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Transcriptomic Analysis of Brassinosteroid Biosynthesis and Signaling Pathway Genes in Healthy and Malformed Tissues of Mango Variety Amrapalli

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In present era, breeding in mango crop are still done through conventional methods which is a serious drawback as it is laborious and time consuming. Mango malformation is a serious disease causing great losses to mango industry. It is difficult to control mango malformation disease through use of any chemicals. Brassinosteroids (BR) are known to have a wide range of functions to biotic and abiotic stresses. No reports are available relating BR level with mango malformation till date. In the present study we analyzed the brassinosteroid level in malformed and healthy buds at different growth stages through transcriptomic approach by using illumina pair-end sequencing. In the present study we observed the transcripts related to BR's, target gene of BR's, biosynthesis, signaling pathways and differentially expressed genes (DEGs). The results indicated that biosynthesis genes were more in malformed tissues (MB-1:5, MB-2:6 & MB-3:7) compared to healthy tissues (HB-1: 5, & HB-2: 5) whereas BR signaling genes were more in healthy tissues (HB-1:24, HB-2: 26) compared to malformed tissues (MB-1:21, MB-2:20 & MB-3: 21). The brassinosteroids signaling pathway genes in healthy tissues increased from bud to panicle formation stages. Differential Expression analysis indicated that BZR-1 a signaling gene was significantly up regulated in healthy panicle formation stage compared to all malformed bud developmental stages. Among the target genes of BR's signaling PIF, MYB-30 TF were higher in number in malformed buds compared to healthy buds at different growth stages. The results could serve as breeding targets for mango crop improvement.

Introduction

Mangifera indica L. (2n=2x=40), a highly heterozygous tropical fruit of the world in Anacardiaceae family belongs to order Sapindales. It is commonly known as "King of Fruits" and is famous worldwide for its color, taste, flavor and aroma (Singh *et al.,* 2016).India being a land of mangoes, more than thousands varieties are grown in different states, which contribute to 50 per

cent of total mango production in world (Kostermans and Bompard, 1993). According to horticulturists and historians, mango originated in India (Singh *et al.,* 2016), which later on spread to South-East Asia, Malaysia and Sumatra. Among the biotic stresses, mango malformation is most destructive, since it causes gross deformations in vegetative and floral tissues of mango (Ploetz,

2001). It is a serious constraint to mango industry (Kumar *et al.,* 2011) and 50-80 per cent fruit yield losses are reported every year (Kumar and Chakrabarti, 1997). The etiology of malformation has been a contentious issue, and a wide range of biotic and abiotic factors have been reported to cause the disease, including viruses, mites and nutritional deficiencies (Ploetz *et al.,* 2002; Nailwal *et al.,* 2006; Jouyban, 2012). Convincing evidence on the involvement of a fungus as the causal agent of malformation has been in the literature for decades (Singh and Dhillon, 1990; Usha *et al.,* 1997; Steenkamp *et al.,* 2000; Ploetz *et al.,* 2002, Nailwal *et al.,* 2006; Chakrabarti, 2011). However, based on Koch's postulate and detailed cytological examination of the infected tissues, it is now established that a fungus, *Fusarium mangiferae* induces the symptoms of mango malformation disease (Britz *et al.,* 2002; Usha *et al.,* 2009; Iqbal *et al.,* 2010). In spite of several decades of incessant research since its recognition in 1891, the etiology of this disease has not been established. Several mango malformation disease management strategies such as use of different chemical pesticides, hormones, pruning diseased panicles etc. met with little or no success (Chakrabarti, 2011).

The plant hormones namely auxin, gibberellin, cytokinin, abscisic acid, ethylene, salicylic acid, jasmonic acid are small signaling molecules controlling various development process in plants (Quecini *et al.,* 2007; Rigal and Ma, 2014), and play important roles in plant–pathogen interactions (Fu *et al.,* 2011). Besides these, a new class of phytohormone, brassinosteroids (BR) isolated from pollen of *Brassica napus* was discovered. Till now, a total of 69 types have been found. These BR's belongs to a polyhydroxylated steroid hormones having active roles in stimulating the plant growth development with a wide range of functions

