein extracts (200 μg) from *B.*

bassiana were incubated with 250 μM of W-5, W-7 or W-12 for 20 min at room temperature before adding substrate, and *in vitro* KIVR activity was measured. Error bars represent SD from three biological replicates ($p < 0.01$, Student's *t* test). The numeric value for 100 % activity was 3.5 U/mg protein.

Fig. 5. BbKIVR peptide suppresses the inhibitory effect of CaM on BbKIVR activity.

A. Gel mobility shift assay showing that the BbKIVR peptide interacts with CaM. The mixtures were electrophoresed in 10 % nondenaturing PAGE. The gels were stained with Coomassie Brilliant Blue. HSP70 peptide (257-KRAVRRLRTACERAKRTLSSS-277) was used as a positive control.

B. CaM was incubated with various concentrations of the peptide for 20 min at room temperature before adding 10 μg of His-BbKIVR and *in vitro* KIVR activity was measured. Error bars represent SD from three technical replicates ($p < 0.05$, Student's *t* test). The numeric value for 100 % activity was 305.5 U/mg protein.

Fig. 6. Light and salt stress affect BbKIVR activity though CaM signaling in *B. bassiana.*

B. bassiana mycelia were grown on the solid SDAY medium for 3 days at 24 °C with 3,000 lux light or without light, and the extracted proteins (200 μg) were subjected to an *in vitro* KIVR activity assay. For the effects of salt and oxidative stresses, *B. bassiana* mycelia were grown on the solid SDAY medium with 0.8 M NaCl or 2 mM H_2O_2 for 3 days. For the effects of CaM inhibitors, *B. bassiana* mycelia were grown on the solid SDAY medium with 250 μM of W-5 or W-7 for 3 days. Error bars represent SD from three biological replicates ($p < 0.05$, $p < 0.01$, Student's *t* test). NS, not significant. The numeric value for 100 % activity was 3.9 U/mg protein.

