Research Article

Overexpression of the Heat Shock-Specific Transcription Factor *HSFA1D* Enhances Thermotolerance in Tobacco Plants

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Abstract | Among different abiotic stresses, heat stress has the most devastating impact on plant growth. Heat shock transcription factors are known to play an important role in regulating heat stress in plants. Tobacco (Nicotiana benthamiana) was transformed with heat shock transcription factor HsfA1d by transfecting leaf discs with Agrobacterium strain GV3101 carrying CaMV35S-YFP::HsfA1d construct. After PCR and confocal-based confirmation, HsfA1d overexpression lines (OX1, OX2 and OX3) were evaluated for their response to heat stress. Overexpression lines on average showed 33.26% less electrolyte leakage after induction of heat stress at 42˚C compared to wild type plants (WT). No significant decrease was recorded in the chlorophyll content of overexpression lines whereas significant decrease was recorded in the chlorophyll content of WT plants following heat stress. Similarly, no significant reduction in the water content of the leaves of overexpression lines was recorded compared to 33.87% reduction in leaf water content of WT plants as a result of heat stress. Furthermore, overexpression lines accumulated significantly higher amounts of proline compared to WT, both at room temperature and at 42˚C. The above results showed that HsfA1d positively regulates thermotolerance in tobacco plants and can be used as target gene for engineering thermotolerance in crops.

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Introduction

Heat stress leads to an array of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and development (Harsh et al., 2016). The elevated temperature is one of the most threatening constraints for agricultural productivity. Thermal stress causes various physiological damages in plants including; scorching of leaves and stems, leaf abscission and senescence, shoot and root growth inhibition or fruit damage, which consequently lead to decreased plant productivity (Vollenweider and Günthardt-Goerg, 2005).

Heat stress also activates genes involved in heat stress pathway. Most of these genes encode heat shock proteins (HSPs) (Kotak et al., 2007). The thermo-labile proteins in plant cells are protected against heat-in-

duced denaturation by many HSPs by acting as chaperones (Waters, 2013). Plants synthesize five major classes of HSPs including small HSPs (smHSPs), HSP60, HSP70, HSP90 and HSP100 (Sun et al., 2002). Based on their sequence homology and cellular localization the smHSPs have been divided into six classes. Three classes (I, II and III) function in the cytoplasm while class IV, V and VI are located in mitochondria, endoplasmic reticulum and chloroplast respectively (Vierling, 1991).

The expression of heat shock-responsive genes in eukaryotes is mainly regulated by heat shock transcription factors (*Hsfs*; Xue et al., 2014). The genome of *Arabidopsis thaliana* encodes 21 *Hsfs*, which can be categorized into three major classes (A, B, C) and 14 groups (A_1-$A_{9,}$ B_1-$B_{4,}$ C_1). A_1 group is important in heat shock response and comprise of 4 *Hsf* genes, *HsfA1a, HsfA1b, HsfA1d and HsfA1e* (Nover et al., 2001). Studies on quadruple knockout *HsfA1a/HsfA1b/HsfA1d/HsfA1e* and four triple knock mutants showed that *HsfA1a, HsfA1b and HsfA1d* are mainly involved in thermotolerance enhancement while role of *HsfA1e* was found to be non-significant (Yoshida et al., 2011). Besides thermotolerance, *HsfA1* group also play role in tolerance to salt, osmotic and oxidative stresses (Liu et al., 2011).

To cope with the detrimental effects of heat, there is a need to develop thermotolerant varieties. Traditional plant breeding along with improved agricultural practices has contributed a lot to crop improvements. Modern biotechnology offers new ideas and techniques to compliment in crop improvement i.e. productivity and nutritional values. Genetically modified (GM) crops have significantly increased yield per unit area since 2006 (James, 2007). In the current study, *HsfA1d* was isolated from *Arabidopsis thaliana* and overexpressed in tobacco to investigate the role of *HsfA1d* in thermotolerance enhancement.

