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Research article

Differential regulation of the banana stress *NAC* family by individual and combined stresses of drought and heat in susceptible and resistant genotypes

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ABSTRACT

Banana, an important tropical fruit crop, often faces drought, heat and its combination during its growth, leading to decreased yields. The combined stresses caused 100% yield loss in Grand Nain (GN) as compared to only 46% in Hill Banana (HB). To understand the response of combined stresses, we studied the stress-responsive *NAC* gene sub-family under individual and combined drought/heat stresses under controlled and field conditions in the stress-sensitive GN (AAA genotype) and stress-tolerant HB (AAB genotype). Under drought, expression of most stress-*NACs* increased with progression of drought in either one or the other genotype with little overlap. Heat stress caused a continuous decline in expression of most genes in HB unlike in GN where many *NACs* were up-regulated although to a lesser scale than for drought. Combination of the two stresses elicited a very different response compared with individual stresses. GN responded strongly to the combined stress with up-regulation of most genes unlike that seen in drought. Surprisingly, *NAC* genes in HB did not respond much to the more severe combination of the stresses despite being up-regulated strongly by drought. The response of the *NACs* to combined field stress was similar to that under controlled conditions. Most of the stress-*NACs* were strongly up-regulated upon treatment with exogenous ABA within 30–60 min, the increase being more prominent in GN. The studies suggest that the B genome in the stress-tolerant HB may counter more drastic combined stresses without taking recourse to the expression of stress NACs.

1. Introduction

Plants have to constantly respond to changing environmental conditions and abiotic stresses that include drought, heat, salinity, cold, flood, wind, etc. Some of these, like drought and heat, can adversely affect normal growth and reproductive development with a strong negative impact on yield in agricultural crops (Barnabas et al., 2008; Li et al., 2009; Prasad et al., 2011). Each stress evokes unique responses in every plant that requires alterations in morphological, physiological and biochemical traits for adaptation. Under field conditions, these stresses may often act in combinations (such as drought and heat, drought and cold, drought and/or heat with high light, heat and salinity etc.) creating even more adverse conditions that target the plant (Mittler, 2006). Unlike isolated stresses provided in laboratory, the onset of field stresses is gradual and associated with many other inter-related changes that not only affect whole plant physiology and biochemistry but also its susceptibility to biotic stresses that may further complicate the responses of the plant (Atkinson and Urwin, 2012; Prasch and Sonnewald, 2013; Suzuki et al., 2014). Since responses to any one of the components of combined stresses may be opposite to that required to combat the other component (Mittler, 2006), the stress combination often has far more disastrous consequences on plant growth and reproduction including agricultural crops especially cereals (Barnabas et al., 2008; Prasad et al., 2011; reviewed by Suzuki et al., 2014). This can greatly impact growth as noted in rice (*Oryza sativa)*, wheat (*Triticum aestivum)* and maize (*Zea mays)* (Perdomo et al., 2015), barley (*Hordeum vulgare)* (Jedmowski et al., 2015) etc. Detailed studies over the last decade and half have shown that the physiological and molecular responses to the combined stresses are unique and therefore difficult to predict from studies on individual stresses (Rizhsky et al.,

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2002, 2004; Mittler, 2006; Rasmussen et al., 2013; Zandalinas et al., 2016; Zhang and Sonnewald, 2017).

A large number of regulatory genes comprising of transcription factors of the AP2/ERF, DREB, NAC, b-ZIP, WRKY, MYC, MYB, bHLH families, respond to individual and combined stresses and initiate a cascade of events that lead to the final response (Agarwal and Jha, 2010). One of these, the NAC (**N**AM, NO APICAL MERISTEM; **A**TAF, ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR; and **C**UC, CUP-SHAPED COTYLEDON) super family of proteins is a large plant-specific transcription factor (TF) family with roles in the development of organs and tissues and various cellular processes (Sperotto et al., 2009; Nuruzzaman et al., 2013). In addition, NAC TFs also govern responses to biotic and abiotic stresses (Nuruzzaman et al., 2013). Several studies document their role in abiotic stress responses in plants like rice (Hu et al., 2006), maize (Shiriga et al., 2014), *Brassica juncea* (Bhardwaj et al., 2015), *G. herbaceum* (Gunapati et al., 2016) and banana (Tak et al., 2017; Negi et al., 2018). Expression of many of the NAC genes such as *ANAC019*, *ANAC055* and *ANAC072* in *Arabidopsis* (Tran et al., 2004), *SNAC1* in transgenic rice (Hu et al., 2006) *GhNAC2* in cotton (Gunapati et al., 2016) etc. bring about drought tolerance and reduction in losses in homologous and heterologous systems, providing evidence of their importance in abiotic stress amelioration (reviewed in Shao et al., 2015). Most of these studies were performed under controlled conditions where plants were subjected to a single stress rather than field conditions where plants face multiple stresses.

Banana is the second most popular fruit consumed worldwide with India as the largest producer (Faostat, 2016; http://www.fao.org/faostat/en/#home). Banana plants are more susceptible to water stress due to the presence of a greater leaf area index and a shallow root system (Turner et al., 2007). Water stress at the flowering stage can be particularly detrimental with bunch weight reducing by 18–42% in different varieties of banana (Ravi et al., 2013). Of the commonly grown triploid banana varieties, the dessert banana (having the AAA genome) is more susceptible to single or combined abiotic stresses compared to those having at least one copy of the B genome (AAB or ABB) as a genetic constituent (Ekanayake et al., 1994; Ravi et al., 2013; Jangale et al., 2019). A transcriptomic study of two banana varieties Saba (ABB) and Grand Nain (AAA) showed induction of bHLH, bZIP, C2H2, ZFHD gene families in the tolerant Saba variety and induced expression of ARFs, ERFs, MYBs, WRKYs and NACs in the sensitive Grand Nain variety (Muthusamy et al., 2016). The NAC family has been studied in banana by Cenci et al. (2014) who proposed forty orthologous groups of NAC genes with groups 3, 5, 6, 1 g and 1 h as those likely to be involved in biotic and abiotic stress responses of banana. Hu et al. (2017) also studied NAC members for expression under different abiotic stress conditions such as cold stress, osmotic stress and salt stresses in two different varieties. However, none of the above studies address the regulatory aspects of banana under actual field conditions where plants are subjected to combined stresses.

We have undertaken studies on combined drought and heat stress responses of field grown banana in the relatively stress tolerant Hill Banana (HB, AAB genotype) and the sensitive Grand Nain (GN, AAA genotype) varieties of banana. Our studies demonstrate major differences in the responses of the two varieties in the regulation of the stress-related members of the NAC family during a combined heat and drought stress under field conditions as compared to individual stresses.

2. Materials and methods

2.1. Plant material

Commercially produced tissue-cultured banana plants of *Musa acuminata* L. AAA Cavendish Group cv. Grand Nain (GN) and *Musa acuminata* L. AAB Group cv. Hill Banana (HB) also known as Virupakshi were grown under controlled conditions and in field conditions with appropriate agronomic practices including irrigation as described (Jangale et al., 2019).

2.2. Database search and sequence retrieval

The *Arabidopsis thaliana NAC* gene sequences were downloaded from database of The Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/). The amino acid sequences of NAC proteins from *Arabidopsis* were used as a query and a local blast search was performed in Banana genome hub (Droc et al., 2013). The banana *NAC* gene sequences were also searched in the Plant Transcription Factor Database (Jin et al., 2017). Common sequences from the two searches were analysed individually for confirmation and presence of NAC domain using the InterProScan program (Jones et al., 2014) and Conserved Domain Database (CCD) search program (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi). We also compared 162 *MaNACs* reported by Cenci et al. (2014) with the 166 *MaNAC* genes we identified and obtained an additional 5 NAC proteins while one (GSMUA_Achr10G27600) was excluded from our study as it failed to show the presence of NAC domain. Three *VOZ* (**V**ascular Plant **O**ne **Z**inc-finger) gene sequences showing similarity to NACs were identified in the banana genome hub and also incorporated in this study.

2.3. Phylogenetic analysis and identification of stress responsive NAC genes

A multiple sequence alignment of all full length banana NAC and VOZ proteins was performed by using ClustalW (http://www.genome.jp/tools/clustalw/) with stress-related NAC proteins from *Arabidopsis*, rice, maize, wheat, barley, *Sorghum bicolor* and VOZ proteins from *Arabidopsis*, rice and maize. The phylogenetic tree of aligned banana NACs with stress-related NACs was constructed using Neighbor-Joining (NJ) method with 1000 bootstrap replicates using MEGA 6 software (Tamura et al., 2013). Within the tree, *MaNAC* genes showing closest similarity to stress-related NACs in *Arabidopsis*, rice, maize, wheat, barley and sorghum were chosen for expression study in response to drought, heat and combination of drought plus heat. All banana *VOZ* genes were taken for expression study as they aligned with stress-related VOZ proteins from *Arabidopsis*.