such as response to environmental biotic and abiotic stresses namely salt and drought stress tolerance, photo-morphogenesis, thermo-tolerance, stomatal development, oxidative stress tolerance, cell death control, disease resistance, herbicide and pesticide tolerance (Vriet *et al.,* 2012; Gruszka, 2013). Brassinosteroids at molecular level enhance metabolism of protein and nucleic acid and also affect gene expression pattern in plants (Khripach *et al.,* 2000). The PGPM (plant growth promoting molecules) brassinosteroids are widely distributed in the plant kingdom from lower to higher plants. The high concentration BR's is mainly found in reproductive organs of the plant with lesser quantity in other vegetative parts of the plants. In plants the BR's are found in pollens (Pachthong *et al.,* 2006), anthers (Watanabe *et al.,* 2000), panicles (Schmidt *et al.,* 1995), seeds, seedlings (Antonchick *et al.,* 2005), shoots (Fukuta *et al.,* 2004), leaves (Sondhi *et al.,* 2008), galls (Ikeda *et al.,* 1983), stems, cambial region (Kim *et al.,* 1990), roots (Yokota *et al.,* 2001), flower buds (Takatsuto 1994), flowers, bran (Abe *et al.,* 1995) and grains (Yokota *et al.,* (1994).

Among the plant hormones, ethylene, jasmonic acid, salicylic acid, are playing crucial roles in governing the plant defense (Kunkel and Brooks, 2002; Genger *et al.,* 2008). Beside these, brassinosteroid also play important role in controlling different diseases especially bacterial (rice), fungal (barley, tomato) and viral disease (tomato, tobacco, cucumber). According to Choudhary *et al.,* (2012) brassinosteroids are involved in complex molecular interaction network, which induce host defense responses upon pathogen attack. The change in the phytohormone biosynthesis and signaling pathways may result in resistance due to combined effect of multiple pathogens. It is necessary to understand the complexity of brassinosteroid and its interaction with other

plant hormone homeostasis during plant–pathogen interactions in malformation disease for developing the hormone-based breeding strategies. Manipulating hormone biosynthesis and signaling pathways result in enhanced resistance to a certain type of pathogens (Holeski *et al.,* 2012).

However, no research findings regarding effect of BR's on malformation are available. Hence the present study was initiated to investigate the effect of BR's biosynthesis and signaling pathways, through transcriptome analysis of healthy and malformed tissues of mango variety Amrapali. This transcriptome approach will help to find the differentially expressed genes of BR's biosynthesis and signaling pathways in healthy and malformed tissues and will be useful to find its relation with mango malformation.

Materials and Methods

Plant material

Mango buds of Amrapalli variety were used to study the transcriptomic analysis of phytohormone biosynthesis and signaling genes. Total five samples (2 healthy and 3 malformed tissues) at different growth stages were collected from the experimental orchard, Division of Fruits and Horticulture Technology, Indian Agricultural Research Institute, New Delhi.

The five samples used for the study are as follows: Single swollen malformed bud stage I (MB-1), Multiple malformed bud stage 2 (MB-2), Multiple malformed bud stage 3 (MB-3), Healthy bud stage 1 (HB-1) and Healthy bud stage 2 (HB-2). Immediately after the sample collection the buds were frozen in liquid N_2 and stored at $-80°C$ upto RNA extraction.

Isolation, qualitative and quantitative analysis of total RNA

Total RNA was isolated from healthy (HB-1 and HB-2) and malformed tissues (MB-1, MB-2 and MB-3) at different growth stages. For each of the five stages we collected three biological replicates and two technical replicates to prepare one pooled RNA sample. Total RNA was isolated by using Trizol method (Hongbao, *et al.,* 2008).

The quality of the isolated RNA was checked on 1% denatured Agarose gel for the presence of 25S and 18S bands. Further, total RNA was quantified using Qubit fluorometer.

Illumina NextSeq 2 x 150 PE (Pair End) library preparations

Illumina deep sequencing platform was used to generate the sequencing data from healthy and malformed tissues of mango cvAmarpali. Illumina TruSeq stranded mRNA Library Preparation Kit was used to prepare the paired-end cDNA sequencing libraries as per its described protocol. Briefly, mRNA was enriched from total RNA followed by fragmentation. The means of the library fragment size distributions for MB-1, MB-2, MB-3, HB-1 and HB-2 stages are 504bp, 467bp, 481bp, 462bp and 480bp respectively. The libraries were sequenced using 2 X 150 PE chemistry on NextSeq-500 and 5 Gb data per sample was generated.

The fragmented mRNA was converted into first strand cDNA, followed by second-strand generation, A-tailing, adapter ligation and finally ended by index PCR amplification of adaptor-ligated library. Library quantification and qualification was performed using DNA High Sensitivity Assay Kit. Trimmomatic (V-0.30) software was used to filter Illumina reads with a Phred quality score below 20; discarding the rest of the sequence and

keeping only pairs where both reads were larger than 40 bp. The transcripts were assembled using Bridger with default parameters. The statistical elements of the assembly were calculated by in house perl scripts.