Materials and Methods

Cloning of *HsfA1d*

Heat shock transcription factor *HsfA1d* was isolated from *Arabidopsis thaliana* and infused in a gateway compatible cloning vector *pUC57GWccdB*, constructed in plant pathology lab, Mid-Florida Research and Education Center, University of Florida USA. *HsfA1d* was then transferred from the entry clone (*pUC57GW-HsfA1d)* into binary destination vector (*pGWB442)* using LR kit (Invitrogen, USA). For LR reaction, the concentration of both *pUC57GW-HsfA1d* and *pGWB442* were adjusted to 20 ng/µl and 40 ng/µl respectively. The LR reaction was incubated at 25 °C for 1 hour and transformed into *stellar* cells using heat shock method followed by plating on LB media supplemented with spectinomycin (50 mg/l). The plates were incubated overnight at 37 °C to observe the colonies. The plasmids were extracted from two colonies using plasmid extraction kit (Qiagen, USA), following manufacturer's instructions. The cloned vector *pGWB442-HsfA1d* were confirmed through digestion with restriction enzyme *NcoI* along with empty vector *pGWB442*, used as control. Electrophoresis was carried out using 1% agarose gel, visualized under ultraviolet (U.V) light and photographed under gel documentation system (Fisher Biotech, USA).

Agrobacterium Mediated Plant Transformation

Explant Preparation: The explant for plant transformation were prepared by sterilizing tobacco seeds in bleach solution (50%) with tween 20 (0.1%) for 10 min with shaking at 230 rpm. The seeds were rinsed 3 times with sterile water and cultured on hormone free MS media. After two weeks the explants were ready for infection.

***Agrobacterium* transformation and Inoculum preparation:** *Agrobacterium GV3101* cells were transformed with *pGWB442-HsfA1d* using heat shock method, followed by plating on LB media supplemented with spectinomycin (50 mg/l) and incubated overnight at 28 °C. One colony of agrobacterium *GV3101* transformed with *pGWB442-HsfA1d,* was inoculated in 5 ml LB media supplemented with spectinomycin (50 mg/l). The culture was grown overnight at 28 °C using 200 rpm. Around 5 ml from overnight culture was added to the flask containing 45 ml LB and incubated at 28 °C for 3 hours at 200 rpm. The culture was centrifuged for 10 min at 3000g. The supernatant was discarded and the pellet was washed twice using 20 ml MMA wash buffer. Finally the pellet was re-suspended in MS media with OD adjusted to 0.6 (OD600 0.6). Acetosyringone (100 mM) was added just before using it for infection.

Infection and co-cultivation: The tobacco leaf discs were infected with inoculum of *GV 3101* harboring pGWB442-*HsfA1d* and incubated for 10 min at 22 °C at 90 rpm. After infection, leaf discs were co cultivated on hormone free MS media supplemented with

100 mM acetosyringone for 3 days under dark.

Selection and regeneration: After co-cultivation, the explants were transferred to MS media supplemented with BAP (2 mg/l), NAA (0.2 mg/l), cefotaxime (200 mg/l) and kanamycin (100 mg/l). The plates were incubated at 25±2 °C under 16 light/8 dark photoperiod. After 2 weeks the explants were sub-cultured. Well-developed calli after 4 weeks were transferred to MS media with NAA (0.2 mg/l) and increased amount of BAP (3 mg/l) for regeneration. Shoots were excised and transferred to hormone free MS media supplemented with kanamycin (100 mg/l). The regenerants were transferred and acclimatized in soil. The putative transgenic plants produced flowers and seeds were collected at maturity.

