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NUCLEAR LOCALIZATION OF *ARABIDOPSIS'S* HEAT SHOCK FACTOR *HSFA1D* USING BIMOLECULAR FLORESCENCE COMPLEMENTATION (BIFC) SYSTEM

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ABSTRACT

Response to thermal stress in plants is mostly regulated by heat shock factors (*hsfs*). Among hsfs, HsfA1d is one of the key players in protecting plants against heat stress. Subcellular localization of gene is prerequisite for its characterization. Bimolecular Fluorescent Complementation (BiFC) provides the most advance and reliable tool to visualize protein-protein interactions and to explore their location in living system. Objective of the present study was to accomplish sub cellular localization of HsfA1d by using BiFC. *HsfA1d* was cloned in-frame with complementary halves of yellow florescent proteins (*YFP*) using gateway cloning system. The BiFC constructs along with positive control were introduced into plant cells through agro-infiltration. The non-florescent fragments of *YFP* tagged with *HsfA1d* produced no signal when they were infiltrated alone in tobacco leaves. The N and C termini of *YFP* fused with *HsfA1d* resulted in florescence upon co-expression in tobacco leaf epidermal cells. The intact YFP tagged with *HsfA1d*, after DAPI staining, was observed under 3 different laser beams of YFP, DAPI and merged. The same florescent signals detected under different channels of laser scanning confocal microscope confirmed the nuclear localization of *HsfA1d*. It is concluded, that *HsfA1d* is localized in the nucleus of cell.

Key words: *HsfA1d*, subcellular localization, yellow florescent proteins, gateway cloning, BiFC

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INTRODUCTION

Proteins interact during every physiological process to perform their biological function. For example, DNA replication, assembly of translation apparatus, regulation of gene expression at different levels, DNA packaging and signal transduction involve protein-protein interaction (Scott *et al.* 2016). Thus, the investigation and characterization of various types of proteins interactions occur in the cell are important to understand the various aspect of cytology. Variety of techniques has been designed for the detection and analysis of proteins interactions both in vitro and in vivo (Xing *et al*. 2016). Every technique has its own pros and cons (Citovsky *et al*. 2006). For example, co-immunoprecipitation is one of the widely used methods to analyze invitro proteins interactions (Zhu *et al.* 2017). However, the requirements for specific antibodies against target proteins make it more expensive and laborious process. Moreover, Co-immunoprecipitation is associated with cell lysis which hinders the subcellular localization of the test proteins (Yang *et al*. 2014). Cross-linking and co-fractionation allows detection of in vivo protein–protein interactions, but the involvement of complex biochemical procedures makes it difficult to apply (Chen *et al.* 2010). Yeast two-hybrid (Y2H) is another assay used to visualize in vivo proteins interactions. The high occurrence of false-positives results and the non-availability of sub-cellular compartments for specific proteins interaction, add to the several drawbacks of this system (Mehla *et al.* 2017). Sos and Ras offers a different version of two hybrid system for detection of protein interaction. In this system, the complementary fragments of target protein (N & C) are fused to the cytoplasmic proteins of interest. The reconstitutions of such fragments during protein interaction degrade the URA3 reporter protein eventually leading to uracil auxotrophy and resistance to 5-fluoroorotic acid. Though, these assays do not need the entry of test proteins to the nucleus, yet drawback lies in allowing the interacting proteins to follow native patterns of subcellular localization (Cruz-Migoni *et al.* 2019). Bimolecular Fluorescent Complementation (BiFC) offers the most advance and novel approach to detect protein interactions directly within the living cell. Proteins interactions occur in any compartment of aerobic cell can be detected by using BiFC (Miller *et al.* 2015). In this assay, the desired protein is tagged with complementary fragments of florescent protein and the plant cells are transformed with both the constructs using *agrobacterium*. The association of non-florescent complementary fragments results in the restoration of florescent signals and eventually detected in the living plant cells (Kerppola, 2008). Initially, BiFC was limited to study the interacting transcription factors belong to bZIP and Rel family (Hu *et al.* 2002). Later on, the use of this assay was extended to detect protein interaction in mammalian (Hynes *et al.* 2004) bacterial (Tsuchisaka and Theologis, 2004) and plants cells (Stolpe *et al.* 2005). In plants, several expression vectors have been constructed to detect protein interaction in epidermal cells of tobacco and *Arabidopsis* (Walter *et al.* 2004). The BiFC vector, that were initially in use, suffered several drawbacks including inadequate cloning site, the requirement of two selectable markers for developing transgenic plants and non-availability of space for the expression of additional reporter genes (Bracha-Drori *et al.* 2004). To come across such problems, a large number of BiFC vectors with expanded MCS have been designed (Walter *et al.* 2004). The expanded MCS helps in tagging target proteins to complementary halves of auto florescent proteins. Maximum diversity in cloning machinery is important as precise association and integration of the interacting proteins is essential for reconstitution of florescent YFP. In addition to, such vector design provides an opportunity for the BiFC expression cassettes to use the *agrobacterium* binary plasmids for co-expression of interacting partners (Lai and Chiang, 2013). Moreover, these vectors help us to incorporate more autofluorescent proteins as reporter genes that may act as internal controls and markers of subcellular compartments. BiFC provides the opportunity to monitor transiently interacting proteins without the aid of any sophisticated equipment. Tobacco (*Nicotiana benthamiana*) offers a model system to be transformed with generated constructs and visualize protein-proteins interaction. *Agrobacterium* uses Ti plasmid (tumor inducing) as vehicle for the transport of target gene into the plant genome. The present study aimed at determining subcellular compartment of *HsfA1d* using gateway compatible BiFC system.

