



Molecular Cloning and Characterization of a Putative *BnHEC3* Gene in Oilseed Rape (*Brassica Napus*)

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Abstract

HECATE3 (HEC3) is an important transcription factor involved in regulating the carpel and transmitting tract development in *Arabidopsis thaliana*. The homologous gene of *HEC3* in *Brassica napus* (designed as *BnHEC3*) was obtained by *in silico* cloning and rapid amplification of cDNA end (RACE) method. *BnHEC3* obtained from cDNA is of 748 bp, containing an open reading frame (ORF) of 546bp and coding a peptide of 181 amino acid residues. Sequence alignment revealed that *BnHEC3* is highly homologous to *HEC3* in *Arabidopsis thaliana*, especially in bHLH region, which is sharing nearly 100% identity, and the polygenetic analysis indicated that *BnHEC3* belongs to atypical bHLH protein family. RT-PCR results showed that *BnHEC3* is mainly expressed in young leave. Southern blotting analysis indicated that *BnHEC3* has at least three copies in *Brassica napus* genome. The transient expression of *BnHEC3-sGFP* in onion epidermic cells showed that *BnHEC3* localized in nuclei. These features implied that *BnHEC3* functions as *HEC3*, and may be involved in the development of female reproductive tissues in *Brassica napus*.

Keywords: *Brassica napus*, Transmitting tract development, bHLH protein, Transcription factor, Subcellular localization

1. Introduction

Properly coordinated development of female reproductive tissues, including stigma, style, septum and transmitting tract, is essential to the successful fertilization. Much of the recent progress has come from studies of model plant *Arabidopsis thaliana*. Rapeseed (*Brassica napus* L.) is an important oil and vegetable crop. However, the mechanism of reproductive development in rapeseed remains elusive. The *Brassica napus* gynoecium, like that of *Arabidopsis*, is a complex organ specialized for seed production and dispersal. It also arises from two congenitally fused carpels and at maturity consists of an apical stigma for pollen capture, a short intervening style and a large central ovary where ovule and seed development occurs. The ovary is divided into two compartments by the septum (Hill and Lord et al., 1989; Okada et al., 1989; Smyth et al., 1990; Sessions and Zambryski et al., 1995; Gremski et al., 2007). The transmitting tract, a specialized tissue which coincides with the path of pollen tubes in the gynoecium, develops in the center of both the style and the septum to increase fertilization efficiency.

During fertilization, pollen grains germinate on the stigma, and then form a tube to deliver the sperms towards the awaiting ovules. The elongation of the pollen tube during pollination is facilitated by differentiation of the transmitting tract tissue, which is composed of highly secretory cells characterized by an extensive extracellular matrix (ECM). And the transmitting tract cells are also undergoing a program of developmentally controlled cell death (Lennon et al., 1998;

Wang et al., 1996; Crawford et al., 2007; Gremski et al., 2007). In the absence of proper transmitting tract differentiation, pollen tube growth is limited and fertility is reduced (Crawford et al., 2007; Gremski et al., 2007).

Through studying on the model plant *Arabidopsis*, a number of genes, including *SPATULA* (*SPT*), *STYLISH1* (*STY1*), *STYLISH2* (*STY2*) and *ETTIN* (*ETT*), have been proved to be involved in patterning the stigma, style, septum and transmitting tract. In recent years, three new genes, *HECATE1* (*HEC1*), *HECATE2* (*HEC2*) and *HECATE3* (*HEC3*), have been identified as important factors for patterning the transmitting tract, which are necessary in the complex program of gynoecium's development. All of these three genes encode closely related basic helix-loop-helix (bHLH) transcription factors with overlapping function. Loss of HEC function leads to defects in the development of the transmitting tract, septum and stigma, and a decrease in fertility. HEC proteins and SPT, another transcription factor belonging to the bHLH family which plays an important role in the septum and stigma development, are likely to cooperatively interact in controlling development (Gremski et al., 2007).

In this study, we cloned the cDNA of putative *BnHEC3* gene (748 bp) from cultivated *B. napus* cv. *Ningyou12* by *in silicon* cloning and the rapid amplification of cDNA ends (RACE) method (Frohman, 1993). And we showed that *BnHEC3* were homologous to *HEC3* with nearly 100% identity in bHLH sequence. Our data suggested that *BnHEC3* may function in the process of transmitting tract development in *Brassica napus*.