Confirmation of transgenic plants: Confocal microscopy was used for confirmation of transgenic plants at cellular level. Leaf segments from putative transgenic and wild type plants were observed under confocal microscope at 20X. Transgenic plants were confirmed at molecular level through PCR. Genomic DNA were extracted from putative transgenic and wild type plants using Qiagen DNeasy plant mini kit. Reaction mixture was prepared by adding, 1µl 10X buffer (10mM), 0.2 µl dNTP (40 mM), 0.3µl each of forward and reverse primer (10 µM), sterilized distilled water (7 µl), genomic DNA (1µl) and 0.05 µl takara taq (5 units/µl). The PCR conditions were, Initial denaturation at 98 °C for 2 min, followed by 30 cycles of polymerization with each one having denaturation (98 °C for 10 sec), annealing (55 °C for 30 sec) and extension (72 °C for 2 min). Sequence of the primers used for the detection of transgene (*HsfA1d*) were:

HsfA1d forward primer: 5-GCCGCCTTCACCATGGATGTGAGCAAAGTAACCAC-3
HsfA1d reverse primer: 5-CTGGGTCACCCTCGATCAAGGATTTTGCCTTGAGGGATC-3.

Heat Treatment and Physiological Analysis of Transgenic Plants

Transgenic plants along with wild type were exposed to heat stress (42°C) in growth chamber for 6 days and evaluated for the following physiological parameters:

Cell membrane stability (CMS): Electrolyte leakage (EL) was measured to evaluate cell membrane stability (Blum and Ebercon, 1981). Leaf discs (0.2 g) were taken and allowed to incubate in 20 ml deionized water for 12 hours on shaker. The initial conductance C_i was recorded by measuring the conductance of solution using a corning conductivity meter 441. Leaf tissues were then autoclaved at 121°C for 15 min. The conductance of solution was measured again as C_{max} after 12 hours of incubation. Leaf EL was calculated using equation:

$$EL\ (\%) = C_i/C_{max} * 100$$

Chlorophyll content: Fresh leaf tissue (0.2 g) was taken and placed in dimethyl sulfoxide for 2 days under dark. The absorbance of solution was determined at 645 and 633 nm using spectrophotometer. Chlorophyll content was determined according to Arnon (1949), using equation:

$$Chlorophyll\ (mg/l) = 20.2\ D_{645} + 8.02\ D_{663}$$

Relative water content: Leaf relative water content was calculated using fresh weight (FW), turgid weight (TW) and dry weight (DW). Fresh weight was measured immediately after leaves were cut off the plants. Then leaves were soaked in distilled water for 12 hours at 4°C until the leaves became fully turgid and then blotted dry to determine TW. Leaf DW was measured after leaves were dried in an oven at 87 °C for 72 hours. Relative water content was calculated using the equation:

$$RWC\ (\%) = (FW\text{-}DW) / (TW\text{-}DW) \times 100$$

Proline content: Proline content was measured by the method of Bates et al. (1973). Fresh leaf samples (0.2 g) were taken in mortar containing 10 ml of 3% sulfosalyclic acid. Leaf samples were grinded with pestle and filtered into test tube. Two ml filtrate was taken in another test tube followed by the addition of 2 ml each of ninhydrine reagent and glacial acetic acid. Samples were boiled in water bath at 100 °C for 1 hour. The reaction was stopped on ice and 4 ml toluene were added to produce 2 layers. Absorbance of the upper layer was measured at 520 nm. Proline content was measured using the equation:

Proline (µmol/g F.W) = [(µg proline/mL x mL toluene)/115.5 µg µmol^{-1}]/[g sample/5]

Results

Cloning of *HsfA1d*

Gateway cloning system provides very efficient

system for cloning of genes (Xu and Li. 2008). The *Arabidopsis* heat shock transcription factor *HsfA1d* was transferred from entry clone (*pUC57GW-HsfA1d)* into binary destination vector (*pGWB442)*, followed by transformation to *stellar* competent cells (Clontech, USA). Restriction digestion of cloned *pGWB442-HsfA1d* produced four expected bands of 8081 bp, 2433 bp, 1316 bp and 391 bp while destination vector *pGWB442* produced the expected 3 fragments of 8081 bp, 2288 bp and 2035 bp (Figure 1). The *pGWB442-HsfA1d*, where *HsfA1d* is cloned in-frame with yellow florescent protein is shown in Figure 2.