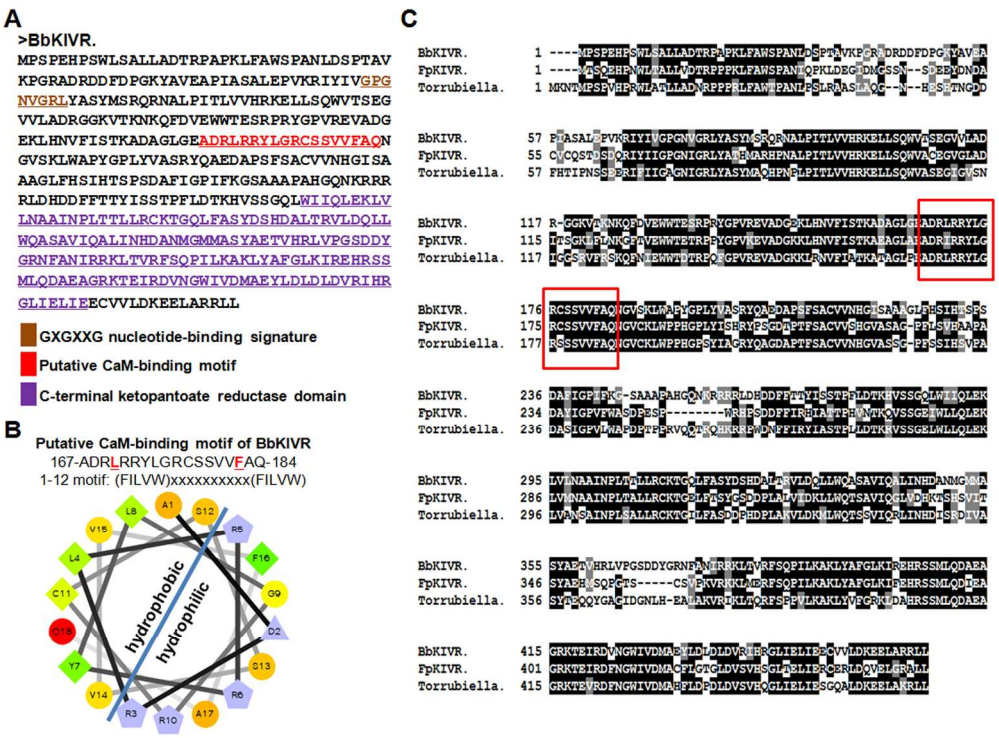

Figure 1

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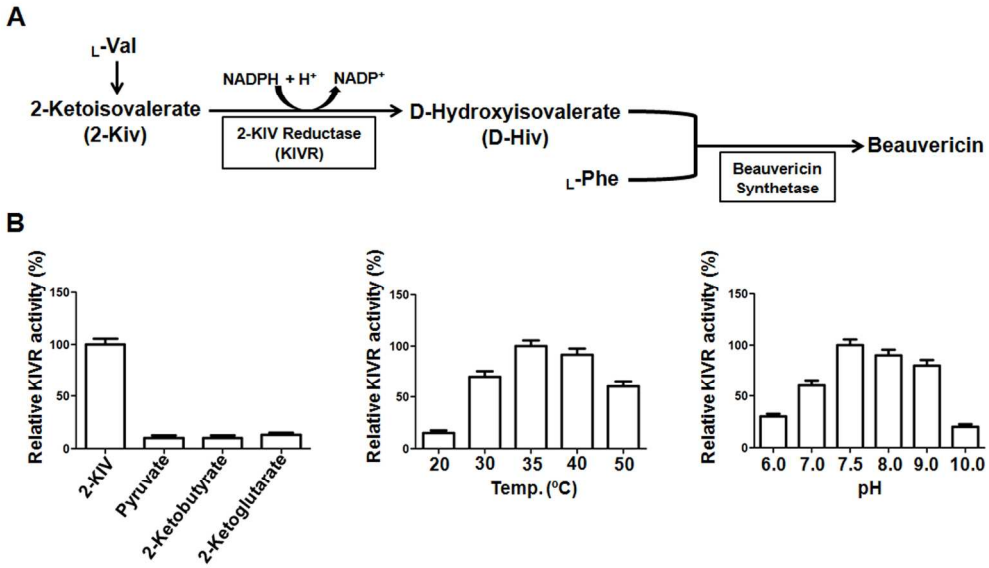

Figure 2

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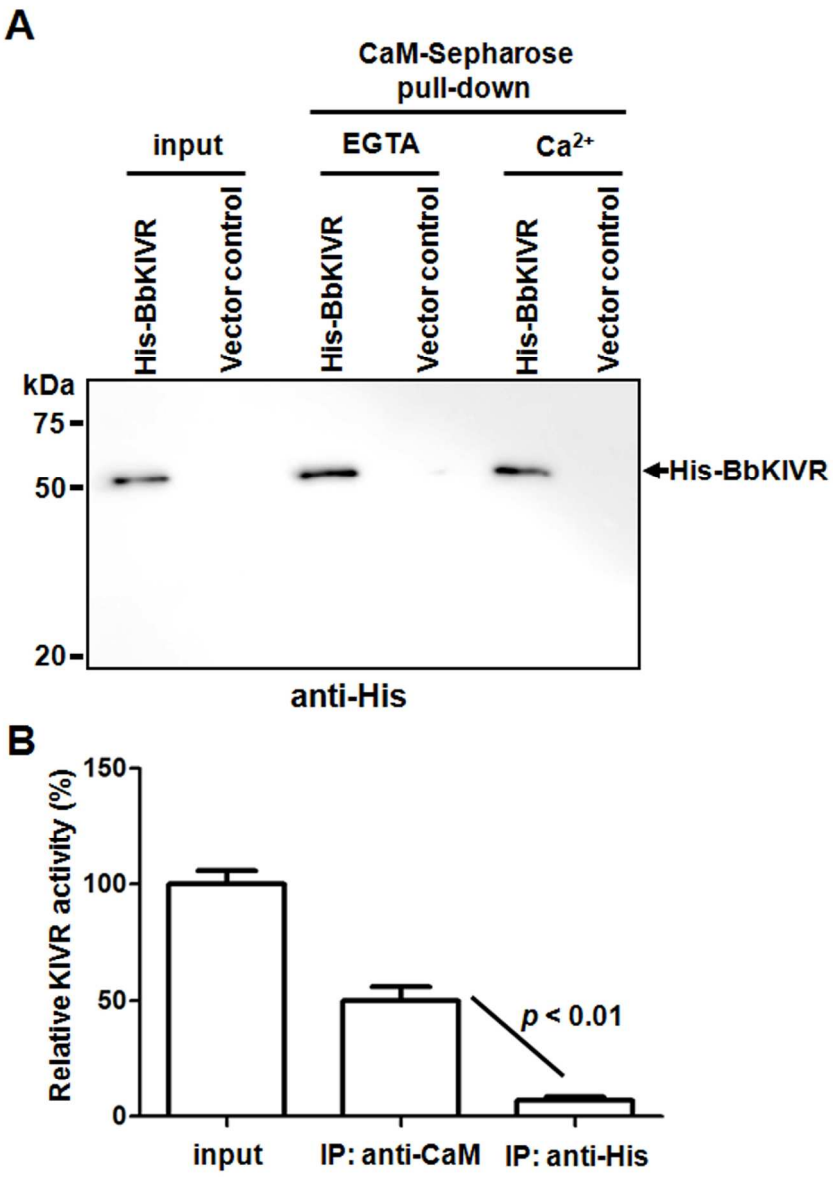