2. Materials and methods

2.1 Plant materials and DNA, RNA extractions

B. napus cv. *Ningyou12* was used in this study. Roots, stems, leaves, floral buds, and siliques (35d after pollination) were harvested at the indicated stages. RNA and DNA were extracted immediately. Total RNA was isolated with Trizol (Invitrogen) reagent according to the manual. Mature leaves were harvested for genomic DNA extracted with CTAB extraction method.

2.2 Cloning of the full-length cDNA of *BnHEC3*

50µg total RNA from flower buds of *Ningyou12* was reverse-transcribed into first-strand cDNAs with the PrimeScript Reverse Transcriptase (Takara) in a 50 µl reaction volume. For screening of the *BnHEC3* from *Brassica*, the cDNA sequence of *HEC3* (NM_121012.1) was used as a query to blast against *Brassica* database in the TAIR (www.arabidopsis.org), and a putative *BnHEC3* EST and a genomic DNA BAC were obtained. The PCR primers (E1/E2, P1/P2 and P3/P4) were designed according to the sequences above.

Primers are as follows:

E1: 5'-CAC TAC TCT TTT CTC CGG AG-3',

E2: 5'-AGC TTG ATC TTG CGG CGG-3',

P1: 5'-ATC ATC ATA ACG ATC CAA TCG G-3',

P2: 5'-ATT GCA CCA TTA CTC ACA CTT TAT-3',

P3: 5'-ATG GCC ATG GAC CAA CAC AC-3',

P4: 5'-TTA AAT CAG TTC ACC TCC TCC TCC-3'

The PCR thermocycling conditions were as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1min, 60 °C for 30 sec, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The *BnHEC3*-EST and partial *BnHEC3* genomic DNA fragments were purified and cloned into pMD18-T vector (Takara) and sequenced. The 5' and 3' FULL RACE Kits (Takara) were used to clone 5' and 3' flanking sequence of the cDNA.

2.3 Bioinformatic analysis of *BnHEC3*

DNA and amino-acid sequences manipulation were performed with DNASTar 5.0 software. Homology analysis was also performed using Blastx, Genedoc, and BLAST Network Service in TAIR (<http://www.arabidopsis.org>) and BLAST Network Service in NCBI (<http://www.ncbi.nlm.nih.gov/>). Expasy database (<http://www.expasy.org/>) was used to analyze the profile of *BnHEC3* protein. The sequences alignment was performed using the program Multalin with the default parameters (<http://prodes.toulouse.inra.fr/multalin/multalin.html>) (Corpet, 1988), and GeneDoc were used for editing sequence alignment. Neighbor-Joining Phylogenetic Tree with bootstrap values was constructed using Mega 3.1.

2.4 Southern hybridization analysis

Six restriction enzymes (*Bam*H I, *Eco*R I, *Kpn* I, *Sac* I, *Xba* I, *Xho* I) were used for Southern blotting. About 10 µg of genomic DNA was digested with 20 unit of restriction enzyme in a final volume of 100 µl at 37°C for 18 h. The cleaved DNA fragments were run on a 0.8% (w/v) agarose gel and transferred to Hybond-N + membranes (Amersham Pharmacia). The *BnHEC3*-EST (360bp) was amplified with E1/E2 by PCR and purified as a probe for southern blotting hybridization. The membranes were hybridized following the standard protocols (Chomczynski, 1992), and then was washed with 2×SSC plus 0.1% (w/v) SDS at 65°C for 30 min and exposed to X-ray film at -70°C.

2.5 Analysis of *BnHEC3* expression

Five micrograms of the total RNA isolated from roots, stems, young leaves (before pollination), mature leaves (35 days after pollination), flowers, and pods of the cultivar *Ningyou12* were reverse-transcribed into first strand cDNA with the PrimeScript Reverse Transcriptase (Takara), respectively. The first strand cDNA mix was used as template and P3/P4 were used as primers for RT-PCR. The PCR were performed as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1min, 60 °C for 30 sec, and 72 °C for 45 sec, and a final extension at 72 °C for 10 min. *BnActin* gene amplified with primers β - Actin F (5'-ATG GCC GAT GGT GAG GAC ATT C-3') and β - Actin R (5'-GGT GCG ACC ACC TTG ATC TTC-3'), was used as an internal control in the experiments.