De novo transcriptome assembly

The next generation sequencing for MB-1, MB-2, MB-3, HB-1 and HB-2 samples were performed using 2x150PEchemistry on the Illumina NextSeq platform and approximately 5-6 GB data was generated per sample. Trimmomatic (V-0.30) software was used to filter Illumina reads with a Phred quality score below 20; discarding the rest of the sequence and keeping only pairs where both reads were larger than 40 bp (Bolger *et al.,* 2014). The transcripts were assembled using Bridger with default parameters. CD-Hit was run on the above mentioned Bridger (Chang *et al.,* 2015) assembled transcripts to get the unigenes. All CDS were predicted from the unigenes using Trandecoder (http://transdecoder.sf.net) with default parameters. Subsequently, the predicted CDS were annotated using BLASTX against NCBI nr database.

Functional annotation and pathway analysis

The predicted CDS were subjected to similarity search against NCBI's non-redundant (nr) database using the BLASTx algorithm. GO sequence distributions helps in specifying all the annotated nodes comprising of GO functional groups.

The platform independent java implementation of the Blast2GO PRO software (Gotz *et al.,* 2008) was used to retrieve associated gene ontology (GO) terms describing biological processes, molecular functions, and cellular components. CDS associated with similar functions are assigned

to same GO functional group. To further differentiate the NCBI nucleotide sequences and assembled sequences at the protein level, COG classification was undertaken to analyse the NCBI sequences. The unigenes sequences were aligned to COG database to classify and predict possible functions. To identify the biological pathways in all samples, the detected CDS were mapped to reference canonical pathways in KEGG using KEGG automatic annotation server (KAAS) (Kanehisa and Goto, 2000).

Differential gene expression analysis

The DESeq package was used to detect significantly DE genes between control and treated samples. DEG analysis was carried out for commonly occurring CDS (based on common NR blast hit accession) among the control and infected samples. The genes were found to be differentially expressed with a log fold-change value $> +2$ for up-regulated genes and log fold-change value < -2 for down-regulated genes (Fig. 1).

Results and Discussion

Identification of brassinosteroid related coding sequences (CDS) and their functional annotation

The transcripts related to brassinosteroid were identified in all five malformed and healthy tissues at different growth stages (MB-1, MB-2, MB-3, HB-1 and HB-2) and their total number varied from 8 (MB-3) to 15 (MB-1). The number of CDS in MB-1, MB-2, MB-3, HB-1 and HB-2 are 15, 12, 5, 8 and 13 respectively (Table 1). The BLASTX was used against the NCBI NR database to get the coding sequences functionally annotated.

The summary of BlastX annotation results are shown in the supplementary table 1. The identified functional categories of coding sequences are brassinosteroid insensitive 1-

associated receptor kinase 1-like protein, Brassinosteroid LRR- receptor kinase-like, brassinosteroid signaling positive regulator family protein and brassinosteroid-6-oxidase 2.

Expression profile of brassinosteroid biosynthesis pathways genes

Brassinosteroids biosynthesis pathway genes (Figure 2) namely DWF-4, CYP-85A2, ROT-3, CPD and DET-4 were observed in three malformed and two healthy tissues during bud to panicle development stages (Table 2). During bud to panicle development stages, the total number of biosynthesis genes showed increasing pattern in malformed tissues whereas in healthy tissues it remained unchanged. The expression of all brassinosteroids biosynthesis pathway genes namely DWF-4, CYP-85A2, ROT-3, CPD and DET-1 were similar at bud stages of healthy and malformed tissue whereas at panicle development stages, CYP-85A2 and CPD showed higher level at MB-3 stage compared to HB-2 stage; while rest of the genes (DWF-4, ROT-3 and DET-1) expressed similarly in healthy and malformed tissues at different growth stages.