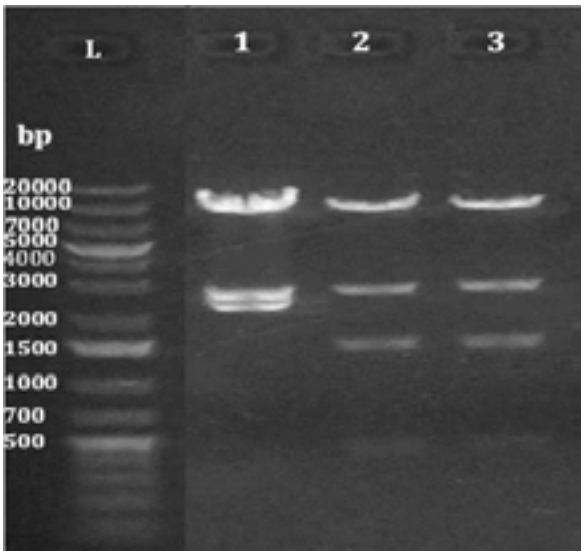

Figure 1: *Restriction digestion of cloned plant expression vector pGWB442-HsfA1d along with their empty control pGWB442, using enzyme NcoI.* **L,** *1kb plus DNA ladder;* **1,** *pGWB442;* **2,** *pGWB442-HsfA1d;* **3,** *pGWB442-HsfA1d*

Plant Transformation

The *Agrobacterium* is a very good tool in hands of molecular biologist to genetically manipulate plant (Gelvin, 2003). *Agrobacterium GV3101* cells were successfully transformed with *pGWB442-HsfA1d* vector. The tobacco explants treated with *agrobacterium* harboring *pGWB442-HsfA1d* proliferated on media

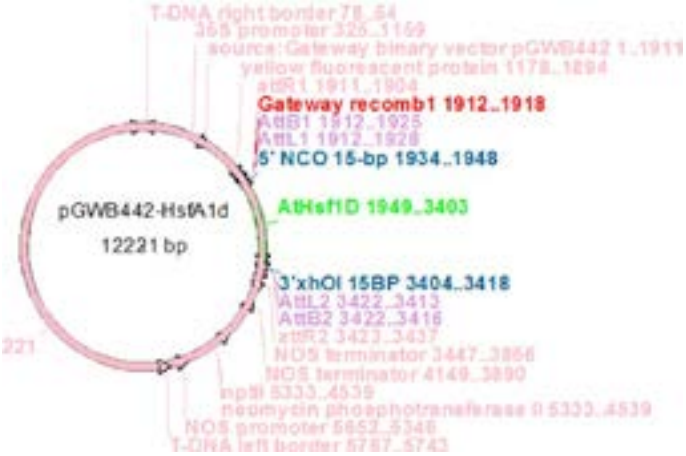

Figure 2: *Diagrammatic representation of pGWB442-HsfA1d vector.*
CaMV35S= *promotor;* **nptII**= *Plant selectable marker;* **neomycin phosphotransferase**= *Bacterial selectable marker:* **YFP**= *yellow florescent proteins,* **HsfA1d**= *Gene of interest*

Figure 3: *Different phases of invitro tobacco plant regeneration and establishment in soil.* **A,** *Swelling of leaf discs;* **B,** *Callus formation;* **C,** *Shoot development;* **D,** *Root formation;* **E,** *Establishment in soil;* **F,** *Flower production*