2.6 Subcellular localization of *BnHEC3*

Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) was used to predict the subcellular localization of *BnHEC3* and *HEC3*. *sGFP* vector, which have a multiple cloning site (MCS) between 35S promoter and *sGFP* gene, was used for constructing *BnHEC3-sGFP*. Briefly, ORF of *BnHEC3* without stop coding (TAG) was amplified with two primers containing *Xba* I and *Bam*H I restrict sites respectively as follows: 5'-tct aga ATG GCC ATG GAC CAA C-3' and 5'-gga tcc AAT CAG TTC ACC TCC T-3'. The PCR thermocycling conditions were as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1min, 58 °C for 45 sec, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR product was purified, ligated to pMD18-T-vector, and sequenced. Then the correct plasmid was digested with *Xba* I and *Bam*H I, and the fragment was gel purified, ligated into the *Xba* I/ *Bam*H I predigested *sGFP* vector to construct *BnHEC3-sGFP*.

The recombination plasmid was transformed into the onion epidermal cells using gene-gun. Then, the onion epidermal cells was cultured under 25 °C and observed under fluorescence microscope after 16-24 hr.

3. Results

3.1 Cloning and structural analysis of *BnHEC3*

HEC3 gene was used as a query to search against the *Brassica* database, thus an EST and a partial genomic DNA were obtained, which are 360bp (AT002234.1) and 848bp (DU105139) in length, respectively. The EST sequence is same to the corresponding region in the partial BAC sequence, so it is named as the *BnHEC3*-EST. A complete open reading frame (ORF) indicated by DNASTar was 546bp. Fragments obtained by PCR from cDNA and genomic DNA are uniform, indicating that *BnHEC3* has no introns (Fig. 1.).

To get the complete sequence of *BnHEC3*, RACE method was used. Two sequences with different lengths in 3' ends were obtained by 3' RACE. The length of the longest sequence amplified from cDNA of *BnHEC3* is of 748 bp with a 23 bp of incomplete 5'-leader sequence and 219 bp of 3'-untranslated region (Fig. 2.). The putative ORF of 546 bp from *BnHEC3* encodes a peptide of 181 amino acids with a predicted molecular weight of 20.3 kDa and a calculated isoelectric point of 5.88 (Protparam: [http:// www.expasy.ch](http://www.expasy.ch)).

Using SMART program (Simple Modular Architecture Research Tool: [http:// smart.embl-heidelberg. de](http://smart.embl-heidelberg.de)), a bHLH domain (amino acid 89-137) was identified in the *BnHEC3*, and an alanine residue (A) was also found at site 9 within the basic region of the *BnHEC3*, indicating that *BnHEC3* is an atypical bHLH protein, whereas typical bHLH proteins have a glutamic acid residue (E) at that site instead (Fisher and Goding, 1992; Buck and Atchley, 2003; Toledo-Ortiz et al., 2003) (Fig. 2.). The extensive protein sequence similarity between *BnHEC3* and *HEC3* was a 30 amino acid N-terminal extension of the region of the bHLH domain. This led to *BnHEC3* being grouped as a subfamily of bHLH proteins (Heim et al., 2003; Gremski et al., 2007). The conservation was primarily restricted to the bHLH region, and the other regions showed also a high degree of similarity among the bHLH proteins comparing (Fig.3).The bHLH domain of *BnHEC3* shared extremely high similarities (nearly 100%) with that of *HEC3* (Fig. 3.). Sequence alignment of the *BnHEC3* with *HECs* and another related bHLH factor INDEHISCENT (IND) revealed that the *BnHEC3* also lack a conserved glutamate at position 13 of the basic domain (Fig.3.). This glutamate has been shown to be important for DNA binding (Ellenberger et al., 1994; Ma et al., 1994; Gremski et al., 2007). The phylogenetic tree showed that *BnHEC3* belongs to the atypical bHLH proteins group with *HEC3*, and also strongly supported the high identity between *BnHEC3* and *HEC3* (Fig. 4.).