Expression profile of brassinosteroid signaling pathways genes

Brassinosteroids signaling pathway (Figure 3) genes namelyBRI-1, BAK-1, BKI-1, BSK, BIN-2, BZR-1/2, TCH-4 and CYCD-3 were found in the mango transcriptome of healthy and malformed tissues (Table 3). The total number of signaling genes were more in healthy tissues (HB-1:24, HB-2: 26) compared to malformed tissues (MB-1:21, MB-2:20 and MB-3: 21). The higher number of brassinosteroids signaling genes observed at healthy bud stage (HBS-1) are BRI-1, BSK, BZR-1/2, and TCH-4 whereas at single swollen malformed bud stage (MB-1), BIN-2 was higher and BKI-1 and CYCD-3 were having similar expression in HB-1 and MB-1

tissues. BSK, BIN-2 and CYCD-3 genes were more during HB-2 stage compared to MB-3 stages whereas BAK-1 was higher at MB-3 stage compared to HB-2 stage and rest of the genes namely BRI-1, BKI-1, BZR-1/2, and TCH-4 were similar in expression in MB-3 and HB-2 growth stages.

DEG's of brassinosteroid biosynthesis and signaling pathways

In healthy and malformed tissues of mango cv. Amrapali, the expression level variations at different growth stages for brassinosteroid biosynthesis and signaling pathway genes was performed through differential gene expression analysis. For better understanding of brassinosteroid interaction with malformation, the six possible combinations (HB-1$_{VS}$ MB-1, HB-1$_{VS}$ MB-2, HB-1$_{VS}$ MB-3, HB-2$_{VS}$MB-1, HB-2 $_{VS}$MB-2, HB-2 $_{VS}$MB-3) were studied (Table 4). We compared the DEGs between the samples within a specific stage of healthy and malformed tissue. The DEG's analysis revealed that out of all brassinosteroid biosynthesis and signaling pathway genes studied, only three genes (CPD: carboxypeptidase-D; CYCD-3: Cyclin Delta-3 (D-type cyclins) and BZR1: Brassinazole-Resistant1**)** were significantly expressed. The total number of upregulated genes for CPD, CYCD-3 and BZR1 were (4, 3, and 3); and down regulated genes were (12, 2 and 0) respectively.

Target genes of brassinosteroids signaling

In the mango transcriptome data of healthy and malformed tissues, we observed 7 different types of target genes of BR's signaling. They were PIF (Phytochrome-interacting factor), REF-6 (Relative of ELF6), SBI-1 (Suppressor of BRI1), PP2A (protein phosphatase 2A), LCMT (leucine carboxylmethyl transferase), IWS-1 (Interacts with SPT6) and MYB30-TF (MYB30 transcription factors). The number of IWS-1

gene was similar in all stages of healthy and malformed tissues; while REF-6 gene was absent in all stages except in MB-1. The target genes PIF, SBI, LCMT, and MYB30-TF were higher in MB-1 when compared to healthy tissues were higher in healthy tissues compared to MB-1 growth stage. During panicle development stage, PIF, MYB30-TF were more in MB-3 compared to HB-2; while SBI, PP2A and LCMT were higher in HB-2 compared to MB-3 (Fig. 4).

Table.1 Number of brassinosteroid transcripts identified in different stages of Healthy and malformed tissue

Functional Categories of coding sequences	MB-1	MB-2	MB-3	HB-1	HB-2
Brassinosteroid insensitive 1-associated receptor kinase 1 -like protein	10	7	1	3	8
Brassinosteroid LRR- receptor kinase-like	1	2	1	1	1
Brassinosteroid signaling positive regulator family protein	2	2	3	3	4
Brassinosteroid-6-oxidase 2	2	1	0	1	0
Total transcripts	15	12	5	8	13

Table.2 Expression of Brassinosteroids biosynthesis pathways gene in Healthy and malformed stages

Stages / Genes	MB-1	MB-2	MB-3	HB-1	HB-2
DWF-4	1	1	1	1	1
CYP-85A2	1	1	2	1	1
ROT-3	1	1	1	1	1
CPD	1	1	2	1	1
DET-1	1	2	1	1	1
Total Gene	5	6	7	5	5

Table.3 Expression of Brassinosteroids signaling pathways gene in Healthy and malformed stages

Stages / Genes	MB-1	MB-2	MB-3	HB-1	HB-2
BRI-1	1	1	1	2	1
BAK-1	2	2	2	2	-
BKI-1	2	2	3	2	3
BSK	7	6	7	8	9
BIN-2	2	2	2	1	4
BZR-1/2	2	2	2	3	2
TCH-4	1	1	1	2	1
CYCD-3	4	4	3	4	6
Total Gene	21	20	21	24	26

Table.4 Differentially expressed genes of brassinosteroid biosynthesis and signaling pathways in six possible combinations of healthy and malformed tissue