MATERIALS AND METHODS

Cloning of *HsfA1d* in BiFC Vectors: *Arabidopsis*'s *HsfA1d* was tagged with N and C termini of *YFP* in BiFC vectors using LR reaction (Supp. 1). The LR reaction for cloning *HsfA1d* in frame with N terminus of *YFP* was initiated by mixing *pUC57GW-HsfA1d* (entry clone) and *pGSA002-nYFP*^{n} (destination vector) in ratio of 4and 1 Fmoles respectively followed by the addition of clonaseII enzyme (0.5 µl) and placed for 1 hour at room temperature. Similarly, for cloning *HsfA1d* in frame with C terminus of *YFP*, the reactants (*pUC57GW-HsfA1d and pGSA002-nYFP*^{c}) were added in 3.5:1Fmoles. The resultant mixture was kept for 1 hour at 25 ºC. Heat shock method was used to transform *stellar* cells with cloning product and applied on spectinomycin (50 mg/l) containing LB plates. The LB plates were incubated overnight at 37 ºC. Plasmids were extracted from transformed bacteria using plasmid extraction kit and subjected to digestion with restriction enzyme *NOCI* for confirmation. The digested DNA fragments were separated through electrophoresis using agarose gel (1%).

Preparation of Chemically Competent *Agrobacteria* (*GV3101*): Single colony of *Agrobacteria* was inoculated in 5 ml nutrient broth and subjected to overnight shaking (200 rpm) at 28 ºC. One ml of the resultant culture was mixed with fresh nutrient broth (50 ml) and grown at 28 ºC till the OD_{600} reached to 0.6. Further bacterial growth was stopped by keeping the culture on ice (30 min) and distributed in 2 sterile tubes equally. The bacterial culture was subjected to centrifugation (4000 g) for 5 min. The pellet was re-suspended in 15 ml $CaCl_2$ (50 mM) and placed on ice for 15 min followed by centrifugation (3800 g) for 5 min. The pellet was re-suspended in 2 ml $CaCl_2$ (50 mM) supplemented with glycerol (15%). Aliquots were made and stored at -80 ºC.

Transformation of competent *Agrobacteria*: Competent agro-bacterial cells **(**10 µl**)**, after thawing, were mixed with plasmid DNA (1 µl) and placed on ice for 8 min. The cells were first subjected to cold treatment (-80 ºC for 5 min) followed by incubation for 5 min at 37ºC and kept on ice for 1 min. Fresh nutrient broth (1 ml) was added to the cells and grown at 28ºC under 200 rpm. The cells were centrifuged (8,000 g) for 1 min and the pellet was re-suspended in nutrient broth (50 µl). Cells were applied to nutrient agar plates with spectinomycin (50 mg/l) and placed for 2 days at 28ºC. In this way, *agrobacteria* were transformed with cloned BiFC vectors along with *pGWB442-YFP-HsfA1d* (positive control).

