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; Prach 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., 2000).
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 (NAM, NO APICAL MERISTEM; ATAF, ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR; and CUC, 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* (Gunapat 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 (Gunapat 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 when 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 triplloid 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 (ABB 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 (CDD) search program (http://www.ncbi.nlm.nih.gov/Structure/bwpsb/bwpsb.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 (Vascular Plant One Zinc-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 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), hence 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:

\[
\text{Percent yield loss} = \left(\frac{\text{Avg. bunch weight of control plants} - \text{Avg. bunch weight of treated plants}}{\text{Avg. bunch weight of control plants}}\right) \times 100
\]

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 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 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 in a 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 ≤ 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
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 sub-domains 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 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.
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 MaNAC34, 37, 38, 95 and 149, 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. MaNAC71 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).

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.
4. Discussion

ABA plays an important role in governing many of these responses. 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 harder 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 168 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
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.
MusaNAC1 by Negi et al. (2018) was recently shown to confer drought tolerance in transgenic banana by influencing the H$_2$O$_2$ 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 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 (BaXJiao) 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 (RSARKKNSLDDLWVLCRIYNKKGGLEK), 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, ADVDRSAKNSRLR-LDDLWVLCRIYNKKGGLE; Table S6). Surprisingly, although 33 other NACs out of 169 also showed presence of this motif it was only as a truncated version (LDDLWVLCRIYNKKGGLEK) that was found in the non-stress NACs. The first part of the motif (ADVDRSAKNSRL) may thus have a specific role in stress NACs. Remarkably, this motif (ADV-DRSAKNSRLRD) 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 NACs (and also the DREBs; 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 dampering 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 cirtange, 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 PSI 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 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. The GN gene expression of DREB genes to the ABA and independent stress responses of the same NACs in HB to ABA suggests an inherent mitigation of the ABA sensitivity of HB to the use of bioinformatic tools.

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.

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