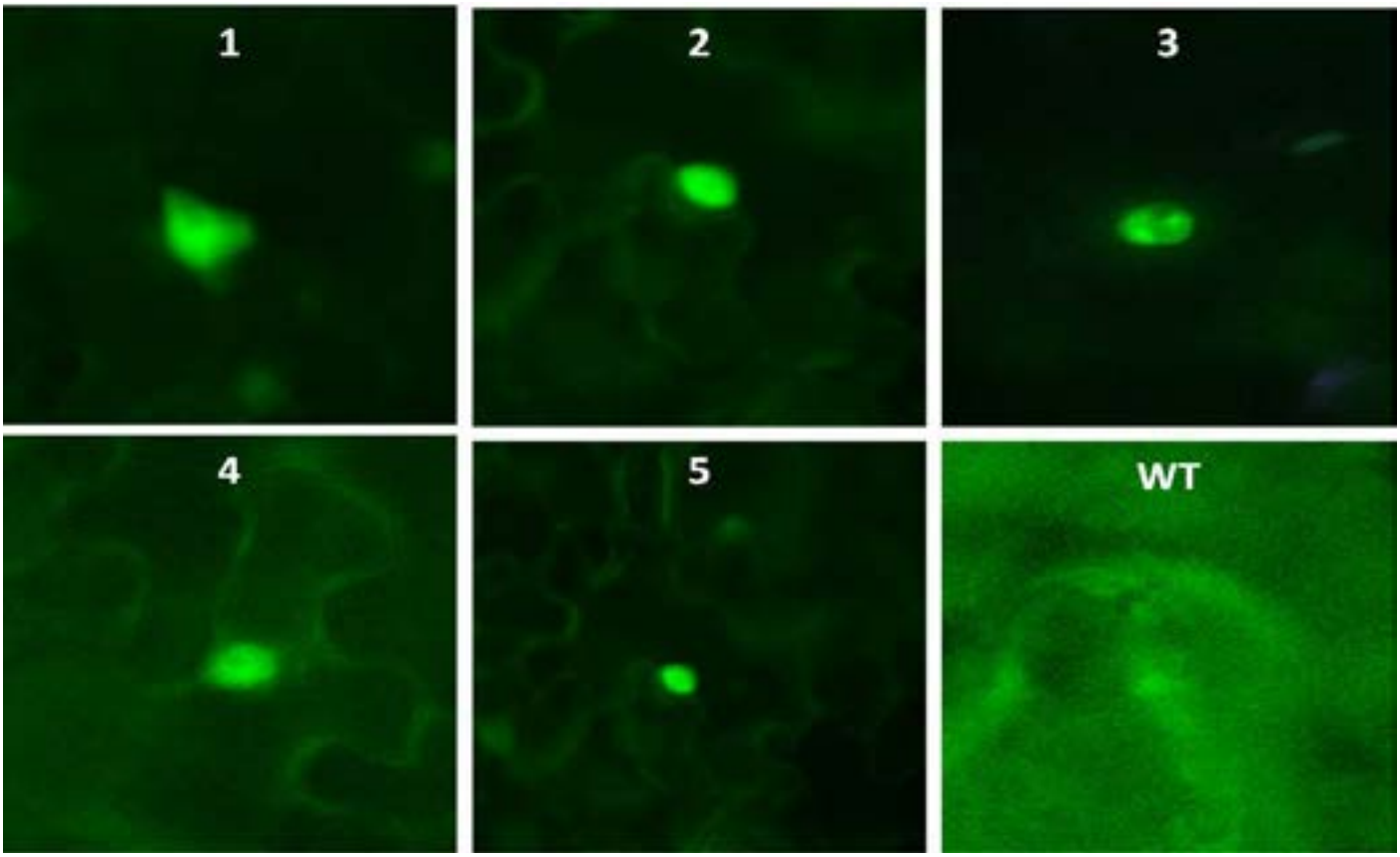

Figure 4: *Expression of yellow florescent proteins in transgenic plants.* **1 to 5,** *Five independent transgenic plant expressing YFP, cloned in frame with HsfA1d;* **WT,** *Wild type*

supplemented with kanamycin (Figure 3A). Well-developed calli were produced on MS media supplemented with BAP (2 mg/l) and NAA (0.2 mg/l) growth regulators after 4 weeks (Figure 3B). The calli started regeneration in 3 weeks on MS media supplemented with BAP (3 mg/l) and NAA (0.2 mg/l; Figure 3C) followed by rooting on hormone free MS media in 2 weeks (Figure 3D). The regenerated putative transgenic plants were acclimatized and established in soil (Figure 3E). The plants produced flowers and seeds were collected at maturity (Figure 3F). The yellow florescent protein cloned in-frame with *HsfA1d* showed florescence when the leaf segments excised from transgenic plants were observed under confocal microscope (Figure 4). PCR with *HsfA1d* specific primers, using transgenic DNA at template, resulted in expected 1458 bp amplified band. No such amplification was observed when wild type DNA was used as template (Figure 5).