3.2 Genomic structure of the *BnHEC3* gene

The genomic DNA was digested by six restriction enzymes, respectively, and subjected to Southern analysis. The 360bp *BnHEC3*-EST was used as a probe. The result was illustrated in Fig. 5. It can be seen that there were three bands (one visible band and two weaker bands) in the first, second and fourth lane. The third lane had only one band but the fifth and sixth lanes had three bands with one weak band and two bright ones. The result of the Southern Blotting suggested that there were at least three copies of *BnHEC3* in the genome of *B. napus*.

3.3 Analysis of the *BnHEC3* expression

The result of RT-PCR showed that the expression of *BnHEC* was in young leaves, mature leaves, floral buds and

siliques rather than in roots or stems, and most strongly in young leaves (Fig. 6.). Flowers are abnormal organs of the leaves, and they are the essential origin of the fruits in many plants including oilseed rape. The expression profiles of *BnHEC3* suggested that *BnHEC3* may be involved in regulating the growth and development of leaves and the formation of flower tissues. It may have similar function as *HEC3*, which can regulate the carpel formation and transmitting tract development in *Arabidopsis*.

3.4 Subcellular localization of *BnHEC3*

As a possible transcription factor, *BnHEC3* may be localized in nuclei, however, there was no nuclear localization sequence detected in *BnHEC3* by Motif Scan. Whereas, a putative nuclear localization sequence containing Arginine-rich region (RRREISEKIRILKR) was predicted in *HEC3* in *Arabidopsis*. Based on the sequence alignment of *HEC3* and *BnHEC3*, *BnHEC3* also contains a motif (RRRISERIRILQR) corresponding to the putative nuclear localization sequence of *HEC3*, which may be the putative nuclear localization sequence of *BnHEC3* (Fig. 2). Further investigate the cellular localization of *BnHEC3*, transient expression of *BnHEC3*-sGFP fusion protein in onion epidermal cells was performed. Two kinds of plasmids for transient expression were constructed (Fig.7.A) and transferred into onion epidermal cells respectively. Fluorescence microscopy showed that green fluorescence was observed all over the cells carrying sGFP plasmid, whereas green fluorescence was observed clearly in nuclei of the cells carrying *BnHEC3*-sGFP plasmid, indicating that *BnHEC3* localized in nuclear (Fig. 7.B).

4. Discussion

The bHLH transcription factors, such as *HEC1*, *HEC2* and *HEC3*, play essential roles in development of female reproductive tissues in model plant *Arabidopsis thaliana*. But their function in Rapeseed (*Brassica napus* L.) is still unknown. In this study, we successfully identified a novel gene, named as *BnHEC3*, which may be involved in similar process in Rapeseed.

We obtained the partially complete cDNA of *BnHEC3* from *Brassica napus* using *in silico* cloning and RACE method. Sequence and phylogenetic analysis indicated that *BnHEC3* is highly homologous to *HEC3* and belongs to atypical bHLH family. We performed transient expression of *BnHEC3*-sGFP in onion epidermal cells, and found it is localized in nuclear, which further prove it to be a possible transcription factor as *HEC3*. Southern blotting of *BnHEC3* showed that *BnHEC3* has at least three copies, suggesting that *BnHEC3* plays an important role in the development process of female reproductive tissues in oilseed rape. Our RT-PCR analysis showed that the *BnHEC3* is mainly expressed in young leaves, indicating it may be involved in the specification of floral organs. And it is also expressed in flowers and fruits, suggesting it may have function in regulating the specification of subsequent different cell types in female reproductive tissues.

Before reaching ovules, pollen tubes must travel through several distinct tissues, including the stigma, the stylar transmitting tract and the septum transmitting tract (Crawford et al., 2007; Gremski et al., 2007). Coordinated development of these tissues is crucial for successful fertilization, and the transmitting tract differentiation, is one of the most important process affecting the pollen tube development (Crawford et al., 2007; Gremski et al., 2007). In *Arabidopsis*, three related bHLH transcription factors, *HEC1*, *HEC2* and *HEC3*, are identified to be required for this process (Gremski et al., 2007). An RNA-null mutant of *HEC3* displayed a loss of fertility and defects in transmitting tract tissue in both septum and style (Gremski et al., 2007). Over-expression of *HEC3* led to the ectopic production of stigmatic tissue, consistent with its requirement for stigma development (Gremski et al., 2007).