2.4. Protein motif analysis of banana NAC genes

The conserved sub-domains in banana NAC proteins and an additional 20 motifs in stress-related NACs from *Arabidopsis*, rice, maize, wheat, barley and sorghum having different functions were identified using the MEME program (http://meme-suite.org/tools/meme; Bailey et al., 2009).

2.5. Stress treatments to plants under controlled conditions and in field conditions

Plant growth conditions and stress treatments were as described by Jangale et al. (2019). For stress treatments under controlled growth conditions, two-month-old fully hardened plantlets with five leaves (grown in 300 cc containers with peat-based growing substrate) were first grown at 25 °C with 16 h light in a growth facility. They were then transferred to controlled environmental conditions (06:00 h to 10:00 h at 30 °C, 10:00 h to 18:00 h at 35 °C and 18:00 h to 6:00 h at 25 °C, relative humidity 80 ± 15%, light 350 μmol m^{-2} s^{-1} for 16 h from 06:00 h to 22:00 h) for seven days prior to experiments. Drought treatment was given by withholding water for ten days. Heat stress was given to plants by raising the temperature of growth chamber to 45 °C for six hours (from 12:00 h to 18:00 h) for eight days, but with regular watering. Combined stress treatment was given for eight days by subjecting plants to drought (water withdrawal) along with heat stress (45 °C) for six hours. Plants were grown in three replicates, each replicate containing 30 plants for drought studies, 40 plants for heat and 40 plants for combined stress studies (Jangale et al., 2019). Each sample consisted of a composite of five plants. Leaf samples undergoing stress treatments were collected daily between 14:00 to 14:30 h and immediately frozen in liquid nitrogen. For the drought experiment, leaves were sampled on alternate days.

For combined stress experiments under field conditions, plants were grown as described (Jangale et al., 2019) in two sets of three replicates, each replicate containing five plants. Before conducting the field experiments, the soil was analysed in consultation with agronomy experts to ensure that the field was not influenced by salt or alkaline stresses. All soil test parameters were in the normal range with a soil pH of 7.48 and electrical conductivity of 0.33 mS/cm as per ISO 10390:1994 (https://www.iso.org/standard/18454.html) and ISO 11265:1994 (https://www.iso.org/standard/19243.html) respectively. After five months growth, plants in both sets were subjected to drought stress by water withdrawal for 12 days (May 2015) when these plants were already experiencing harsh summer conditions (noon temperatures ranging between 39 °C and 43 °C, humidity ranging between at 14%–26%; Table S1), henceforth called 'combined stress'. Leaf samples (3rd fully opened leaf from top) were collected from the day of water withdrawal on alternate days and frozen in liquid nitrogen. Sampling was carried out between 14:00 to 14:30 h since the time coincided with peak temperatures along with water stress (Jangale et al., 2019). In the other set, both control and treated plants were watered regularly after 12 days of drought stress and percent survival rate was measured after one month by counting the number of plants that survived. The yield from the plants that survived was estimated at the time of harvesting and percent yield loss calculated by using formula given below:

Percent yield loss = (Avg. bunch weight of control plants)-(Avg. bunch weight of treated plants)/ (Avg. bunch weight of control plants)*100

2.6. RNA isolation, cDNA preparation and qRT-PCR analysis

RNA was isolated and cDNA prepared essentially as described by Jangale et al. (2019). QRT-PCR analysis was carried out using SsoFast EvaGreen Supermix (Bio-Rad, USA) on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). Gene specific primers for qRT-PCR were designed by using Primer3 web version 4.0.0 (http://primer3.ut.ee/) and are shown in Table S2. The primer efficiency was checked before running qRT-PCR. Three serial dilutions (1:4) of pooled cDNA were used as template for qRT-PCR to check primer efficiency. The standard curve of quantity of template (log values) against the Ct values resulted slope and "r" value > 0.99 for all primers. Standard curve for *MaNAC51* is given (Fig. S1) and percent efficiency for all primers was calculated as per BioRad qRT-PCT guidelines (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5279.pdf) and provided in Table S3. Primer efficiency in range of 90–105% is considered to be appropriate for gene expression studies. Five candidate reference genes namely *RPS2*, *MaEF-1*, *UBQ*, *TUB* and *ACT* were selected based on previous studies (Chen et al., 2011; Podevin et al., 2012). These genes were tested with cDNA samples from D1 to D8 and data analysed using Genorm software (Vandesompele et al., 2002). Under our experimental conditions, *MaEF-1* gave the lowest M value (average expression stability) as compared to the other four (Fig. S2) and was used as a reference gene. Relative expression values were calculated using delta CT method (Livak and Schmittgen, 2001).

2.7. Analysis of cis-elements in promoter region of MaNAC genes and ABA treatment

Upstream regions (−3000 bp) of stress responsive *MaNAC* genes were downloaded from banana genome hub and analysed for the presence of stress-related regulatory elements using the PlantCARE database (Lescot et al., 2002). To study ABA response in GN and HB, leaf discs (10 mm diameter) of two month old plants were taken. Three biological replicates each having 16 leaf discs (10 mm in diameter) were soaked in 1 μM ABA solution and samples were collected at 0, 15, 30, 60, 120 and 240 min (Jangale et al., 2019) after ABA treatment for RNA/cDNA preparation as described above. Control (without ABA) was also run in biological triplicates and samples were collected at the same time points.

2.8. Statistical analysis

The relative expression levels determined in stressed samples were compared with those of the day 0 (D0, before treatment) using the Dunnett (two sided) one-way ANOVA test at $P \leq 0.05$.

3. Results

3.1. Effect of combined drought and heat stress on banana productivity

Combined drought and heat stresses are a common feature in fields in tropics where banana are grown. In order to understand the extent of severity of combined drought and heat stresses in the field in the two banana genotypes, GN and HB, plants were first subjected to this stress combination as described in materials and methods. Thereafter, plants were watered regularly and survival rate studied one month after re-watering. None of the GN plants survived the stress while 80% of HB plants survived after re-watering (Fig. 1). Yield loss in these plants was measured after harvesting the banana bunches and the gross fruit weight compared with control plants which were not subjected to combined stress. As there was no survival in GN, the yield loss was 100% in GN as compared to only 46% in HB. This showed that GN plants were highly susceptible to combined stresses while HB plants were relatively tolerant, with a reduced yield loss.

3.2. Identification and phylogenetic analysis of drought and heat related NAC TF family genes in banana

The large NAC gene family contains a subset of stress-associated NACs that play a role in abiotic stresses. To identify the stress-associated NACs in banana, the complete NAC family consisting of a total of 166 NAC genes was identified from the banana genome hub and plant transcription factor databases for study. In addition, three VOZ genes were also chosen for the analysis since the VOZ genes possess a C-terminal region that is similar to the NAC domain and these negatively

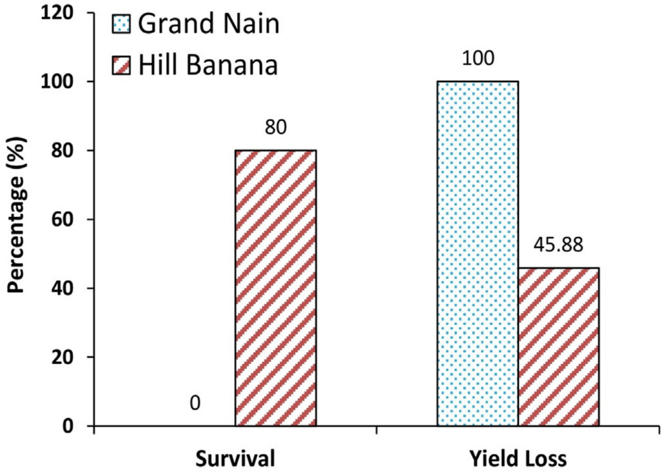

Fig. 1. Survival rate and yield loss in Grand Nain (GN) and Hill Banana (HB) after combined field stresses of drought and heat. Percent survival was calculated by counting plants showing growth one month after watering and yield loss calculated by weighing fruit yield obtained in treated and control plots.