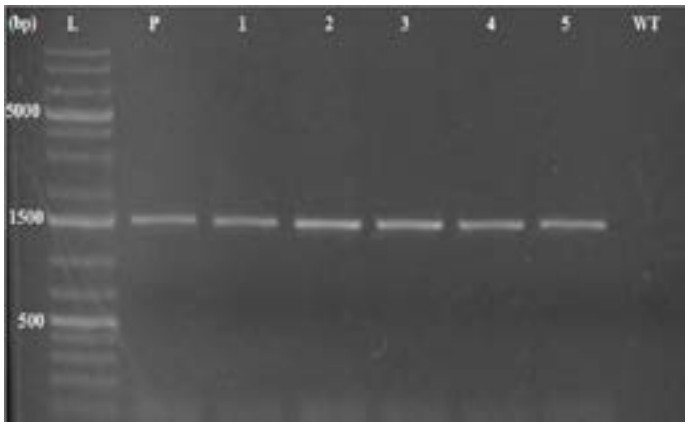

Figure 5: *PCR based amplification of genomic DNA extracted from transgenic plants along with control using HsfA1d specific primers.* **L,** *1kb plus DNA ladder;* **P,** *positive control;* **Lane 1 to 5,** *PCR amplification of genomic DNA extracted from 5 independent transgenic lines with HsfA1d primers;* **WT,** *Wild type*

Physiological analysis of transgenic plants

Heat stress (42°C) caused significantly higher electrolyte leakage from wild type (33.26%) as compared to transgenic plants. No significant difference in electrolyte leakage of transgenic and wild type was recorded at room temperature (Figure 6).

Similarly under heat stress, chlorophyll content was significantly decreased (3 folds) in wild type compared to transgenic plants (Figure 7). Results showed that no significant difference (3.15%), in relative water content of wild type and transgenic plants were recorded at room temperature. However, the transgenic plants were found to retain significantly higher amount (36.8 %) of water as compared to wild type under heat stress conditions (Figure 8). Results exhibited 2 fold increase in proline content of transgenic compared

to wild type plants under heat stress. However, only 1.25 fold increase in proline content of transgenic and wild type plants were recorded at room temperature (Figure 9).

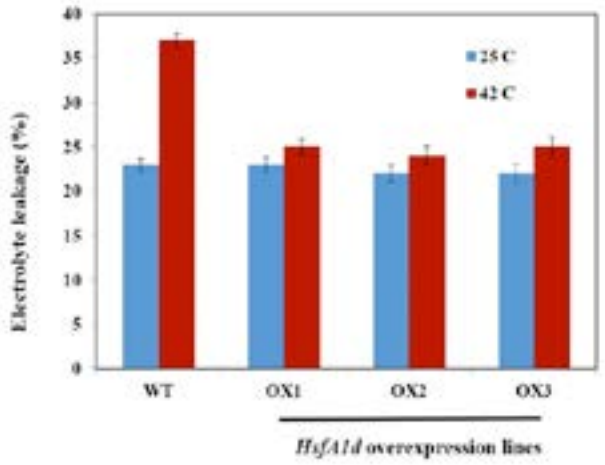

Figure 6: *Effect of heat treatment on electrolyte leakage of HsfA1d overexpression lines,* **WT**= *Wild type*