Figure 3

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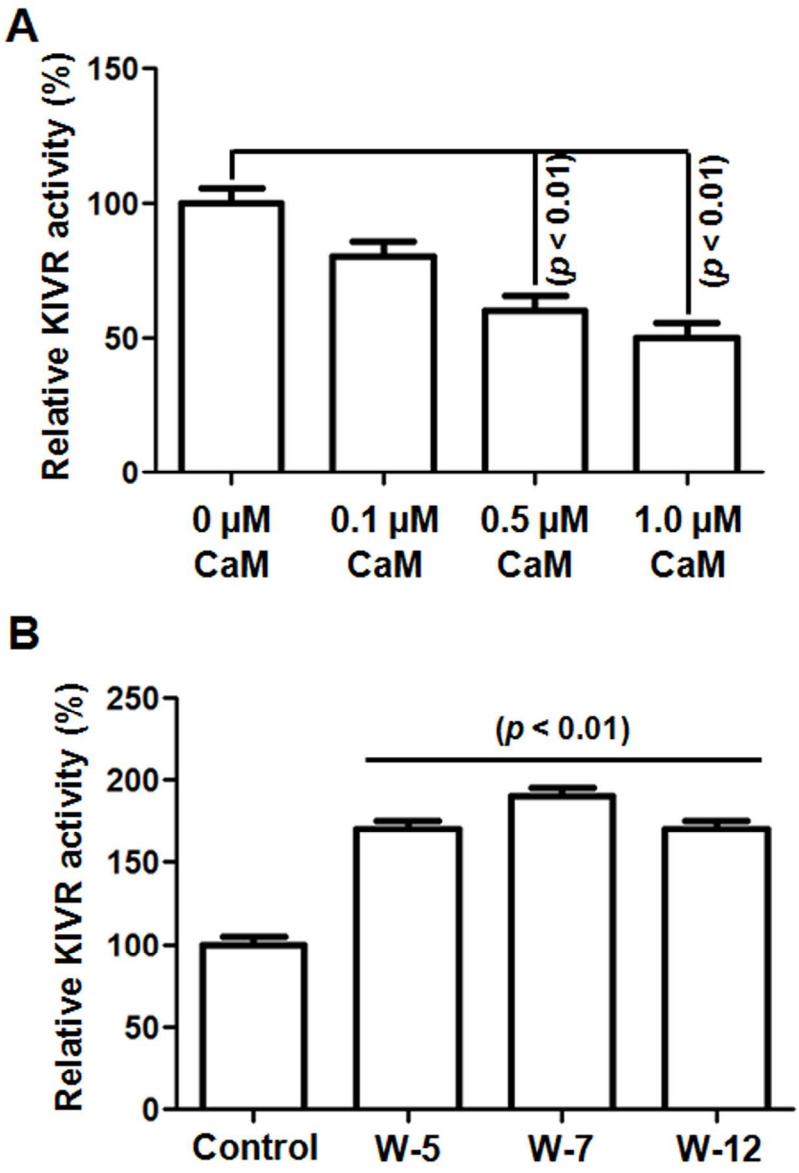

