Chapter 5

Cloning and characterization of CabHLH1 transcription factor
5.1 Introduction

Plant defense responses are controlled by dynamic and variable gene expression changes which lead to reprogramming of many cellular functions. The outcome of the induced defense response seems to be finely tuned by cross-talk between various signaling pathways (Koornneef and Pieterse, 2008) and regulatory elements in the promoters of the defense-related genes, resulting in quantitative and/or kinetic effects on the resistance response (Katagiri, 2004). Transcription factors (TFs) are essential components of these signaling pathways and act by regulating the expression of genes encoding PR proteins and enzymes involved in the synthesis of defense related compounds. A number of transcription factors and effectors have been characterized that are critical in the circuitry controlling signal sensitivity and transduction in defense responses, and several may provide a point for crosstalk and signal integration (Chen and Zhu, 2004). These transcription factors belong to different families, for example, Zinc finger, AP2 domain, homeodomain leucine zipper, WRKY, Myb, bHLH etc. and have been shown to play role in plant defense (Eulgem et al., 2005).

Basic helix-loop-helix (bHLH) transcription factors represent a family of proteins that contains a bHLH domain, a motif involved in DNA binding and dimerization (Murre et al., 1989). Members of bHLH protein superfamily have been found to have an ever increasing number of functions in essential physiological and developmental processes in animals as well in plants (Quail, 2000; Ledent and Vervoort, 2001; Toledo-Ortiz et al., 2003; Sonnenfeld et al., 2005). The bHLH domain contains approximately 60 amino acids, with two functionally distinct regions, the basic region and the HLH region. The basic region is located at the N terminus of the bHLH domain and functions as a DNA-binding motif. It consists of approximately 15 amino acids, which typically include six basic residues (Atchley et al., 1999). The HLH region contains two amphipathic α helices with a linking loop of variable lengths. The amphipathic α helices of two bHLH proteins can interact, allowing the formation of homodimers or heterodimers (Nesi et al., 2000). Some bHLH proteins have been shown to bind to sequences containing a consensus core element called the E box (5'-CANNTG-3'), with the G box (5'-CACGTG-3') being the most common form. In addition, the nucleotides flanking the core element may also have a role in binding specificity (Massari and Murre, 2000; Robinson et al., 2000). According to their
phylogenetic relationships, DNA-binding motifs, functional properties and presence or absence of additional domains, known bHLH proteins from eukaryotes have been divided into six main groups named as group A to F (Atchley and Fitch, 1997). It has been suggested that an ancestral HLH sequence most probably came from group B and proteins of this group are the most prevalent type of bHLH proteins in animals. In Arabidopsis also, the G-box-binding bHLH proteins (part of group B) are the most abundant group (Toledo-Ortiz et al., 2003). Many additional domains have also been identified in various bHLH proteins most common of which are PAS, orange and leucine zipper domains. These additional domains are mostly present at the carboxy-terminal of the bHLH region. However, the position of the bHLH and additional domains within the sequence of the protein varies widely between different families. Compared to animals, only a small number of plant bHLH proteins have been characterized functionally. In plants, the R gene product Lc, which is involved in the control of anthocyanin synthesis in maize, was the first plant protein reported to possess a bHLH motif (Ludwig et al., 1989). In Arabidopsis 162 bHLH-encoding genes have been identified from the analysis of genome sequences (Bailey et al., 2003; Heim et al., 2003; Toledo-Ortiz et al., 2003). Also, around 167 bHLH encoding genes were identified in rice genome (Li et al., 2006). Sequence analysis suggests that most of the plant bHLH proteins belonged to Group B which binds to the G-box sequence CACGTG (Atchley and Fitch, 1997).

Family of bHLH proteins is known to perform diverse regulatory functions. The known molecular properties of bHLH proteins suggest a general mechanism by which such regulation may be accomplished. This mechanism involves (1) generation of a high degree of complexity and diversity in transcriptional regulatory activity through variation in the DNA sequence motif recognized by individual bHLH proteins (2) the capacity to combinatorially amplify the spectrum of possible specific protein–DNA interactions through selective heterodimerization between bHLH proteins with different DNA sequence recognition specificity and (3) the capacity to interact with a network of transcriptional coactivators, corepressors, and signaling molecules through selective protein–protein interactions (Toledo-Ortiz et al., 2003).