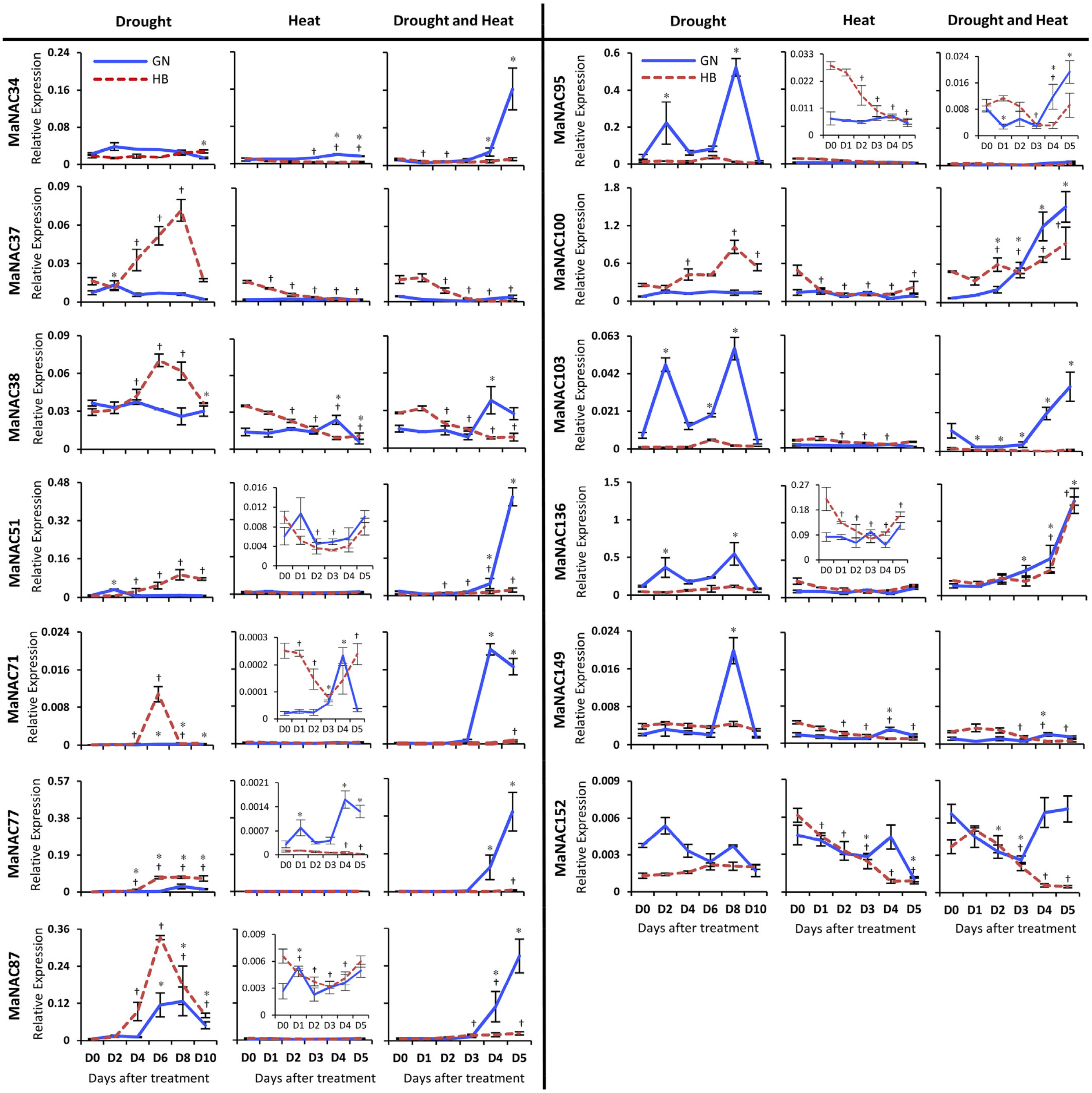

Fig. 2. qRT-PCR based differential expression analysis of *MaNAC* genes in leaves of greenhouse grown plants. Vertical axis indicates relative fold expression values calculated using delta Ct method in leaves of two-month-old GN and HB plants grown under controlled conditions. The reference gene *MaEF* was used for normalization of gene expression. Horizontal axis indicates the duration of treatment in days for drought (D0 - D10), heat (D0 to D5) and combined stresses (D0-D5). Data are mean ± SEM. * and † indicate significant expression with respect to D0 in GN and HB, respectively at significance level P < 0.05.

regulate drought stress in *Arabidopsis*. A considerable sequence similarity of the C-terminal region of VOZ with NAC domain of NAC proteins was observed in banana (Fig. S3). Thereafter, the 169 genes (166 NAC + 3 *VOZ)* were analysed for coding sequences (CDs) and protein length, isoelectric point (pI) and molecular weight (MW) as provided in Table S4.

To understand the evolutionary relationship, functional diversity and to identify putative abiotic stress-related NAC transcription factors in banana, a phylogenetic tree was constructed using ClustalW alignment between banana NAC proteins and drought-responsive NAC and VOZ proteins from seven other plant species namely rice, sorghum, maize, wheat, barley, stiff brome and *Arabidopsis*. Unlike Cenci et al. (2014), where an analysis of both biotic and abiotic stress-related NACs was performed in three plants namely rice, *Arabidopsis* and grapes, we restricted our analysis to abiotic stress-related NACs but extended it to a larger group of primarily monocots, as described above. The information about drought-responsive NAC proteins is provided in Table S5.

Phylogenetic analysis using MEGA 6.0 grouped the banana NAC proteins into 16 groups (A to P) (Fig. S4). The largest clade "J" had 21 NAC proteins while the smallest clade "E" had 2. The remaining clades from A to P (excluding E and J) contained 9, 8, 12, 19, 3, 4, 5, 10, 19, 20, 4, 9, 15 and 3 NAC members respectively. Most abiotic stress-responsive NAC proteins from *Arabidopsis*, rice, wheat, barley, maize, sorghum and stiff brome clustered together in a separate clade "C" of abiotic stress-responsive NACs with 16 out of 21 members. This clade also contained 12 banana NAC proteins namely MaNAC29, MaNAC34, MaNAC37, MaNAC51, MaNAC69, MaNAC71, MaNAC77, MaNAC87, MaNAC100, MaNAC103, MaNAC136 and MaNAC152. VOZ proteins of *Arabidopsis*, rice and maize in phylogenetic tree also grouped together to form a separate clade "P" of VOZ proteins, containing three banana VOZ proteins namely MaNAC38, MaNAC95 and MaNAC149. Five proteins (MaNAC43, MaNAC73, MaNAC107, MaNAC133 and MaNAC137) formed monophyletic clades, not included in any of the 16 groups. As the abiotic stress-responsive NACs of most other plants clustered in clade "C" and clade "P" we chose NAC members in these two groups for further individual and combined stress expression analysis.

3.3. Protein motif analysis of NAC genes

The NAC domain at N-terminus of NAC proteins consists of five subdomains namely A, B, C, D and E. The banana NAC proteins were analysed for the presence of these five sub-domains using MEME software. It was found that out of 169 banana NAC proteins, 131 (75.73%) contained all five sub-domains; 15 consisted of A, C, D and E; 12 consisted of A, D and E; 5 consisted of A, B, D and E; 2 consisted of B, D and E; 2 consisted of D and E; 1 consisted of A, B, C and E and 1 consisted of A, C and E sub-domains (Fig. S5). Of the five, sub-domain E was most highly conserved while A and D were relatively highly conserved in all banana NACs. NAC proteins that failed to contain all five sub-domains can be considered as NAC-like proteins as previously described in rice (Fang et al., 2008) and *Brachypodium distachyon* (You et al., 2015). Banana VOZ proteins (*MaNAC38*, *MaNAC95* and *MaNAC149*) did not display any sub-domain as they only share sequence similarity with the NAC domains observed in Fig. S3.

Besides the five sub-domains, we searched 20 other motifs in stress-related NACs of banana and few NAC proteins from *Arabidopsis*, rice, maize, wheat, barley and sorghum. Fig. S6 shows the variations in these motifs in different NAC proteins in different species. Details of motifs identified are given in Table S6. We found that motif no.13 appeared primarily in drought responsive *NAC* genes of rice, maize, wheat, barley, sorghum and banana suggesting that its presence may be associated with stress tolerance in these *NAC* proteins.

3.4. Differential expression of NAC genes in drought tolerant and susceptible genotypes under individual and combined stresses under controlled conditions

In order to obtain an insight into the regulation of the stress-NACs to abiotic stresses that are commonly experienced by banana in the tropics, an experiment was laid out with two-month-old banana plants of GN and HB under controlled (glasshouse) growth conditions. The plants were exposed separately to drought and to heat or in a combination of both to understand the responses of *NAC* genes to these stresses in both the genotypes. Clear differences in the expression patterns were observed between drought and heat stress responses in both the genotypes (Fig. 2). Of the 15 NACs that grouped along with the stress-responsive NACs in the phylogenetic study, expression of two, namely *MaNAC29* and *MaNAC69,* was not detected by qRT-PCR in any of the stress treatments. Under drought stress alone, of the 13 stress NACs, *MaNAC51, 77, 87, 95, 100, 103, 136,* and *149* showed significant changes in transcript levels, being up-regulated. For most of these genes, the up-regulation was mainly seen on day 8 and ranged from 5- to 300-fold. For genes *MaNAC95, 103, 136* and *152,* two distinct peaks were observed on days 2 and 8 while for *MaNAC77, 87* and *149* a single prominent peak was observed on day 8. Interestingly, HB showed a far greater response to individual drought stress under controlled conditions than GN for most stress-NAC genes. This was particularly apparent in *MaNAC34, 51, 71, 77, 87*and *100* which showed a transient but prominent up-regulation in 6–8 days. For all these genes, the drought-related increase in transcript levels was at least 3 to 10-fold higher in HB than in GN indicating that HB was apparently more sensitive to drought compared to GN. *MaNAC100* and *MaNAC136* were found to be most drought-responsive with the highest transcript abundance in GN and HB, respectively, in response to drought. Interestingly, for most genes except *MaNAC77* and *87,* the response to drought was only seen in one of the genotypes, either GN or in HB, but not in both. This indicated that despite a common stress, downstream responses elicited by the stress were regulated differently by expression of a different set of NAC genes in HB and GN.