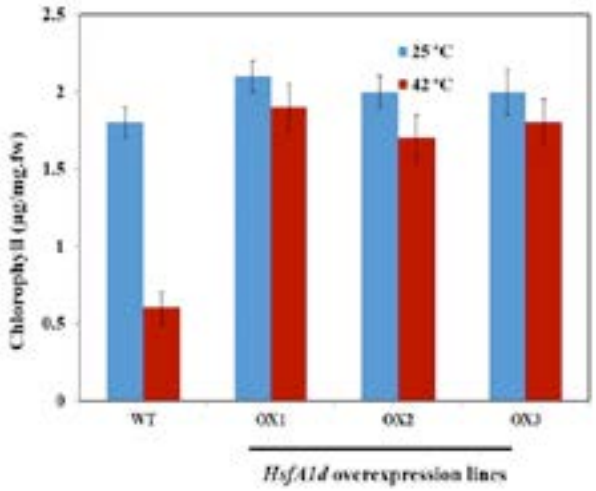

Figure 7: *Effect of heat treatment on Chlorophyll content of HsfA1d overexpression lines (OX1, OX2 and OX3), WT= Wild type*

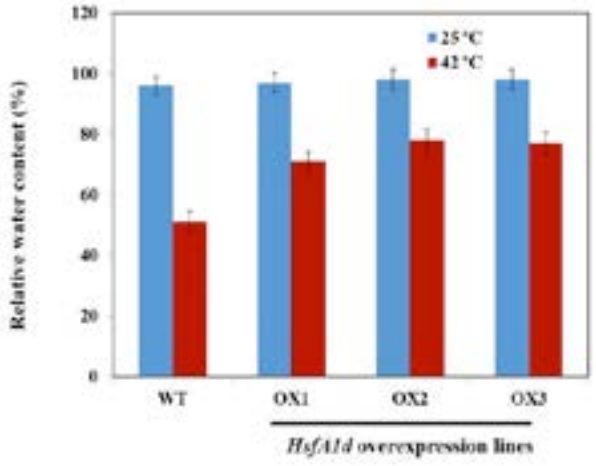

Figure 8: *Effect of heat treatment on Relative water content of HsfA1d overexpression lines (OX1, OX2 and OX3), WT= Wild type*

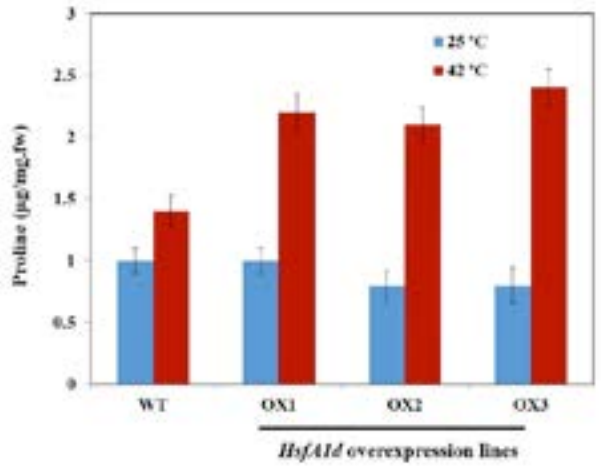

Figure 9: *Effect of heat treatment on Proline content of overexpression lines (OX1, OX2 and OX3), WT= Wild type*

Discussion

Under natural field conditions, plants are exposed to a plethora of biotic and abiotic stresses. Abiotic stresses are the primary cause of crop loss worldwide, reducing crop productivity by an estimated 50% annually (Rodziewicz et al., 2014). Among abiotic factors; heat stress has the most detrimental impacts on plant growth.

In the present study *HsfA1d* was isolated from *Arabidopsis thaliana* and overexpressed in tobacco using agrobacterium mediated transformation approach. Successful transformation and regeneration potential, exhibited by *Nicotiana benthamiana* in the current study, strengthened the earlier reports (Valenzuela et al., 2005). The young age of explant (2 weeks old) and bacterial optical density (0.6) provided conducive environment for agrobacterium to infect the explant (Guo et al., 2014). The addition of acetosyringone (100 mM) provided a stimulus for excision of *agrobacterium* T-DNA and its integration into the plant genome (Kavitah et al., 2010). The successful elimination of *agrobacterium* achieved in the current study, through the application of cefotaxime (200 mg/l), are in line with Ishida et al. (1996). The application of cefotaxime in such concentration was found to be lethal for agrobacterium causing no damage to explant. Efficient selection of transformed cell achieved in the study conducted, through the addition of kanamycin (100 mg/l), strengthened the earlier reports (Opabode, 2006). The kanamycin resistant gene, present in transformed cells, produced protein which detoxified the effect of kanamycin.