Therefore, *BnHEC3*, as the homologous gene of *HEC3* in *Brassica napus*, may also play essential roles in the development of female reproductive tissues. Our study paved the way for subsequent research on *BnHEC3* in *Brassica napus*. By further study on it, we may finally get much more understanding about the fertility mechanism in rapeseed, and the knowledge may be useful to the agricultural production.

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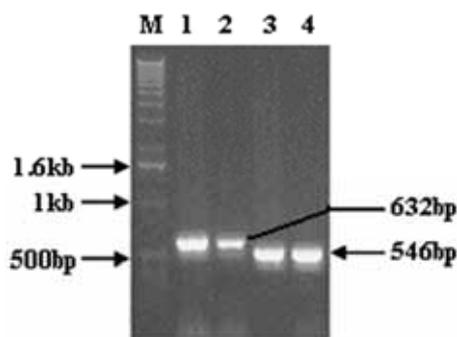


Figure 1. The fragments are amplified by PCR (P1/P2, P3/P4) from cDNA and genomic DNA of *BnHEC3*, respectively.

M: DNA marker; 1 and 3: fragments amplified from genomic DNA of *BnHEC3*. 2 and 4: fragments amplified from cDNA of *BnHEC3*.

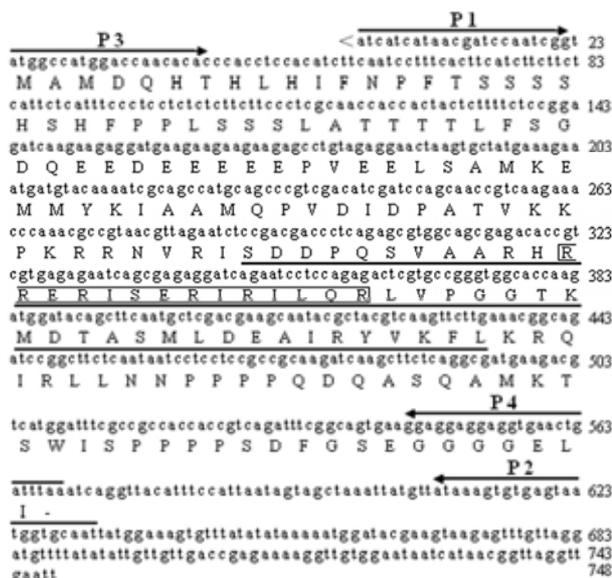


Figure 2. cDNA sequence of the *BnHEC3* gene and deduced amino acid sequence.

“<” in front of cDNA sequence showed that the incomplete 5'- end sequence of cDNA of *BnHEC3*. bHLH domain signature region of *BnHEC3* is underlined. A putative nuclear localization sequence was marked with rectangular box. Primers (P1/P2 and P3/P4) were indicated with the arrows.



Figure 3. Multiple sequence alignment of the bHLH region from *BnHEC3* and other bHLH family proteins from *Arabidopsis*.

The proteins aligned are: *BnHEC3*, *HECATE3* (*HEC3*), *HECATE2* (*HEC2*) and *HECATE1* (*HEC1*) (Gremski et al., 2007) and *INDEHISCENT* (*IND*) (Liljegren et al., 2004). The black arrow head marks an alanine that replaces the conserved glutamate carried by most other *Arabidopsis* bHLH proteins. Different grey level represents the different percentage of similarity.