Transient Transformation of Tobacco Leaves

Inoculums preparation: Single colony of transformed A*grobacteria* was added to nutrient broth (5 ml) with spectinomycin (50 mg/l) and subjected to overnight shaking (200 rpm) at 28 ºC. The resultant culture was centrifuged (3000 g) for 5 min and the pellet was re-suspended in autoclaved water (20 ml) followed by re-centrifugation, using same rpm and time. The pellet was re-suspended in MMA solution (5 ml) and kept for 2 hours at 25 ºC. Final volume of inoculums was adjusted to 10 ml with OD_{600} of 0.6 using MMA solution. Equal volumes (5 ml) of agro inoculums harboring pGSA002-NYFP-HsfA1d and pGSA002-CYFP-HsfA1d were mixed.

Infiltration of tobacco leaves: For BiFC, two weeks old tobacco seedlings (Sup. 1) were divided into 4 groups. Each group of seedlings was infiltrated with 5 ml of different constructs i.e *pGWB442-YFP-HsfA1d* (positive control), *pGSA002-NYFP-HsfA1d* (negative control), *pGSA002-CYFP-HsfA1d* (negative control) and mixture of *pGSA002-NYFP-HsfA1d+ pGSA002-CYFP-HsfA1d* (test). The infiltrated seedlings were exposed to dark for 40 hours and observed for florescence. The florescent leaf segments (test samples) were stained with DAPI (4′,6-diamidino-2-phenylindole) reagent for 20 min and re-observed under 3 different channels ((YFP, DAPI and merged), using confocal microscopy.

Confocal microscopy: One cm of infiltrated leaf segment was placed on microscopic slide with upside down and about 30 µl H2O was added. The leaf piece was covered with cover glass and fixed using adhesive tape on both sides. Olympus laser scanning confocal microscope was used for images.

RESULTS

Heat shock factor (*HsfA1d)* was tagged with complementary halves of *YFP* using gateway cloning system (LR reaction; Fig 1A & B).

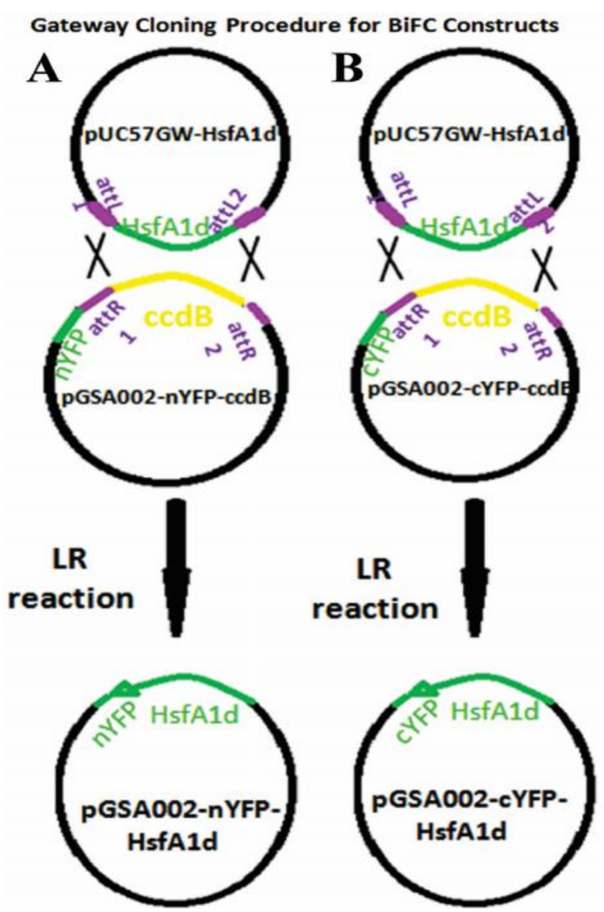

Figure1. Gateway procedure (LR reaction) for cloning *HsfA1d* in frame with, (A) N terminus of *YFP* and (B) C terminus of *YFP*.

E. coli competent cells were transformed with product of the both LR reactions (Supp.2). Three fragments (10702, 1121 and 391 bp) were observed upon digestion of *pGSA002-nYFP-HsfA1d* with restriction enzyme *NcoI* while the empty vector (*pGSA002-nYFP*), used as control, resulted in 2 fragments (10557 and 1840 bp). Similarly, restriction digestion-based confirmation of *pGSA002-cYFP-HsfA1d* with *NcoI* produced 3 fragments (10702, 797 and 391 bp) while 2 fragments (10557 and 1516 bp) were observed when the control vector (*pGSA002-cYFP*) was subjected to *NOCI* as shown in the Supp.3. The BiFC vectors where *HsfA1d* has been cloned in frame with N & C termini of *YFP* shown in the Figure 2A & B.