In animals they act as regulatory factors in different processes such as neurogenesis, cardiogenesis, myogenesis, and hematopoiesis (Jones, 2004). In plants also, bHLH proteins participate in regulating a broad range of growth and developmental
processes at all phases of the life cycle including floral organogenesis, hormone responses, and light signaling. In organogenesis, a bHLH protein was found to be involved in trichome development (Payne et al., 2000) while another gene, SPATULA, which encodes a bHLH transcription factor, was found to be involved in carpel and fruit development in Arabidopsis (Groszmann et al., 2008). In light signaling, bHLH transcription factors are shown to take a center stage through phytochromes (Duek and Fankhauser, 2005). In addition, bHLH proteins have been shown to positively mediate ABA signaling in Arabidopsis (Li et al., 2007). Some bHLH genes were found to be expressed in response to brassinosteroids and were required for normal growth of the plant (Friedrichsen et al., 2002). Beyond their role in normal growth and developmental processes, bHLH proteins are shown to play role in stress responses also. In this direction, a bHLH transcription factor in rice was shown to be involved in signal transduction during cold stress (Wang et al., 2003). Further, a bHLH transcription factor namely JINI was found to mediate jasmonate regulated defense response in Arabidopsis (Lorenzo et al., 2004).

In the present study, the transcript profiling during Fusarium wilt in chickpea led to the identification of a bHLH transcription factor having a unique caspase domain. The gene was found to be differentially expressed and it was of great interest to clone the gene and study its role in defense response. Here we present the expression study of the CabHLH1 transcription factor in response to Fusarium and many plant hormones. We also demonstrate its tissue specific expression, copy number and subcellular localization.

### 5.2 Material and methods

#### 5.2.1 Cloning of full length CabHLH1

Full length cDNA clone of CabHLH1 was obtained by performing 3' and 5' RACE using gene specific primers and RACE kits (Invitrogen). The gene specific primers were designed from the sequence of EST clone (CaF1_WIE_34_F11). 3'RACE was performed using gene specific primer (bHLH-3'F) and UAP primer provided with the 3'RACE kit as detailed in section 4 of appendix III. 5'RACE was performed by using two gene specific primers (bHLH-5'R1 and bHLH-5'R2) and AP from the kit as detailed in section 5 of appendix III. The amplified products were run on 1% agarose gel, purified with gel extraction kit (Qiagen) and the purified products were then
cloned in the pGEM-T Easy vector. The 3' and 5' RACE products cloned in pGEM-T were named as p3'CabHLH1 and p5'CabHLH1 respectively. The clones were sequenced using standard procedure of sequencing. For the amplification of full length clone, gene specific primers were designed from the full length nucleotide sequence as obtained from alignment of the 3'CabHLH1, 5'CabHLH1 and the EST sequences. The full length cDNA clone was amplified by PCR using cDNA as template and the gene specific primer pair (bHLH-F1 and bHLH-R1). The PCR product was run on 1% agarose gel, purified by gel extraction kit (Qiagen) and subsequently cloned into the pGEM-T Easy vector. The cloned product was named as pCabHLH1. The sequence of the primers is given in table 2 of appendix II. The PCR was carried out as described in section 6 of appendix III, and the annealing temperature for this primer pair was kept at 60°C. For long term storage, the bacterial cultures were grown overnight and 80% sterile glycerol was added so as to obtain a final concentration of 15% and stored in -80°C.

5.2.2 Amplification of CabHLH1 from genomic DNA

The genomic clone of CabHLH1 was isolated using genomic DNA as template and bHLH-F1 and bHLH-R1 gene specific primer pair (see table 2 of appendix II) so as to amplify genomic ORF. For this primer pair, annealing temperature was kept at 60°C. Genomic DNA was isolated as described in section 2 of appendix III. The PCR was carried out as described in section 6 of appendix III.

5.2.3 Genomic southern

Genomic southern was performed in order to find out the copy number of CabHLH1 in chickpea. 10μg of genomic DNA samples were digested with restriction enzymes HindIII, NotI, NcoI and EcoRV. The digested samples were separated on 0.8% agarose gel, denatured and blotted on to Genescreenplus membrane (Amersham). For hybridization, ORF region of CabHLH1 was amplified using bHLH-F1 and bHLH-R1 primer pair and pCabHLH1 as template. The amplified product was run on 1% agarose gel and purified by gel extraction and this purified product was further used for preparing the 32P-dCTP labelled probe using random labelling kit (NEB). The detailed description of southern blot preparation and hybridization is given in section 14 of appendix III.
5.2.4 Northern blotting

Northern-blot analysis was performed to determine the expression pattern of CabHLH1 in response to Fusarium wilt and its tissue specific expression. For this, 20μg of RNA samples were separated on a 1.5% formaldehyde-agarose gel and then blotted onto Genescreenplus membrane (Amersham). The EST clone of CabHLH1 was amplified from the plasmid containing this clone using M13 forward and reverse primers. The primer sequence is given in table 2 of appendix II and the PCR detail in section 6 of appendix III. The amplicon was run on 1% gel, purified by gel extraction and the purified product was used for preparing 32P-dCTP labelled probe by random labelling using random labelling kit (NEB). The detailed description of northern blot preparation is given in section 15 and that of hybridization in section 14 of appendix III.