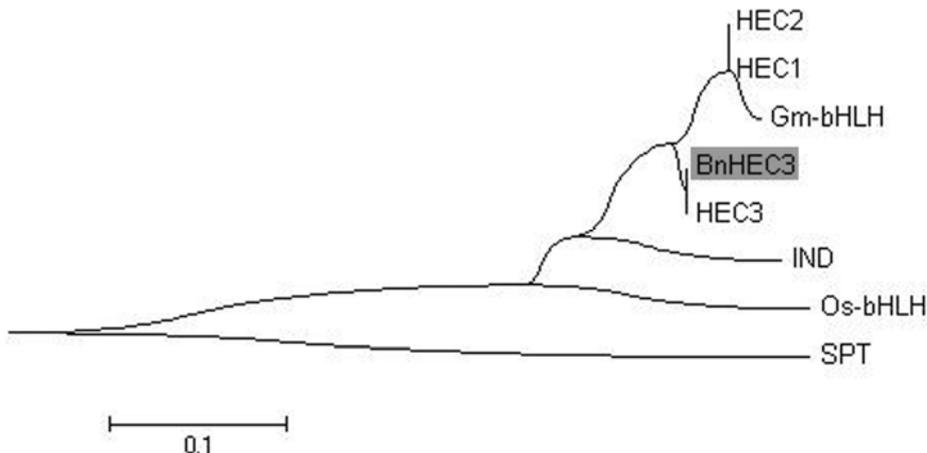


Figure 4. Neighbor-Joining Phylogenetic Tree of the bHLH Domains of bHLH family proteins.

The Phylogenetic Tree showed the phylogenetic relationship among the bHLH family proteins, including *BnHEC3*, *HECATE3* (*HEC3*), *HECATE2* (*HEC2*), *HECATE1* (*HEC1*), *SPATULA* (*SPT*), *INDEHISCENT* (*IND*), *Gm-bHLH* (*Glycine max-bHLH*) and *Os-bHLH* (*Oryza sativa Japonica Group-bHLH*). It is obvious that *BnHEC3* and *HEC3* are highly homologous.

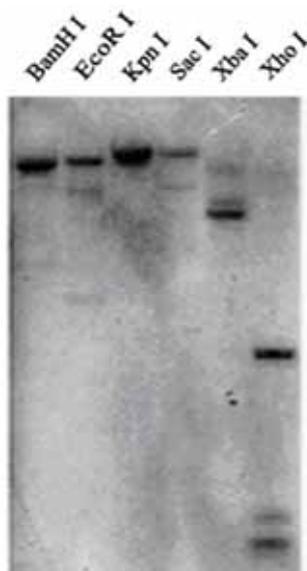


Figure 5. Southern blotting of *BnHEC3* gene in *Brassica napus* genome.

Genomic DNA was digested with *Bam*H I (line 1), *Eco*R I (line 2), *Kpn* I (line 3), *Sac* I (line 4), *Xba* I (line 5) and *Xho* I (line 6), respectively, followed by hybridization with the probe of *BnHEC3*-EST.

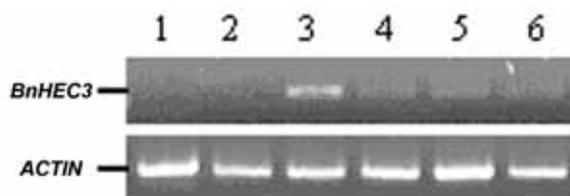


Figure 6. Expression profile of *BnHEC3* was analyzed by RT-PCR.

1: roots; 2: stems; 3: young leaves; 4: mature leaves; 5: floral buds s; 6: siliques (35d after pollination). β -ACTIN was used as control.

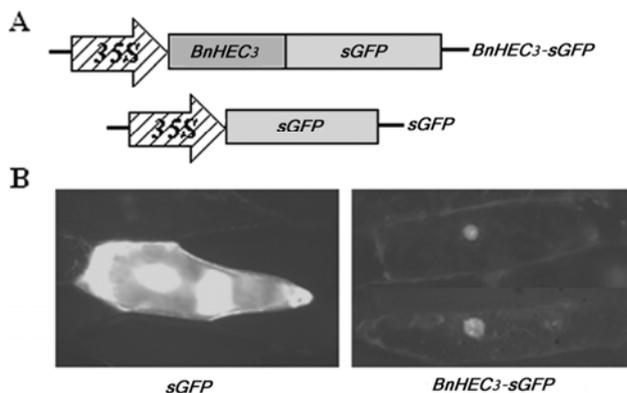


Figure 7. Subcellular localization of *BnHEC3* in onion epidemic cell.

(A) Schematic diagram of recombination plasmid. (B) Fluorescent microscopy analysis of subcellular localization of *BnHEC3*. It shows the onion epidemic cells that carrying *sGFP* plasmid (left) and carrying *BnHEC3* –*sGFP* plasmid (right).