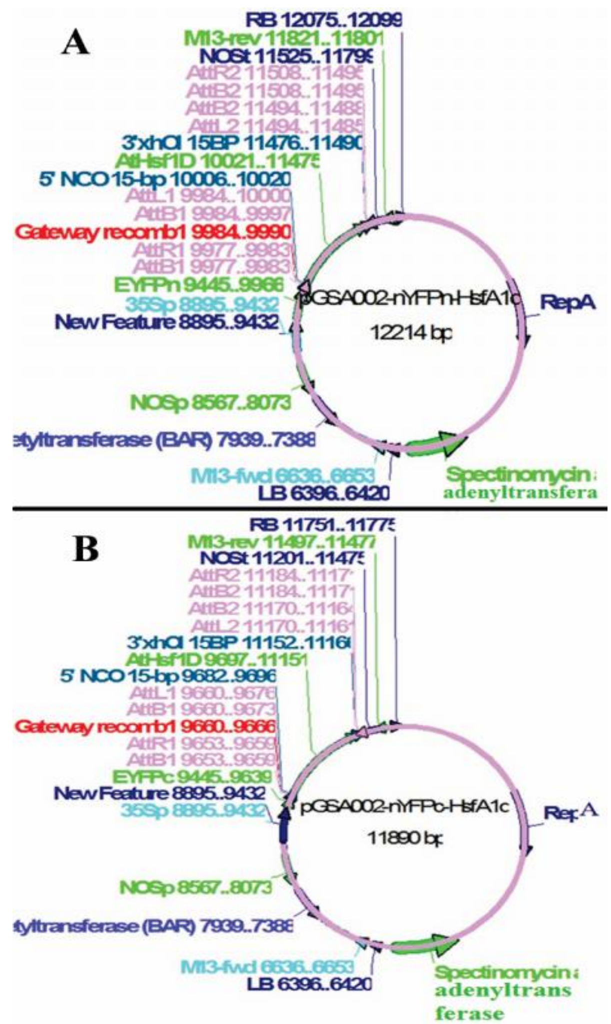

Figure 2. Cloned BiFC vectors, (A) *pGSA002-nYFPn-HsfA1d* and (B) *pGSA002-nYFP^c-HsfA1d*.
CaMV35S = Promoter, Spectinomycin adenyletransferase= bacteria marker, NOS = terminator sequence, *EYFPn*= N terminus of *YFP*, *EYFPc* = C terminus of *YFP*,*HsfA1d*= gene of interest

Competent *Agrobacterial* cells were successfully transformed with *pGSA002-nYFP-HsfA1d and pGSA002-*

cYFP-HsfA1d (Supp. 4). Tobacco leaves infiltrated *pGWB442-YFP-HsfA1d,* used as positive control*,* shown florescence under confocal microscope (Fig. 3A) while no florescence was recorded when *pGSA002-nYFP-HsfA1d and pGSA002-cYFP-HsfA1d* were expressed alone in tobacco leaf samples (Fig. 3B & C). Similarly, florescence was observed when *pGSA002-nYFP-HsfA1d and pGSA002-cYFP-HsfA1d* were co-expressed in tobacco leaves (Fig 3D).

The tobacco epidermal cells, detected positive for florescence, revealed florescence under observing 3 laser beams (YFP, DAPI and merged) using confocal microscopy (Fig. 4).

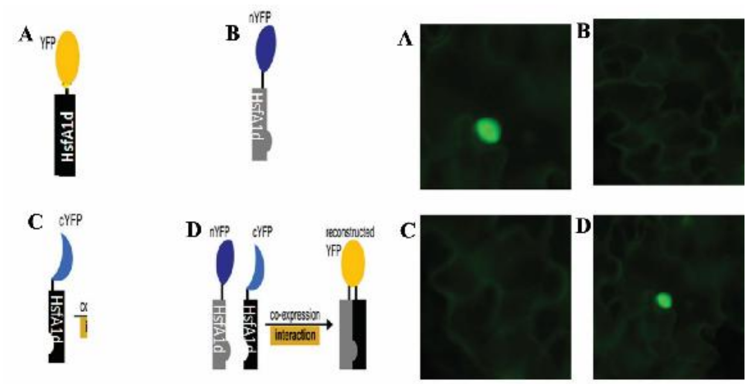

Figure 3. BiFC based detection of *HsfA1d* in *N. benthamiana* leaf epidermal cells, (A) *YFP* tagged with *HsfA1d* (positive control), (B) *HsfA1d* tagged with N terminus of *YFP* (negative control), (C) *HsfA1d* tagged with C terminus of YFP (negative control) and (D) Co-expression of N and C termini of *YFP* tagged with *HsfA1d* (test) Scale bar = 10 µm.