5.2.5 Quantitative real time PCR

For quantitative RT-PCR, two week old chickpea seedlings were treated with various hormones and the tissue collected at various time points after treatment. Total RNA was extracted using TRIzol reagent and used for cDNA synthesis by High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). The cDNA was diluted 10 times and qRT-PCR was performed in triplicates in ABI 7500 sequence detection system using SYBR Green Master Mix (Applied Biosystems) and gene specific primers bHLHRT-F and bHLHRT-R (see table 1 of appendix II). The relative quantification method (ΔΔ-CT) was used to evaluate quantitative variation between the replicates examined. The amplification of 18S RNA was used as an endogenous control to normalize all data.

5.2.6 Subcellular localization

The subcellular localization of CabHLH1 was studied by performing transient expression assay in onion epidermal cells. For this CabHLH1 was fused in frame with 5’ terminus of GFP reporter gene in pCAMBIA-1302. The fusion construct was named as pCAMBIA-CabHLH1 and was prepared by amplifying CabHLH1 from pCabHLH1 using pCambHLH-F and pCambHLH-R primer pair (see table 2 of appendix II) and cloning in BgIII and SpeI sites of vector as described in section 17 of appendix III. The fusion construct of CabHLH1-GFP was bombarded on to the onion.
peels which were then incubated for 24 hours before visualizing in confocal microscope.

5.3 Results and discussion

5.3.1 Cloning of full length *CabHLH1* gene

Full length *CabHLH1* was amplified by 5' and 3' RACE using RACE kits (Invitrogen) and following manufacturer’s instructions. For 3' RACE, cDNA was synthesized using oligo dT primer from the kit (AP primer). Using this cDNA as template, 3' end of *CabHLH1* was amplified using UAP primer of the kit and the gene specific primer bHLH-3’F designed from the sequence of the EST (CaF1_WIE_34_F11). The amplified product of approximately 0.65 kb (Figure 5.1) was run on the gel, eluted and subsequently cloned in pGEM-T Easy vector. The clone was sequenced by standard procedures of sequencing and confirmed by the presence of overlap with the sequence of partial clone.

For 5' RACE two gene specific primers were designed namely bHLH-5’R1, and bHLH-5’R2. bHLH-5’R1 was used for making cDNA for the 5'RACE. cDNA was purified with the S.N.A.P. column provided with the kit and the purified cDNA was proceeded for TdT tailing. Tailed cDNA was proceeded for the direct PCR amplification using bHLH-5’R2 primer. The PCR product was diluted (1:100) and 2μl was taken for the secondary PCR using the same primer. The amplified product of ~0.3kb (Figure 5.2) was eluted from gel and cloned in pGEM-T Easy vector. The clone was sequenced by standard procedures of sequencing and confirmed by the presence of overlap with the existing EST sequence. 5' and 3' RACE resulted in a 750 bp long cDNA clone consisting of an open reading frame (ORF) of 687 bp (Figure 5.3).