We next performed an analysis of the expression of the *NAC* genes in response to heat. Heat treatment caused very different effects in both genotypes. GN seemed to respond strongly to heat with a significant up-regulation of its genes like *MaNAC34, 38, 71, 77* and *87*. *MaNAC77* and *MaNAC87* were the most responsive with an up-regulation on day 1 itself, *MaNAC87* responding more prominently of the two (Fig. 2). The others showed an increase on day 4. In contrast to GN, HB responded to heat treatment with a rapid decline in expression of almost all the genes studied. The decrease started from day 1 itself and continued until day 5 except for *MaNAC71* and *87* where an increase at day 5 was observed. In most cases, the steady state levels decreased by 30–70% of the pre-treatment levels in HB.

The combined stresses of drought and heat triggered a major change in the expression patterns of the *NAC* genes with a response that differed from the individual stresses. In GN, the expression of genes like *MaNAC34, 38, 51, 71, 77* and *100,* which did not respond much to the individual stresses of drought and heat, showed a considerable increase by day 5. For *MaNAC87,* the increase was higher and earlier than that observed for individual stresses while for *MaNAC103, 136* and *152* there was an alteration in the pattern of increase with a suppression of the earlier peak in these genes. *MaNAC95* showed a suppression of the expression that was seen under individual stresses. For the rest, the expression did not change much. For HB, the responses were very different from those of GN. The strong up-regulation in HB upon individual drought stress was lost upon its combination with heat with most genes being down-regulated or not showing much change. The only exceptions were *MaNAC100* and *136* which showed an earlier increase upon combined stresses, somewhat similar to that seen in drought. Collectively, these results showed that under controlled conditions, individual stress-associated *NAC* family genes responded differently to individual drought and heat stresses and their combination and that the regulation was strongly dependent on the genetic background on the presence or absence of the B genome (Ravi et al., 2013; Jangale et al., 2019).

In earlier studies, it was observed that banana cultivars possessing at least one copy of the B genome as a genetic constituent were tolerant to abiotic stresses than cultivars possessing the triploid A genome (Ravi et al., 2013; Jangale et al., 2019).

3.5. Regulation of the NAC gene family under combined drought and heat stresses in field grown plants

Under natural field conditions plants are not exposed to measured isolated stresses but have to respond to a complex array of factors both abiotic and biotic, simultaneously. Within abiotic stresses, plants growing under the hot tropical summers are often exposed to water stress due to rapid water loss from the soil and high vapour pressure deficit (VPD) during dry summers. In order to obtain an idea about how these might affect responses in banana, a detailed expression study was performed in 5-month-old field grown banana plants (prior to initiation of flowering) in both varieties. These plants, growing at peak summer temperatures of 39–43° (and therefore already experiencing prolonged heat stress), were subjected to an additional 10 days water stress as described.

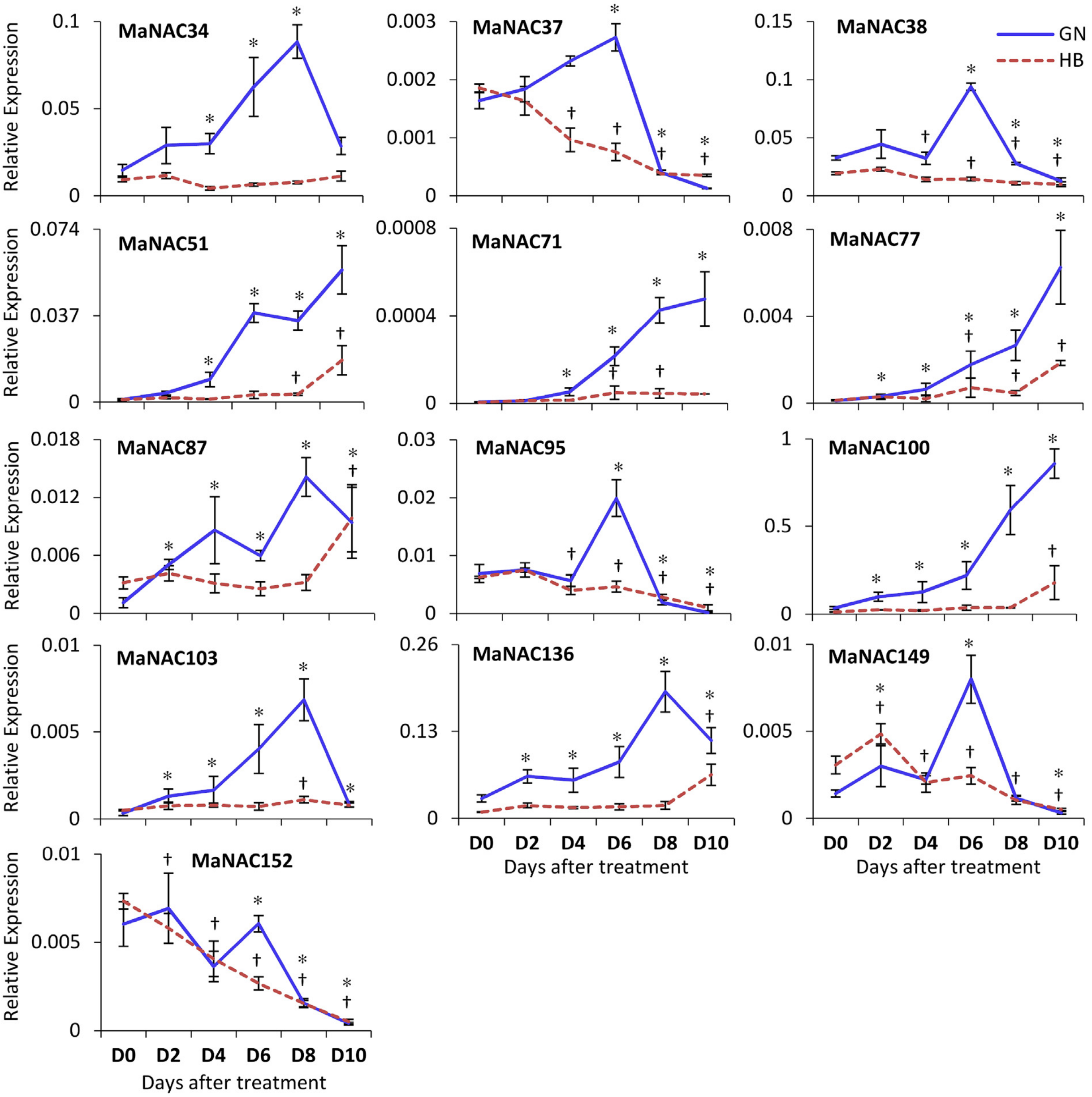

Fig. 3. QRT-PCR based differential expression of *MaNAC* genes in leaves of five-month-old field grown plants subjected to combined drought and heat stresses. Vertical axis indicates relative fold expression values calculated using delta Ct method in leaves of five-month-old GN and HB plants. Horizontal axis indicates the duration of drought treatment in days (D0 to D10) to plants already experiencing hot summer conditions. The reference gene *MaEF* was used for normalization of gene expression. Data are mean ± SEM. * and † indicate significant expression with respect to D0 in GN and HB respectively at significance level of $P < 0.05$.