Figure 4

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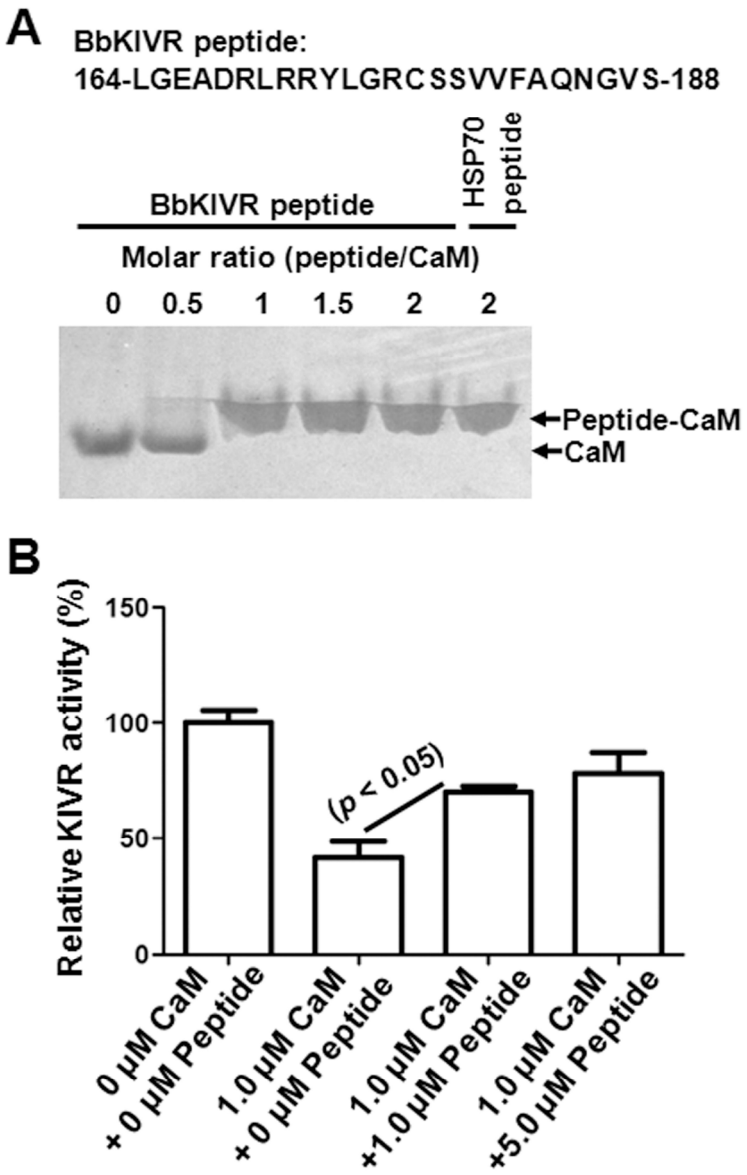

Figure 5

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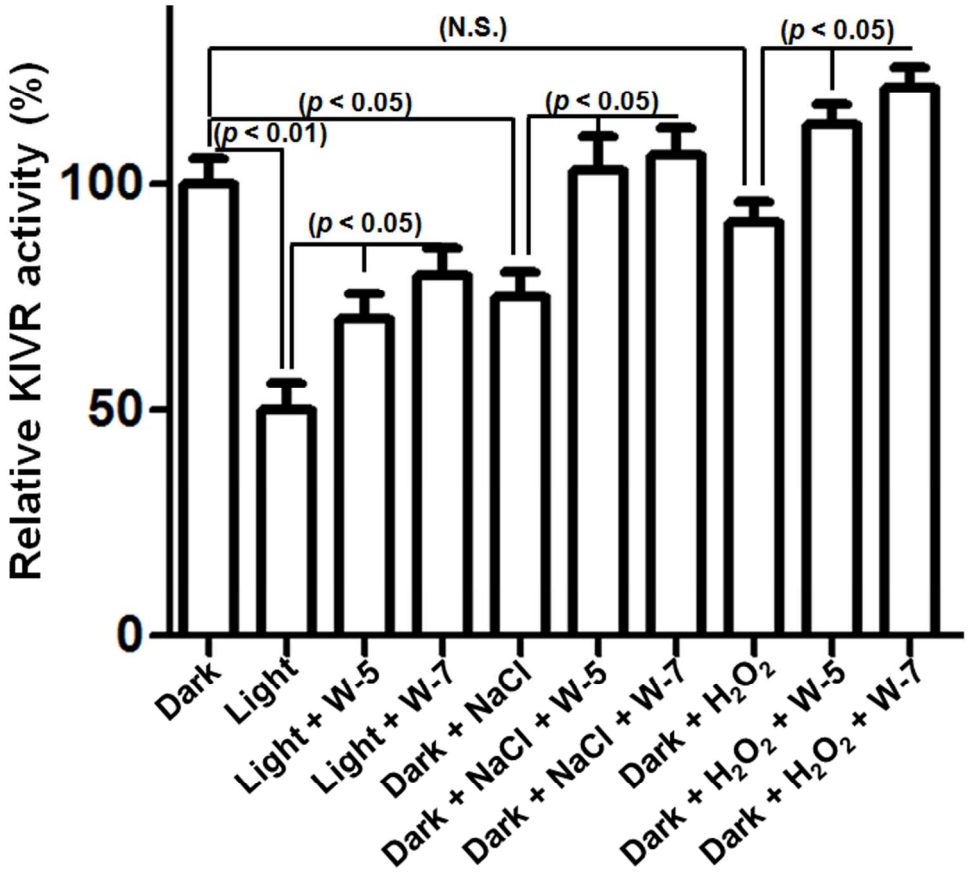

Figure 6

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