5.3.2 *In silico* analysis of *CabHLH1* encoded protein sequence

The predicted protein product of *CabHLH1* comprises 228 amino acid residues with a calculated molecular mass of about 25.193 kDa and isoelectric point of 6.63. The schematic representation of the gene structure is given is figure 5.4A. *In silico* analysis with ExPASy ProtParam tool revealed the presence of approximately 14% basic, 14 % acidic and 76 % neutral amino acids. The homology search against GenBank database showed that *CabHLH1* had maximum homology with an unnamed protein of *Vitis vinifera* and bHLH transcription factor of *Nicotiana*. It also showed
Figure 5.1: 3' RACE of CabHLH1. (A) Flow diagram depicting 3' RACE procedure. Oligo dT adapter primer (AP) was used to bind the polyA tail of the mRNA. Reverse transcriptase generates single stranded cDNA using mRNA as template. Gene specific primer (bHLH-3'F) and UAP primer specific to 3' adapter were used to amplify the target 3'dDNA end sequence. (B) 1% agarose/EtBr gel showing 3' RACE product of CabHLH1. Lane 1 represents 1Kb ladder and lane 2 the PCR product. (C) Map of pGEM-T Easy vector used to clone the amplified product. (D) Diagrammatic representation of 3' RACE product of CabHLH1 cloned in pGEM-T easy vector and the construct named as p3' CabHLH1.
Figure 5.2: 5' RACE of *CabHLH1*. (A) Flow diagram depicting 5' RACE procedure. (B) 1% agarose/EtBr gel showing 5' RACE product of *CabHLH1*. Lane 1 represents 1Kb ladder and lane 2 the PCR product. (C) Map of pGEM-T Easy vector used to clone the amplified product. (D) Diagrammatic representation of 5' RACE product of *CabHLH1* cloned in pGEM-T vector and the construct named as p5' *CabHLH1*. 
Figure 5.3: Isolation of full length cDNA clone of CabHLH1. (A) Schematic representation of the alignment of sequence of partial clone (EST) and that of 3' and 5'RACE products and the primer position for full length cloning. (B) 1% agarose/EtBr gel showing full length cDNA clone. Lane 1 indicates 1Kb ladder and lane 2 PCR product. (C) Map of pGEM-T Easy vector used for cloning the PCR product. (D) Diagrammatic representation of full length clone of CabHLH1 cloned in pGEM-T Easy vector and the construct is named as pCabHLH1.
homology to an unknown protein of *Medicago* and IAA-Leu resistant DNA binding protein of *Arabidopsis*. The deduced protein sequence encodes a transcription factor with bHLH domain represented by amino acid 70 to 119 and a caspase domain represented by amino acids 94 to 169 (Figure 5.4B, 5.4C). The presence of caspase domain in *CabHLH1* raises intriguing possibilities of mechanism and additional aspects of function of this gene. Caspases represent a family of cysteine proteases that have key roles in apoptosis. Further, caspases are known to form dimers and hence it would be interesting to speculate that *CabHLH1* might form homo or hetero dimers through the caspase domain and perform downstream function. Recent progress in caspase research indicated that these proteins are important not only in apoptotic but also in non apoptotic processes. It was shown that caspases independent of their enzymatic action also signal through protein-protein interaction and perform role in innate immunity in animals (Kuranaga and Miura, 2007). Based on these evidences, it could be speculated that *CabHLH1* might play a regulatory role in plant immunity by executing apoptotic or non apoptotic function.

5.3.3 Multiple sequence alignment and phylogenetic analysis of *CabHLH1*

In order to compare the amino acid sequence of *CabHLH1* with related protein sequence from other plants, we did multiple sequence alignment using MAFFT program LINSI algorithm. The results showed that there was considerable conservation along the amino acid sequence of orthologs from various organisms particularly in the stretch of amino acids which represented the bHLH domain (Figure 5.5). Earlier studies in *Arabidopsis* and rice have also revealed that the signature bHLH region across the members of bHLH family is highly conserved (Toledo-Ortiz *et al.*, 2003; Li *et al.*, 2006).

In order to evaluate the evolutionary relationship of *CabHLH1*, its amino acid sequence was aligned with sixteen orthologs of bHLH proteins from other organisms and a phylogram was generated using MAFFT program neighbour joining conserved method. The analysis showed that *CabHLH1* shared same lineage as that of unnamed protein of *Vitis vinifera*, bHLH protein from *Nicotiana*, unknown protein from *Medicago* and IAA-Leu resistant DNA binding protein of *Arabidopsis* (Figure 5.6). All these proteins were found to be falling closer to each other in the tree. *CabHLH1* was also related, although, distantly to bHLH transcription factors from various other
**Figure 5.4:** cDNA and deduced amino acid sequence of *CabHLH1*. (A) Diagrammatic representation of gene structure of *CabHLH1*. (B) Domain analysis. (C) Nucleotide and the amino acid sequence. Nucleotides in lower case indicate the 5' UTR and 3' UTR. Those in upper case indicate complete ORF with the start and stop codons and the deduced amino acid sequence. Nucleotides highlighted in sea green colour indicate the helix loop helix domain and those with blue color indicate caspase domain. The green region signifies the region shared by the two domains.
Figure 5.5: Sequence alignment of *CabHLH1* with the sequences of orthologs from other plants. The multiple sequence alignment was done using MAFFT program LINSI algorithm.
Figure 5.6: Phylogenetic tree showing evolutionary relationship between CabHLH1 and other similar proteins. The tree was generated using MAFFT program neighbour joining conserved method.
plants like oryza, maize, Brassica etc. Further, CabHLH1 was placed quite far away from its human ortholog. Interestingly, CabHLH1 was more closely related to its ortholog from *Vitis* and *Nicotiana* rather than its legume counterpart.