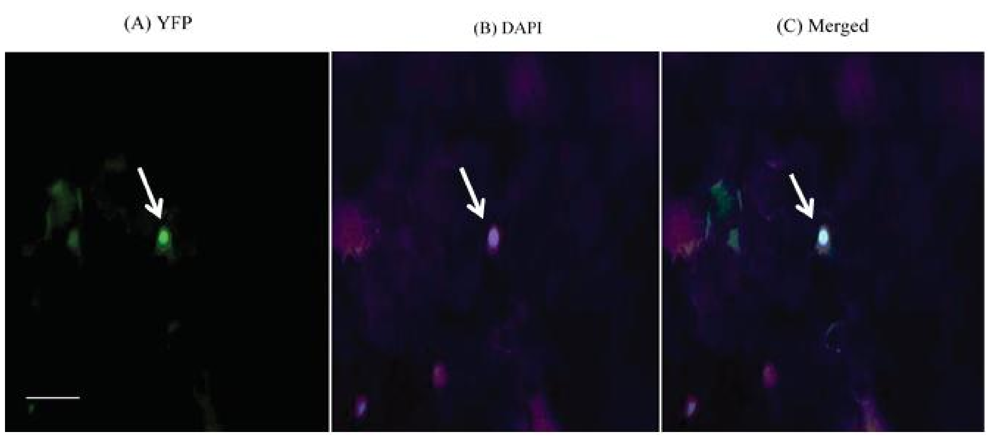

Figure 4. Nuclear localization of *HsfA1d* in *N. benthamiana* leaf epidermal cell, Detection of interacting N and C termini of *YFP* tagged with *HsfA1d* under, (A) *YFP* channel, (B) DAPI channel and (C) Merged channel

DISCUSSION

The integration of Gateway cloning system and BiFC provide powerful tool to the researchers for understanding protein interactions in the cells. Gateway cloning technique allows the genetic engineers to rapidly design a variety of vectors with high degree of precision (Karna *et al.* 2018) while the BiFC helps in detection of protein-protein interaction and their subcellular localization (Gookin and Assmann, 2014).The attachment (att) sites lies on the vectors provide a platform for recombination that ultimately leads to gene shuffling (Reece-Hoyes and Walhout, 2018). In the current study, recombination at the attachment sites present on entry clone (aaL1, attL2) and destination vector (attR1, attR2) helped in cloning target gene through LR reaction. The BiFC vectors (*pGSA002-nYFP-HsfA1d* and *pGSA002-cYFP-HsfA1d*) constructed in the present study strengthened the earlier reports of Karimi *et al.* (2007), that gateway accurately clone gene cassettes into multiple vectors. *Agrobacterium* mediated infiltration was successfully used for introduction of BiFC vectors into the tobacco plant. These results are in line with the reports of Li *et al.* (2005). The mentioned results are also in agreement with Leckie and Stewart (2011) that *agrobacteria* extend valuable source for the transport of target gene into host plant. Epidermal cells of 4 weeks old *Nicotiana benthamiana* were transformed with *HsfA1d* tagged with *YFP*. Similar results exhibited by Schweiger and Schwenkert (2014), further endorsed the utility of *Nicotiana benthamiana* as model system for transient expression of the target gene. The young age of explants, inoculums OD (0.6) and post infiltration period (42 hours) contributed in successful delivery of *HsfA1d* fused with auto-florescent protein into tobacco (Bernaudat *et al.* 2011). Among advanced techniques, BiFC represents one of the most powerful tools for detection and studying protein interactions in living cell (Citovsky *et al*. 2006). The foundation of this assay lies on reconstitution of fluorescent complex of two complementary fragments of *YFP* fused to the interacting molecules (Schütze *et al*. 2009). In the current study, the co-expression of cloned BiFC vectors (*pGSA002-HsfA1d-nYFP*and *pGSA002-HsfA1d-cYFP*) in tobacco cells, extended opportunity for non-florescent C and N termini to reconstitute YFP and hence signals were detected. Similar results were also shown by Horstman *et al*. 2014. No florescence was observed when these vectors were expressed alone, as negative control, in the host cells. These results are in agreement with the reports of Peter *et al.*, (2017). Signals were detected when *pGWB442-HfsA1d* tagged with intact YFP was expressed in epidermal cells as positive control. The leaf segments already detected positive for YFP were stained with DAPI and examined under 3 distinct laser beams (DAPI, YFP, merged) for florescence. As DAPI only binds to the nuclear DNA, therefore appearance of florescent proteins tagged with target gene, on the same location under 3 different laser beams accomplished that *HsfA1d* lies in nuclear compartment of the cell. Hernández-Sánchez *et al.* (2015), ended up with same results upon exposing the host tissues to nuclear dye (DAPI) for subcellular localization of the target gene.