In GN, the earliest response was observed in genes *MaNAC77, 87, 100, 103* and *136* wherein transcript levels began increasing significantly by day 2 itself, suggesting that these were the most sensitive of the NACs to drought. Expression in these genes continued to increase with progressive drought with peak levels being 20–50 fold higher than at the start of the treatment, except *MaNAC136* which increased 8-fold. For the genes *MaNAC87, 103* and *136,* peak expression was attained at day 8 followed by a decrease by day 10 (Fig. 3). Others genes like *MaNAC34, 37, 38, 95* and *149* responded later and showed a transient but prominent increase at day 6 followed by a decrease from day 8/10. In all these genes, the increase ranged from 2 to 6 fold. *MaNAC7*1 had an unusual pattern showing a decrease over the first six days followed by an increase on days 8/10. *MaNAC152* showed a continuous decrease with progression of drought. Of all the genes, *MaNAC100, MaNAC136* and to a lesser extent *MaNAC34* and *MaNAC38* showed the greatest transcript abundance (seen as higher delta Ct ratios) in response to combined stresses. These genes also showed a higher background and induced transcript abundance under individual stresses in GN, especially with respect to drought (Fig. 2). Interestingly, Hill banana did not seem to respond much to drought under the hot field conditions just as in case of controlled conditions. Most of the genes did not undergo any change in expression during the progression of drought under the hot summer conditions. The only *NAC* genes that responded to drought were *MaNAC51, MaNAC77, MaNAC87, MaNAC100* and *MANAC136* and even in these genes the response was only observed on day 10 and the increase was much less compared to GN. The exception was *MaNAC149* which increased at day 2 (earlier than GN which increased at day 6) but decreased thereafter. *MaNAC95* and *MaNAC152* showed a continuous decrease in response to the combined drought and heat stresses (Fig. 3).

Table 1
Analysis of *cis*-elements in promoter regions of stress-responsive *MaNAC* genes.

cis-elements	ABRE (ABA response)	DRE (dehydration, low-temp, salt stresses)	LTR (low-temperature stress)	MBS (drought stress)	TC-rich repeat (Defense and stress)	HSE (heat shock element)	WUN (wound response)	Total
MaNAC34	10	3	1	2	0	0	0	16
MaNAC37	7	1	1	1	0	0	1	11
MaNAC38	1	0	0	1	0	0	2	4
MaNAC51	6	2	0	1	2	0	3	14
MaNAC71	8	0	0	4	0	0	0	12
MaNAC77	16	2	0	4	0	0	1	23
MaNAC87	13	2	1	4	0	0	1	21
MaNAC95	14	2	0	6	0	0	1	23
MaNAC100	15	2	0	1	1	0	1	20
MaNAC103	10	2	2	6	0	0	0	20
MaNAC136	14	2	2	3	0	0	1	22
MaNAC149	4	2	1	4	1	0	1	13
MaNAC152	8	1	1	1	0	0	0	11
Total	126	21	9	38	4	0	12	210

3.6. Identification of stress-related cis-elements and regulation of the NAC genes by ABA

The responses to abiotic stresses are driven by stress-associated transcription factors through recognition of specific *cis* elements in the promoters of target genes that allow binding of the TFs to the promoter. ABA plays an important role in governing many of these responses. To obtain an idea about the possible regulation of the *NAC* genes by various factors, we analysed the promoter regions of stress-related *MaNAC* genes (3000 nt upstream of the translation initiation codon) for the presence of stress response associated *cis*-elements such as ABREs (abscisic acid responsive element), DRE (dehydration and salt stress responsive), TC-rich repeats (defense and stress responsive element), MBSs (MYB binding site involved in drought induction), WUNs (wound-responsive element), LTRs (low temperature responsive element) and HSEs (heat shock responsive element). We found existence of a total of 126 ABRE *cis*-elements in the promoter regions of all *MaNAC* genes followed by MBS (38), DRE (21), WUN (12), LTR (9) and TC-rich (4). Surprisingly, none of the promoters showed the presence of HSE element within the region studied (Table 1). Of the thirteen stress-responsive *NAC* genes studied in banana, two *MaNAC77*and *MaNAC 95* possessed the highest number of stress related *cis*-elements in their promoters. *MaNAC77* possessed 16 ABREs, four MBS, two DREs and one WUN but no stress elements for LTR, TC-rich and HSE while *MaNAC95* possessed 14 ABREs, six MBS, two DRE and one WUN but no stress elements for LTR, TC-rich and HSE like *MaNAC77*.

Since many of the responses to abiotic stresses such as drought, salt, cold, etc are governed by ABA, we next studied whether ABA influenced the expression of stress-related *NAC* genes. Leaf discs were exposed to 1 μM ABA for up to 4 h and expression of the *NAC* genes studied at 15, 30, 60, 120 and 240 min. As observed, most genes with the exception of *MaNAC37, 38, 71* and *103* showed a clear increase in transcript levels in GN in response to ABA (Fig. 4). The majority of these genes responded rapidly to ABA by a transcriptional increase within 30–60 min of ABA treatment. In most cases, the increase was transient lasting for an hour or less. *MaNAC152* showed a relatively delayed response with a peak at 2–4 h.

Most of these genes were also up-regulated by ABA in HB. However, the scale of increase was considerably low in HB for most genes and also slightly delayed compared to GN. The genes *MaNAC37, 38, 71* and *103* which did not respond much to ABA treatment in GN were also unresponsive in HB (Fig. 4). The studies showed that ABA was an important regulator of the *NAC* genes.

4. Discussion

Banana, one of the most important tropical fruit crops, is constantly under the threat of multiple field stresses. The most common stress combination of drought and heat that is experienced in tropics, is also one of the most devastating of all in plants (Suzuki et al., 2014). This is largely in part due to the opposite response strategies required by the plant to counter the effects of individual drought and heat stresses.

To obtain a better insight into how individual and combined drought and heat stresses are regulated in banana, we chose two varieties, namely GN (AAA genome) and HB (AAB genome), that differ in their genetic constitution and their inherent responses to stress. The presence of the B genome appears to be a key component in HB, imparting it with better physiological adaptation to field stresses and making it hardier than GN (Ravi et al., 2013; Jangale et al., 2019). We had previously shown that stomata in GN are larger in size and tend to remain open even as the plant experiences an increase in mid-day temperature thereby losing greater amount of water while stomata in HB are smaller and close during mid-day (Jangale et al., 2019). Similar differences in regulation have been observed between other AAA and AAB varieties (Ekanayake et al., 1994). Also, GN responds to multiple stresses with a greater increase in reactive oxygen species as compared to HB probably leading to a more serious oxidative damage under drought and combined drought and heat stresses in GN than in HB (Jangale et al., 2019). The B genome thus seems to activate different physiological responses for the survival of the plant.

The NAC TF family has a major role in abiotic stress responses (Puranik et al., 2012; Nuruzzaman et al., 2013). Since NAC family members are missing from multi-cellular algae that are progenitors of land plants, it has been suggested that these may have arisen after transition of plants from water to land as a response to counter abiotic stresses associated with terrestrial growth (You et al., 2015). This has been corroborated by several studies on *NAC* family genes in plants like rice (Hu et al., 2006), common bean (Wu et al., 2016), cotton (Gunapati et al., 2016), etc. In view of the importance of *NAC* genes (particularly the stress *NAC* genes) in abiotic stress responses, we first identified a subset of 15 abiotic stress-related *NAC* genes (from 166 *MaNAC* genes and 3 VOZ genes; this study and Cenci et al., 2014) through phylogenetic analysis of the banana NAC gene family with abiotic stress-related NAC members from 6 monocots and *Arabidopsis*. We show that this subset responds to individual drought and heat stresses and their combination under controlled conditions as well as field conditions. The responses, however, vary considerably between GN and HB. Under individual stresses in controlled conditions, drought and heat separately regulated different sets of genes in GN and HB (Fig. 2). Under drought, most of the selected genes underwent an increase in expression with progression of drought. HB, the more stress-tolerant genotype, responded more prominently to individual drought stress than GN with a 3- to 10-fold higher expression of most of the selected *NAC* genes. Interestingly, the expression of one of these, *MaNAC87,* named as