Callus induced in the present study, by adding BAP (2 mg/l) and NAA (0.2 mg/l), are in line with Dhaliwal et al. (2004) that high concentration of cytokinine (BAP) and low concentration of auxin (NAA) cause callus induction. The application of such growth regulators in specific concentration resulted in dedifferentiation of cells and thus callus was produced. The results recorded in the current study for shoot induction were in agreement with Dhaliwal et al. (2004). The enhanced rate of cell division, achieved through the application of BAP (3 mg/l) and NAA (0.2 mg/l), facilitated shooting. Root induction with hormone free MS media in the present study, were in line with Ali et al. (2007). Confirmation of transgenic plants at cellular level, achieved through the use of confocal microscopy. The expected 1458 bp band amplified by PCR strengthened the reports of Kalenahalli et al. (2013), that PCR can be used effectively for confirmation of transgenic plant at molecular level.

The significant increase in electrolyte leakage (33.26 %) of wild type compared to transgenic plants, under heat stress, are in line with Xu et al. (2010). Heat stress results in the expansion of pores present in cell membrane of wild type plant and thus more electrolyte leakage occurred. On the other hand, the cell membrane of transgenic plants were protected against the heat stress induced damage resulted in less electrolyte leakage. The significant decrease in chlorophyll content (3 fold) exhibited by wild type plants compared to transgenic plants, under heat stress, were in agreement with reports of Xu et al. (2014). The biosynthesis of chlorophyll is prone to the devastating effect of heat (Tewari and Tripathy, 1998). In case of transgenic plants, the enzymes involved in biosynthesis of chlorophyll are protected against heat stress by Hsfs and thus no significant decrease in chlorophyll content was recorded. The heat stress increases the rate of transpiration, reducing the relative water content of the plant (Kumar et al., 2013). The high relative water content (36.8 %) shown by transgenic plants compared to wild type strengthened the earlier reports of Xu et al. (2014). The HSPs regulated by Hsfs minimize the loss of water by protecting the cell membrane and cell wall against the heat induced damage, helping the transgenic plants to retain more water under heat stress. In the current study, the significant increase in proline content shown by transgenic plant (2 fold) compared to wild type under heat stress, are in line with Xue et al. (2010). The *HsfA1d* induces the production of HSP, which acts as molecular chaperones and protect the enzymes involved in biosynthesis of proline (Cvikrová et al., 2012). Thus high proline is accumulated under heat stress in case of transgenic plants. These results strengthened the earlier reports that plants release proline as osmoprotectant under stress condition and thus can be used as excellent stress marker.

Conclusion

The synthesis of entry clone through infusion reaction and preparation of binary destination vector through LR reaction provide efficient system for vector construction. The growth regulators (BAP and NAA) can be efficiently used to get the plant regenerated. The plants transformed with *HsfA1d* were found to retain more water and accumulate more proline under heat stress. The transgenic plants were concluded to have efficient protective system, causing less electrolyte leakage and less chlorophyll damage under heat stress. Based on different physiological parameters studied, the incorporation of *HsfA1d* is recommended in heat sensitive crops for engineering of thermotolerance

Acknowledgments

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Conflict of Interest

There exist no issue of conflict of interest.

Authors' Contribution

The project was designed by Zamarud Shah in consultation with Safadr Hussain Shah. The research work was conducted by Zamarud Shah under the supervision of Gul Shad Ali. The research article was drafted by Zamarud Shah while Dr. Asad jan helped in reviewing the article.

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