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Author's contribution: Zamarud Shah conducted the main experiment and collected data. Gul Shad Ali supervised the research. Arshad Iqbal and Hussain Shah helped in manuscript writing. Muhammad Zulfiqar Ahmed and Masroor Hussain contributed in figure setting and data analysis. Ashrf El-Sayed and Nisar Ahmad helped in making the revised manuscript.

Conflict of interest: The authors declare that they have no conflict of interest

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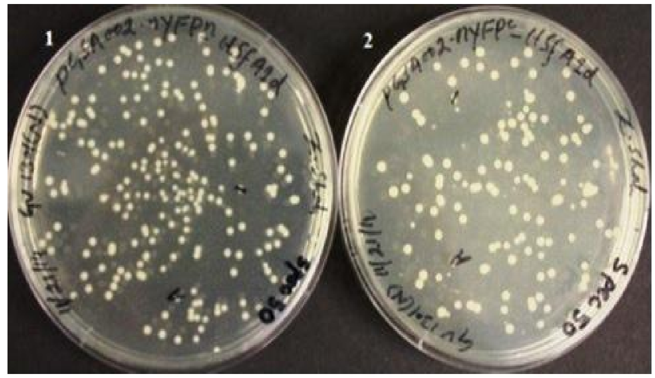
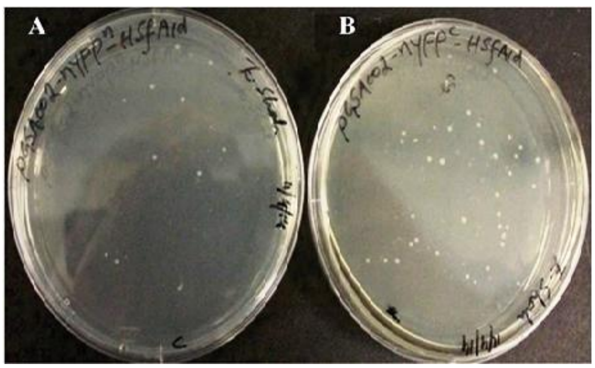
SUPPLEMENTARY DATA

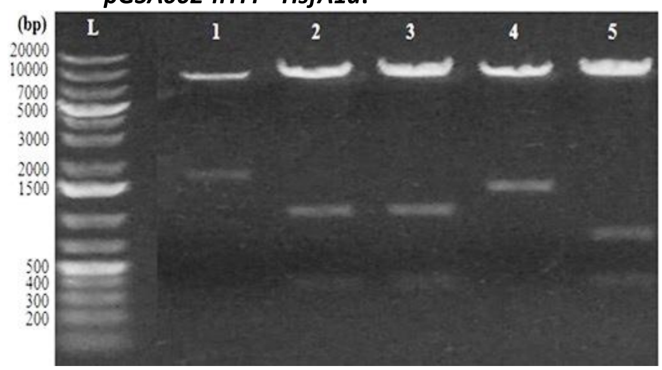
Supp 1. Tobacco leaves used for infiltration of *GV3101* harboring *BiFC constructs*



Supp 3. *Agrobacterium GV3101* cells transformed with, (a) *pGSA002- nYFP*^{n}*-HsfA1d* and (b) *pGSA002-nYFP*^{c}*-HsfA1dHsfA1d nYFP*^{n}*-HsfA1d* and (b) *pGSA002-nYFP*^{c}*-HsfA1d*.



Supp 2. *E. coli* cells transformed with, (a) *pGSA002-nYFP*^{n}*-HsfA1d* and (b) *pGSA002-nYFP*^{c}*-HsfA1d*



Supp 4. Restriction digestion of cloned BiFC vectors *pGSA002-nYFP*^{n}*-HsfA1d* and *pGSA002- nYFP*^{c}*-HsfA1d* along with their empty vectors (control) using enzyme *NcoI*.