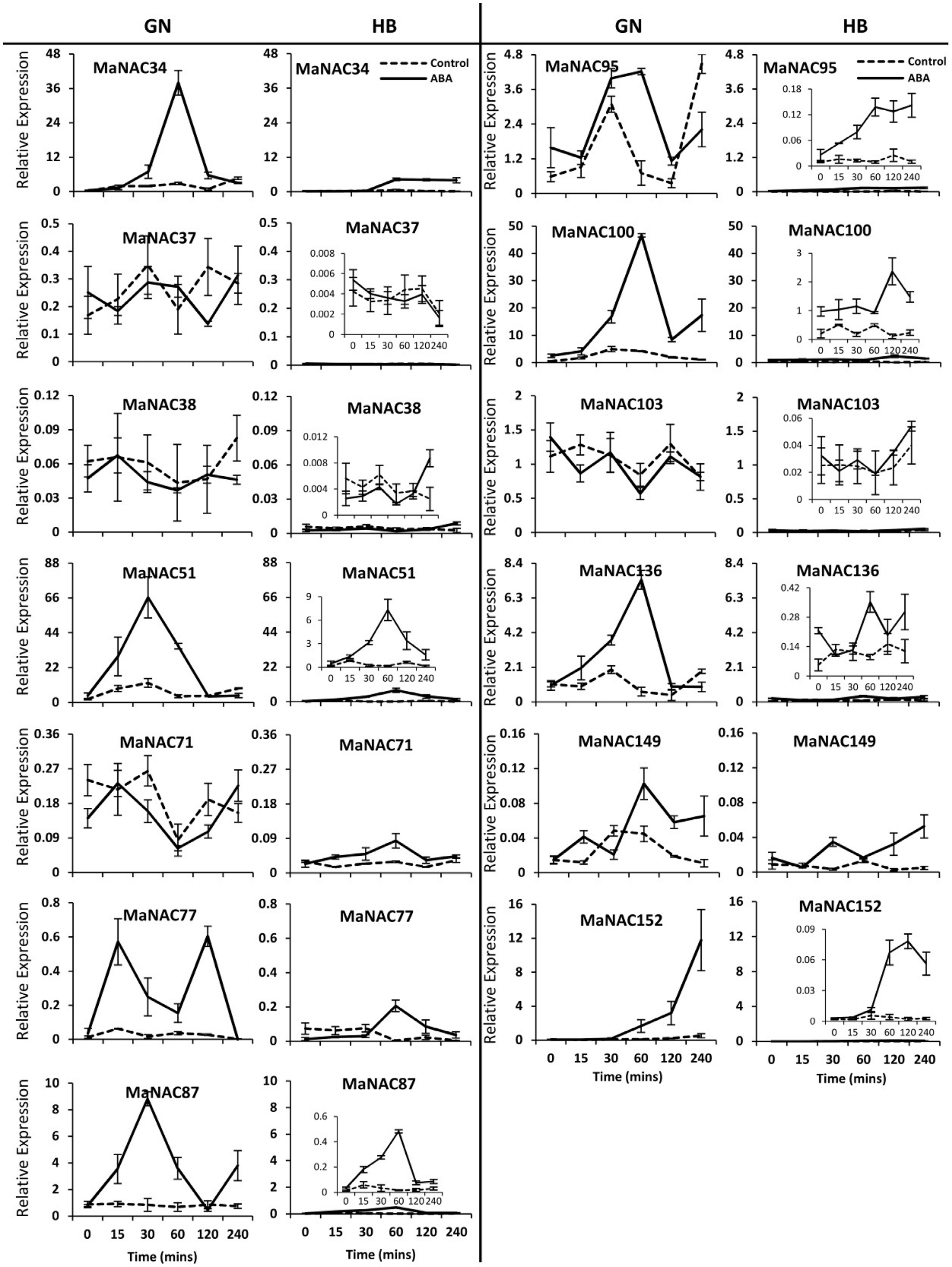

Fig. 4. Regulation of *MaNAC* genes by ABA. For ABA treatment, GN and HB leaf discs (10 mm in diameter) were soaked in ABA solution (1 μM) or without ABA (control) for up to 4 h. X-axis represents time of ABA exposure in minutes and Y-axis represents relative fold change in gene expression. Data are mean ± SEM.

MusaSNAC1 by Negi et al. (2018) was recently shown to confer drought tolerance in transgenic banana by influencing the H_2O_2 content of the guard cells and modulating stomatal closure (Negi et al., 2018). This gene is also up-regulated in combined stresses in HB and GN. However, except for *MaNAC87,* there was not much overlap in the genes that were up-regulated in GN and HB under drought stress, with different genes undergoing up-regulation in the two genotypes. This indicated that the same stress activated different sets of controls in the two genotypes leading to regulation of different NAC genes and, in all likelihood, different responses as in rice (Garcia-Morales et al., 2014, 2018) and soybean (Hussain et al., 2017). In many respects, the responses of *NAC* genes in HB to drought are similar to those of the banana *DREB* genes (Jangale et al., 2019) in that these also showed a greater response to drought in the HB genotype compared to GN under controlled conditions. Similar results were also made by Hussain et al. (2017) who observed a higher expression of stress related *GmNAC* genes in the drought-tolerant soybean cultivar than in the sensitive cultivar.

Greater differences were observed between GN and HB responses when subjected to heat stress. Interestingly, heat stress caused a rapid and continuous decline in expression of most genes in HB, an effect that is opposite to that seen upon drought. Thus, the same *NAC* genes that are activated by drought seem to be detrimental to the heat stress response in HB. GN however, did not show such a collective down–-regulation of genes. Instead, quite a few *NAC* genes were up-regulated in GN even by heat, although the scale of up-regulation was about 10 times lesser than that seen for drought in GN. This shows clear differences in how a rise in temperature is perceived and responded to, by two different genotypes in banana. Heat stress responses are known to differ from those of drought in that heat stress amelioration requires higher transpiration for leaf cooling through opening of stomata while the drought stress response requires closure of stomata to conserve water (Rizhsky et al., 2002, 2004; Prasch and Sonnewald, 2013; Zhang and Sonnewald, 2017). These opposite effects may therefore require different responses that are more clearly seen in the stress-tolerant genotypes such as HB. Since the presence of the B genome differentiates the two genotypes, this is a clear indication that the B genome of banana (present in HB) is responsible for bringing about a change in perception of the stresses that ensures better survival. Salicylic acid (SA) has been proposed to play a role in thermotolerance in plants (Dat et al., 1998) and even in alleviation of combined triple stress of drought, heat and salt (Torun, 2019). Hence, responses of the more efficient genotype (as controlled by the B genome) may hint towards differential regulation of the SA pathway, although this would require further studies. Nevertheless, it should be noted that the opposite regulation between heat and drought in HB seems to be specific to the NAC genes since it was not observed as prominently for the DREB genes upon heat stress in HB (Jangale et al., 2019).

Combination of the two stresses elicited a response that differed from that of the individual stresses. Moreover, the responses also showed differences between the two genotypes. GN, the more sensitive genotype, responded strongly with most genes being up-regulated within 4–5 days of combined stress. The response was contrary to that observed for drought where most of these genes showed very little response to individual stresses in GN under drought. Surprisingly, NAC genes in HB did not respond much to the more severe combination of the stresses despite being up-regulated strongly by drought. With the exception of *MaNAC100* to some extent, most genes were unresponsive to the combined stresses. The observations were similar to those made by Rizhsky et al. (2004) in *Arabidopsis*, where the responses shown by *Arabidopsis* plants in combination of drought and heat stress were different to those exposed to drought or heat stress individually. Strikingly, similar observations were also made by Rasmussen et al. (2013) where a large proportion of the genes that responded to individual stresses barely showed any change in transcripts upon a combination of stresses. This clearly shows that responses to stress combinations are regulated by different pathways.

The response of the *NAC* genes to combined stresses under controlled conditions in GN and HB was similar to the combined stress response observed in the field. Here too, GN showed a much higher sensitivity to the combined stresses, unlike HB, which seemed to not respond to the stress as in case of its response to combined stress under controlled conditions. The exception was *MaNAC100* which increased in combined stresses in the field and under controlled conditions. The expression of *MaNAC51, 77* and *87* also increased a little although the abundance was very low compared to other transcripts. In this respect, the behavior of *NAC* genes in both the genotypes to these stresses is quite similar to that of the DREBs (Jangale et al., 2019). The higher expression of both these two stress-responsive families to combined stresses in GN, but not in HB, is indicative of a fundamental difference between the genotypes in their response to the more severe drought and heat combination. That said, some NACs are commonly expressed upon stresses in both genotypes (Figs. 2 and 3) and may direct specific stress-responsive actions. Some amongst these, like *MaNAC100* and *136,* are rather abundant and changes in their expression may be more relevant to the overall stress response compared to that mounted by other far less abundant NACs, provided there is equivalence between transcript levels and translation efficiency.

Besides drought and heat, *NAC* genes are also expressed in other stresses. A comparative expression analysis of the *MaNACs* from a banana transcriptomic data in osmotic, cold and drought conditions (Muthusamy et al., 2016; Hu et al., 2017; Table S7) showed that eight *NAC* genes that were also selected in our studies, namely *MaNAC34*, *51*, *77*, *95*, *100*, *103* and *136,* were induced in abiotic stresses such as cold and osmotic stresses. Of these *MaNAC51, 77, 95* and *136* were up-regulated in the AAB genotype but only *MaNAC51* and *136* were up-regulated in GN, an AAA genotype (Muthusamy et al., 2016). All these four genes and *MaNAC34* were also up-regulated by cold stress in AAA (BaXiJiao) and ABB (Fen Jiao) genotypes while *MaNAC34* and *51* were down-regulated in AAA genotypes (Hu et al., 2017) indicating that the *NAC* genes were also responsive to other stresses. Interestingly, *Os01g66120,* the rice orthologue of *MaNAC136* (which is induced in the drought-sensitive GN under water stress), was also induced in the sensitive rice cultivar Tres Rios under oxidative stress of polyethylene glycol (Garcia-Morales et al., 2018). Likewise, homologues of two banana *NAC* genes, *MaNAC71* and *MaNAC87* in soybean, *GmNAC065* and *GmNAC073,* respectively were also found to be induced under individual water stress in the drought tolerant variety (Hussain et al., 2017; Table S8).