5.3.4 Genome organization of *CabHLH1*

We aimed at obtaining initial information about the genome organization (copy number and intron number and size) of *CabHLH1* gene. In this direction, we performed PCR using genomic DNA from chickpea as template. Primers were designed from 5’ and 3’ ends of *CabHLH1* cDNA in order to amplify the ORF. The PCR gave a band of same size as that of the cDNA clone suggesting that *CabHLH1* is an intronless gene (Figure 5.7A). Earlier reports on presence and distribution of introns in bHLH family proteins has shown that 80% of the members of this family in *Arabidopsis* and rice contain introns and the intron position is conserved even though the number varies (Toledo-Ortiz *et al.*, 2003; Li *et al.*, 2006). However, in this study the absence of intron suggests that this gene family might differ in genome organization in legumes. Interestingly, a *myc* gene in mouse was found to be intronless and acted as a strong inducer of apoptosis (Sugiyama *et al.*, 1998).

Southern transfer of chickpea genomic DNA digested separately with various restriction enzymes was also performed and hybridized with ORF region of *CabHLH1* cDNA as probe under high stringent conditions. *HindIII*, and *NotI* are non-cutters of *CabHLH1* and gave double and single bands respectively after hybridization. The single site cutters *EcoRV* and *NcoI* gave three bands each (Figure 5.7B). Taken together, these results suggest that *CabHLH1* is present in more than one copies in chickpea.

5.3.5 Expression of *CabHLH1* in response to *Fusarium* wilt

Expression pattern of *CabHLH1* in response to *Fusarium* wilt in chickpea was studied by RNA gel blot. The experiment was performed with susceptible (JG-62) and resistant (WR-315) genotypes of chickpea. The results showed that *CabHLH1* gene had some basal expression in both the genotypes indicating its requirement in normal developmental processes. However, the transcript level increased in response to *Fusarium* infection suggesting that the gene is regulated by biotic stress (Figure 5.8A and 5.8B). The expression reached maximum level within 6 hours of *Fusarium* infection indicating its role in early phase of defence. However, the expression did not
Figure 5.7: Genome organization of *CabHLH1*. (A) PCR amplified product of *CabHLH1* using genomic DNA as template and ORF end primers. Lane 1 and 2 indicate 1Kb ladder and PCR product respectively. (B) Southern blot hybridization analysis for predicting the copy number. Each lane was loaded with 10μg of chickpea genomic DNA digested with indicated restriction endonucleases and the blot was hybridized with ^32^P-labeled probe prepared from full length cDNA clone of *CabHLH1*. Size markers in Kb are indicated on left.
show any major difference in susceptible and resistant genotypes. This suggested that although this gene might be involved in defense response against *Fusarium* wilt, its expression kinetics does not bear any direct effect on susceptibility or resistance of a plant. Moreover, as suggested by the expression pattern, *CabHLH1* might be involved in very early events and might be followed by a long series of other important events that would in turn have a direct effect on making a plant susceptible or resistant to pathogen attack. Also this gene might be involved in basal defense.

### 5.3.6 Analysis of tissue-specific expression of *CabHLH1*

In order to know about the tissue specific expression of *CabHLH1*, RNA gel blot was performed with RNA isolated from root, stem and leaf tissue of *Fusarium* infected chickpea plant. The blot was hybridized with probe prepared from cDNA clone of *CabHLH1*. The results showed that the gene was expressed in all the three tissue types, however, the expression was more in root followed by stem and leaf (Figure 5.8C). This suggested that the gene plays regulatory role against wilt in multiple organs, however, root being the first organ to come in contact with the fungus expresses this gene to a higher level.