The considerable change in expression of the stress NACs in banana in response to drought, heat and their combinations made us wonder whether any structural features in these NACs allowed them to regulate certain targets. Earlier, Nuruzzaman et al. (2013) identified a conserved 28-amino acid motif (RSARKKNSLRLDDWVLCRIYNKKGGLEK), named as WVLCR motif, outside the NAC domain in proteins of stress NAC group of monocots (rice) and dicots (*Arabidopsis*). We observed that the 13 banana stress NACs had this motif (motif 13, ADVDRSARKKNSLRLDDWVLCRIYNKKGGLEK; Table S6). Surprisingly, although 33 other NACs out of 169 also showed presence of this motif it was only as a truncated version (LDDWVLCRIYNKKGGLEK) that was found in the non-stress NACs. The first part of the motif (ADVDRSARKKNSL) may thus have a specific role in stress NACs. Remarkably, this motif (ADVDRSARKKNSLRLD) was present in most stress-responsive NAC genes of banana, rice, maize, wheat, barley and sorghum indicating a specific role.

Another interesting feature of the *NAC* genes chosen for study was the ABA responsiveness. Most of the genes studied showed a substantial increase in expression upon treatment with exogenous ABA within 30–60 min. Although the increase was seen in both GN and HB, the increase was much more prominent in GN. In this context, it is of interest to note that most of the gene promoters had several ABA responsive elements such as ABREs as well as MBS and DRE elements that would collectively allow the genes to effectively respond to ABA and other ABA mediated stresses such as drought, osmotic stresses and salt stresses (Table 1). Indeed, as is evident from the present studies and those from others (Muthusamy et al., 2016; Hu et al., 2017) these genes are also activated in other stresses including cold. The number of the *cis* elements does not necessarily correlate with the strength of expression, indicating that not all elements observed may be involved in TF binding. However, the higher expression of *MaNACs 34, 51, 87, 95, 100, 136* and *152* after ABA treatment could be due to the presence of DRE elements that function as coupling elements for ABREs. Despite expression of some NACs in heat, no heat stress elements were observed in the genes even up to 3000 nt upstream of the start codon, suggesting that these responses may be governed by others as yet uncharacterized *cis* elements. Surprisingly, the ABA-mediated increase in expression of the NACs was considerably muted in HB. Considering that drought and abiotic stress response pathways are governed in large part by ABA, the greater sensitivity of GN *NAC*s (and also the *DREB*s; Jangale et al., 2019) suggest that the GN stress response operates largely through the ABA-dependent pathway. Incidentally, stomata in GN were also more sensitive to ABA (Jangale et al., 2019). In contrast, the reduced sensitivity of the same NACs in HB to ABA suggests an inherent mitigation of the ABA sensitivity of HB in response to these stresses suggesting that HB may depend more on ABA-independent pathways for its survival. This would mean that the B genome component may activate ABA-independent pathways for more severe stresses and that this may require dampening the ABA-dependent responses. In this respect, it is interesting to note that in citrus, Zandalinas et al. (2016) showed that the *Cleopatra* mandarin, a more drought tolerant variety, showed greater susceptibility to heat whereas *Carrizo citrange*, a drought susceptible variety, showed greater tolerance to heat. A combination of the two stresses affected *Carrizo* to a lesser extent compared to *Cleopatra* mandarin due to a more efficient control on transpiration and reduced oxidative damage in *Carrizo* (Zandalinas et al., 2016, 2017). The stress combination was associated with a decrease in ABA levels/signaling compared to water stress alone leading to opening of stomata and reduced PSII damage in *Carrizo*. Instead SA accumulated as a response to provide thermotolerance in citrus.

What thus seems apparent from the present studies on NACs and DREBs (Jangale et al., 2019) and other studies (Rizhsky et al., 2002, 2004; Rasmussen et al., 2013; Zandalinas et al., 2019) is that the conventional logic of assigning efficacy of stress tolerance with higher expression of *DREBs, NACs* or other stress-related transcription factor genes can be misleading. Sensitive genotypes such as GN, despite higher expression of *NACs* and *DREBs* are unable to mount an efficient stress response as seen from complete loss of survival (Fig. 1). On the other hand, efficient genotypes such as HB seem to counter more drastic stresses without taking recourse to the expression of stress-associated DREBs and NACs. Instead, they mount a different physiological response that apparently bypasses the need for these TFs as evident from lack of expression of these genes in HB upon combined stresses, both under controlled conditions as well as field conditions. The differences in the responses of HB are, in all likelihood, due to the B genome. Considering that the B genome accounts for only a third of the HB component, this indicates dominance of the B genome over the A genome as far as stress responses are concerned. The success of the B genome in subverting the combination of stresses and providing greater survival (Fig. 1) suggests that it possesses genes that may effectively manage multiple stresses. Identifying these will be crucial to development of future banana varieties to tide over the water crisis and increasingly warmer climates that slowly engulf most parts of the world.

5. Conclusion

We show that drought and heat stresses individually elicit distinct responses through activation of different NAC family genes in two banana genotypes while the more severe combination of these stresses changes the response under controlled conditions as well as in the field. The more efficient B genome in HB seems to alter the response in a manner that largely bypasses the need for the expression of the NAC family.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.10.040.

Author contribution

AA., PVS and BK conceived and designed the research. RSC and BLJ conducted all the experiments and performed bioinformatics analysis. RSC, BLJ, BK, APS analysed the data and APS, BK, PVS wrote the manuscript. All authors read and approved the manuscript.

References

Agarwal, P.K., Jha, B., 2010. Transcription factors in plants and ABA dependent and independent abiotic stress signalling. Biol. Plant. 54, 201–212.

Atkinson, N.J., Urwin, P.E., 2012. The interaction of plant biotic and abiotic stresses: from genes to the field. J. Exp. Bot. 63, 3523–3543.

Barnabás, B., Jäger, K., Fehér, A., 2008. The effect of drought and heat stress on reproductive processes in cereals. Plant Cell Environ. 31, 11–38.

Bailey, T.L., Boden, M., Buske, F.A., Frith, M., Grant, C.E., Clementi, L., Ren, J., Li, W.W., Noble, W.S., 2009. Meme suite: tools for motif discovery and searching. Nucleic Acids Res. 37 (Suppl. l_2), W202–W208.

Bhardwaj, A.R., Joshi, G., Kukreja, B., Malik, V., Arora, P., Pandey, R., Shukla, R.N., Bankar, K.G., Katiyar-Agarwal, S., Goel, S., Jagannath, A., Kumar, A., Agarwal, M., 2015. Global insights into high temperature and drought stress regulated genes by RNA-Seq in economically important oilseed crop Brassica juncea. BMC Plant Biol. 15, 9.

Cenci, A., Guignon, V., Roux, N., Rouard, M., 2014. Genomic analysis of NAC transcription factors in banana (*Musa acuminata*) and definition of NAC orthologous groups for monocots and dicots. Plant Mol. Biol. 85, 63–80.

Chen, L., Zhong, H., Kuang, J., Li, J., Lu, W., Chen, J., 2011. Validation of reference genes for RT-qPCR studies of gene expression in banana fruit under different experimental conditions. Planta 234, 377–390.

Dat, J.F., Lopez-Delgado, H., Foyer, C.H., Scott, I.M., 1998. Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. Plant Physiol. 116, 1351–1357.

Droc, G., Larivière, D., Guignon, V., Yahiaoui, N., This, D., Garsmeur, O., Dereeper, A., Hamelin, C., Argout, X., Dufayard, J.-F., Lengelle, J., Baurens, F.-C., Cenci, A., Pitollat, B., D'Hont, A., Ruiz, M., Rouard, M., Bocs, S., 2013. The Banana Genome Hub. Database 2013, bat035.

Ekanayake, I.J., Ortiz, R., Vuylsteke, D.R., 1994. Influence of leaf age, soil moisture, VPD and time of day on leaf conductance of various *Musa* genotypes in a humid forest-moist savanna transition site. Ann. Bot. 74, 173–178.

Fang, Y., You, J., Xie, K., Xie, W., Xiong, L., 2008. Systematic sequence analysis and identification of tissue-specificor stress-responsive genes of NAC transcription factor family in rice. Mol. Genet. Genomics 208 (6), 547–563.

García-Morales, S., Gómez-Merino, F.C., Trejo-Téllez, L.I., 2014. NAC transcription factor expression, amino acid concentration and growth of elite rice cultivars upon salt stress. Acta Physiol. Plant. 36, 1927–1936.

García-Morales, S., Gómez-Merino, F.C., Trejo-Téllez, L.I., Tavitas-Fuentes, L., Hernández-Aragón, L., 2018. Osmotic stress affects growth, content of chlorophyll, abscisic acid, Na +, and K +, and expression of novel NAC genes in contrasting rice cultivars. Biol.

Plant. 62, 307–317.

Gunapati, S., Naresh, R., Ranjan, S., Nigam, D., Hans, A., Verma, P.C., Gadre, R., Pathre, U.V., Sane, A.P., Sane, V.A., 2016. Expression of GhNAC2 from *G. herbaceum,* improves root growth and imparts tolerance to drought in transgenic cotton and *Arabidopsis*. Sci. Rep. 6, 24978.

Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q., Xiong, L., 2006. Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc. Natl. Acad. Sci. 103, 12987–12992.

Hu, W., Ding, Z., Tie, W., Yan, Y., Liu, Y., Wu, C., Liu, J., Wang, J., Peng, M., Xu, B., Jin, Z., 2017. Comparative physiological and transcriptomic analyses provide integrated insight into osmotic, cold, and salt stress tolerance mechanisms in banana. Sci. Rep. 7 43007.

Hussain, R.M., Ali, M., Feng, X., Li, X., 2017. The essence of NAC gene family to the cultivation of drought-resistant soybean (Glycine max L. Merr.) cultivars. BMC Plant Biol. 17, 55.

Jangale, B.L., Chaudhari, R.S., Azeez, A., Sane, P.V., Sane, A.P., Bal, Krishna, 2019. Independent and combined abiotic stresses affect the physiology and expression patterns of DREB genes differently in stress susceptible and resistant genotypes of banana. Physiol. Plant. 165, 303–318.

Jedmowski, C., Ashoub, A., Momtaz, O., Brüggemann, W., 2015. Impact of drought , heat , and their combination on chlorophyll fluorescence and yield of wild barley (*Hordeum spontaneum*). J. Bot., Le 2015, 120868–120877.

Jin, J., Tian, F., Yang, D.C., Meng, Y.Q., Kong, L., Luo, J., Gao, G., 2017. PlantTFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. Nucleic Acids Res. 45, D1040–D1045.

Jones, P., Binns, D., Chang, H.Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A.F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S.Y., Lopez, R., Hunter, S., 2014. InterProScan 5: genome-scale protein function classification. Bioinformatics 30, 1236.

Lescot, M., Dehais, P., Thijs, G., Marchal, K., Moreau, Y., Peer, Y., Rouze, P., Rombaut, S., 2002. PlantCARE, A database of plant *cis*-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. Nucleic Acids Res. 30, 325–327.

Li, Y., Ye, W., Wang, M., Yan, X., 2009. Climate change and drought: a risk assessment of crop-yield impacts. Clim. Res. 39, 31–46.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.

Mittler, R., 2006. Abiotic stress, the field environment and stress combination. Trends Plant Sci. 11, 15–19.

Muthusamy, M., Uma, S., Backiyarani, S., Saraswathi, M.S., Chandrasekar, A., 2016. Transcriptomic changes of drought-tolerant and sensitive banana cultivars exposed to drought stress. Front. Plant Sci. 7, 1609.

Negi, S., Tak, H., Ganapathi, T.R., 2018. A banana NAC transcription factor (*MusaSNAC1*) impart drought tolerance by modulating stomatal closure and H2O2 content. Plant Mol. Biol. 96, 457–471.

Nuruzzaman, M., Sharoni, A.M., Kikuchi, S., 2013. Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. Front. Microbiol. 4, 248.

Perdomo, J.A., Conesa, M.À., Medrano, H., Ribas-Carbó, M., Galmés, J., 2015. Effects of long-term individual and combined water and temperature stress on the growth of rice, wheat and maize: relationship with morphological and physiological acclimation. Physiol. Plant. 155, 149–165.

Podevin, N., Krauss, A., Henry, I., Swennen, R., Remy, S., 2012. Selection and validation of reference genes for quantitative RT-PCR expression studies of the non-model crop Musa. Mol. Breeding 30, 1237–1252.

Prasad, P.V.V., Pisipati, S.R., Momčilović, I., Ristic, Z., 2011. Independent and combined effects of high temperature and drought stress during grain filling on plant yield and chloroplast EF-Tu expression in spring wheat. J. Agron. Crop Sci. 197, 430–441.

Prasch, C.M., Sonnewald, U., 2013. Simultaneous application of heat, drought, and virus to *Arabidopsis* plants reveals significant shifts in signaling networks. Plant Physiol. 162, 1849–1866.

Puranik, S., Sahu, P.P., Srivastava, P.S., Prasad, M., 2012. NAC proteins: regulation and role in stress tolerance. Trends Plant Sci. 17, 369–381.

Rasmussen, S., Barah, P., Suarez-Rodriguez, M.C., Bressendorff, S., Friis, P., Costantino, P., Bones, A.M., Nielsen, H.B., Mundy, J., 2013. Transcriptome responses to combinations of stresses in *Arabidopsis*. Plant Physiol. 161, 1783–1794.

Ravi, I., Uma, S., Vaganan, M.M., Mustaffa, M.M., 2013. Phenotyping bananas for drought resistance. Front. Physiol. 4, 9.

Rizhsky, L., Liang, H., Mittler, R., 2002. The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiol. 130, 1143–1151.

Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Devletova, S., Mittler, R., 2004. When defense pathways collide. The response of *Arabidopsis* to a combination of drought and heat stress. Plant Physiol. 134, 1683–1696.

Shao, H., Wang, H., Tang, X., 2015. NAC transcription factors in plant multiple abiotic stress responses: progress and prospects. Front. Plant Sci. 6, 902.

Shiriga, K., Sharma, R., Kumar, K., Yadav, S.K., Hossain, F., Thirunavukkarasu, N., 2014. Genome-wide identification and expression pattern of drought-responsive members of the NAC family in maize. Meta Gene 2, 407–417.

Sperotto, R.A., Ricachenevsky, F.K., Duarte, G.L., Boff, T., Lopes, K.L., Sperb, E.R., Grusak, M.A., Fett, J.P., 2009. Identification of up-regulated genes in flag leaves during rice grain filling and characterization of OsNAC5, a new ABA-dependent transcription factor. Planta 230, 985–1002.

Suzuki, N., Rivero, R.M., Shulaev, V., Blumwald, E., Mittler, R., 2014. Tansley review Abiotic and biotic stress combinations. New Phytol. 203, 32–43.

Tak, H., Negi, S., Ganapathi, T.R., 2017. Banana NAC transcription factor MusaNAC042 is positively associated with drought and salinity tolerance. Protoplasma 254, 803–816.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Torun, H., 2019. Time-course analysis of salicylic acid effects on ROS regulation and antioxidant defense in roots of hulled and hulless barley under combined stress of drought, heat and salinity. Physiol. Plant. 165, 169–182.

Tran, L.P., Nakashima, K., Sakuma, Y., Simpson, S.D., Fujita, Y., Maruyama, K., Fujita, M., Seki, M., Shinozaki, K., Yamaguchi-Shinozaki, K., 2004. Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. Plant Cell 16, 2481–2498.

Turner, D.W., Fortescue, J.A., Thomas, D.S., 2007. Environmental physiology of the bananas (*Musa* spp.). Braz. J. Plant Physiol. 19, 463–484.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 research 0034-1.

Wu, J., Wang, L., Wang, S., 2016. Comprehensive analysis and discovery of drought-related NAC transcription factors in common bean. BMC Plant Biol. 16, 193–206.

You, J., Zhang, L., Song, B., Qi, X., Chan, Z., 2015. Systematic analysis and identification of stress-responsive genes of the NAC gene family in *Brachypodium distachyon*. PLoS One 10, e0122027.

Zandalinas, S.I., Balfagón, D., Arbona, V., Gómez-Cadenas, A., 2017. Modulation of antioxidant defense system is associated with combined drought and heat stress tolerance in citrus. Front. Plant Sci. 8, 953.

Zandalinas, S.I., Rivero, R.M., Martínez, V., Gómez-Cadenas, A., Arbona, V., 2016. Tolerance of citrus plants to the combination of high temperatures and drought is associated to the increase in transpiration modulated by a reduction in abscisic acid levels. BMC Plant Biol. 16, 105.

Zandalinas, S.I., Sengupta, S., Burks, D., Azad, R.K., Mittler, R., 2019. Identification and characterization of a core set of ROS wave-associated transcripts involved in the systemic acquired acclimation response of *Arabidopsis* to excess light. Plant J. 98, 126–141.

Zhang, H., Sonnewald, U., 2017. Differences and commonalities of plant responses to single and combined stresses. Plant J. 90, 839–855.