### 5.3.7 Expression of *CabHLH1* in response to various hormonal treatments

We investigated the expression of *CabHLH1* gene in response to various hormonal treatments by quantitative real time PCR. We observed increased *CabHLH1* transcript accumulation in response to SA, transcript level reaching its maximum at 6 hours after treatment. The expression also increased during progressive time points after JA and ACC treatment with maximum level at 12 hours after treatment. brassinosteroid and NO also induced its expression, however, in case of NO, there was an initial downregulation of the *CabHLH1* followed by upregulation at 3 and 6 hours after the treatment. The expression again showed downregulation at 12 hours post treatment. Further, the gene was found to be downregulated in response to ABA except a slight increase at later time points (Figure 5.9). These results suggest that *CabHLH1* is induced in response to all the hormones which are known to play role in plant defense pathways, thereby further confirming its role in plant immunity. Earlier studies have also shown the modulation of expression of bHLH genes in response to various hormones. For example, Myc transcription factors belonging to bHLH class are known to play key role in jasmonate signaling (Boter *et al.*, 2004). bHLH genes are
Figure 5.8: RNA blot analysis indicating expression pattern of *CabHLH1* gene. (A) expression in response to *Fusarium* infection in susceptible (JG-62) and (B) resistant (WR-315) genotypes of chickpea. 20μg of total root RNA isolated from 25-day old chickpea seedlings harvested at various time points after *Fusarium* infection were separated by 1.5% agarose gel. (C) expression in different tissues. For tissue specific expression RNA was isolated from different tissues. The northern blot was hybridized with a cDNA fragment of *CabHLH1* as mentioned in materials and methods. Ribosomal RNAs at lower panels represent loading controls. The graphs indicate fold expression in terms of band intensity.
Figure 5.9: Real time PCR showing relative transcript level of *CabHLH1* in response to various hormones. Transcript levels were normalized by 18S transcript level. Error bars indicate SD of three real time PCR experiments.
also shown to be involved in brassinosteroid mediated responses (Friedrichsen et al., 2002).

5.3.8 Subcellular localization of CabHLH1

In order to investigate the subcellular localization of the CabHLH1 in the cell, pCAMBIA 35S-CabHLH1-GFP was constructed (Figure 5.10). The expression of the fusion gene CabHLH1-GFP was driven by the 35S promoter of cauliflower mosaic virus (CaMV-35S). The plasmid construct was named as pCAMBIA-CabHLH1. The plasmids containing vector control DNA and fusion gene were introduced into onion (Allium cepa) epidermal cells by particle bombardment. Upon observation under confocal microscope, we found predominant nuclear localization of the fusion protein of CabHLH1-GFP, whereas the GFP protein alone from the vector control was distributed in almost all cellular organelles (Figure 5.11). The nuclear localization of CabHLH1 was in confirmation with earlier results which showed that the group of bHLH proteins are present in nucleus where they bind to DNA and perform their regulatory functions (Massari and Murre, 2000).

5.4 Conclusion

Transcription control of plant defense responses is very well documented and members of different families of transcription factors are known to play role in such regulation. Several members of bHLH family of TFs are also implicated in defense. Here we cloned a member of bHLH family (CabHLH1). The gene harboured a bHLH domain like other members of this family, however, it also had a caspase domain which made it different from rest of its counterparts. It was also quite surprising to find the gene intronless. Preliminary analysis regarding the genome organization of the gene as conducted by southern hybridization showed that the gene is present in more than one copies in chickpea. The gene was found to be induced in response to Fusarium wilt in chickpea and its expression was also altered in response to hormones which are usually known to play role in defense pathways. The gene was localized in nucleus. Taken together, these results suggest a possible role of this gene in plant defense against vascular wilt.
Figure 5.10: Construction of pCAMBIA-CabHLH1. (A) Map of pCAMBIA vector used for cloning. (B) PCR amplification of CabHLH1 gene with cloning sites containing sequence for restriction enzymes BglII and SpeI. Lane 2 represents the PCR product. (C) Digestion of pCAMBIA vector and CabHLH1 amplicon with BglII and SpeI. Lanes 2 and 3 represent uncut and digested pCAMBIA vector respectively. Lane 4 represents digested CabHLH1 amplicon. (D) Ligation of CabHLH1 in pCAMBIA. (E) Confirmation of positive clones by digestion. Lane 2 represents digested product of pCAMBIA-CabHLH1. (F) Confirmation of positive colonies by colony PCR. Lanes 2, 3, and 4 represent PCR products obtained from three different colonies. The construct pCAMBIA-CabHLH1 was bombarded in onion cells.
Figure 5.11: Subcellular localization of CabHLH1. Onion epidermal cells bombarded with (A) empty vector for control (B) pCaMBIA-CabHLH1-GFP. The GFP fluorescence was detected by using Confocal microscope. The right panel shows the corresponding phase contrast image.