Stages	Expression	CPD	CYCD-3	BZR1
HB-1 vs MB-1	Up-Regulated	0	0	0
	Down-Regulated	2	1	0
HB-1 vs MB-2	Up-Regulated	1	0	0
	Down-Regulated	2	0	0
HB-1 vs MB-3	Up-Regulated	1	0	0
	Down-Regulated	1	1	0
HB-2 vs MB-1	Up-Regulated	0	1	1
	Down-Regulated	4	0	0
HB-2 vs MB-2	Up-Regulated	2	1	1
	Down-Regulated	3	0	0
HB-2 vs MB-3	Up-Regulated	0	1	1
	Down-Regulated	0	0	0
Total	Up-Regulated	4	3	3
	Down-Regulated	12	2	0

CPD: carboxypeptidase-D; CYCD-3: Cyclin Delta-3 (D-type cyclins) and BZR1: Brassinazole-Resistant1

Fig.1 Flow chart indicating the transcriptomic analysis of BR's in Healthy and malformed tissue of mango cultivar Amrapalli
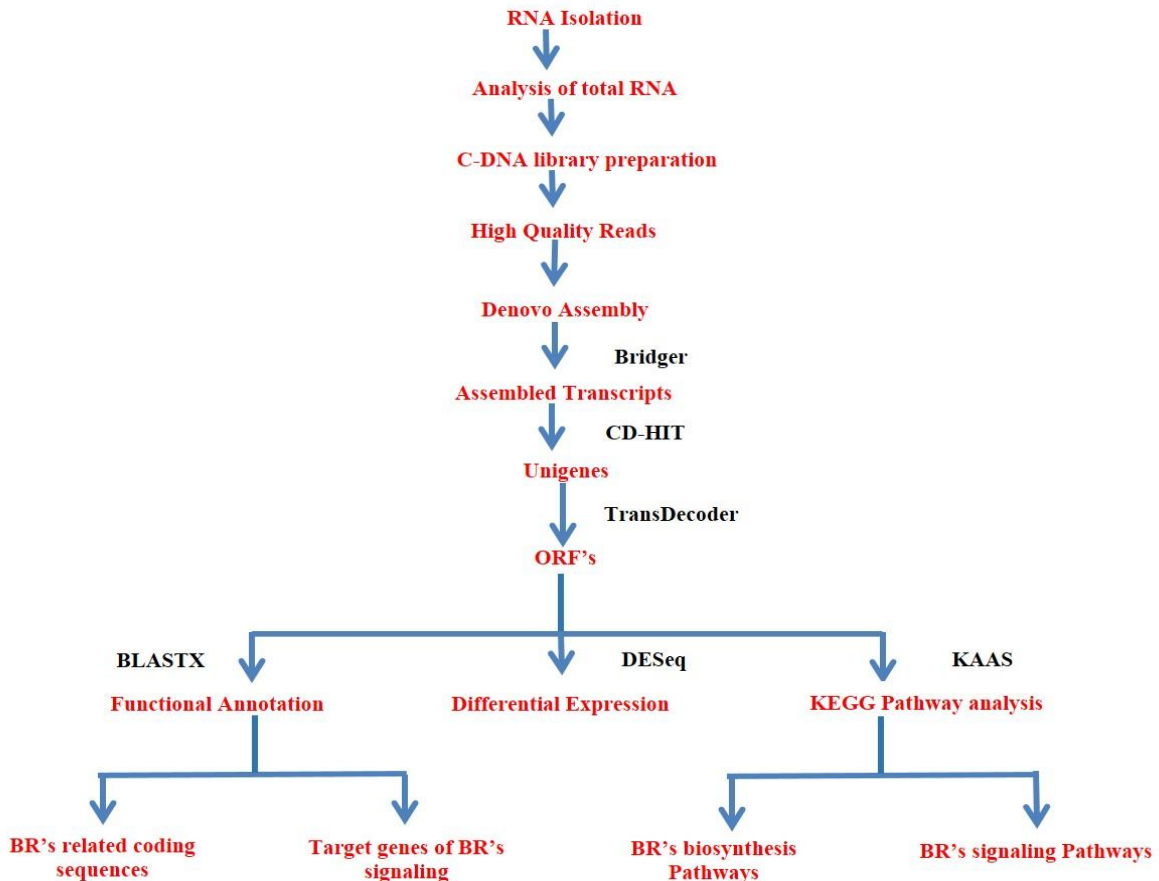
Fig.2 Proposed BR biosynthesis pathway genes in healthy and malformed tissue of mango cultivar Amrapalli which follow the Late C-6 Oxidation pathway (Chung and Choe, 2013)
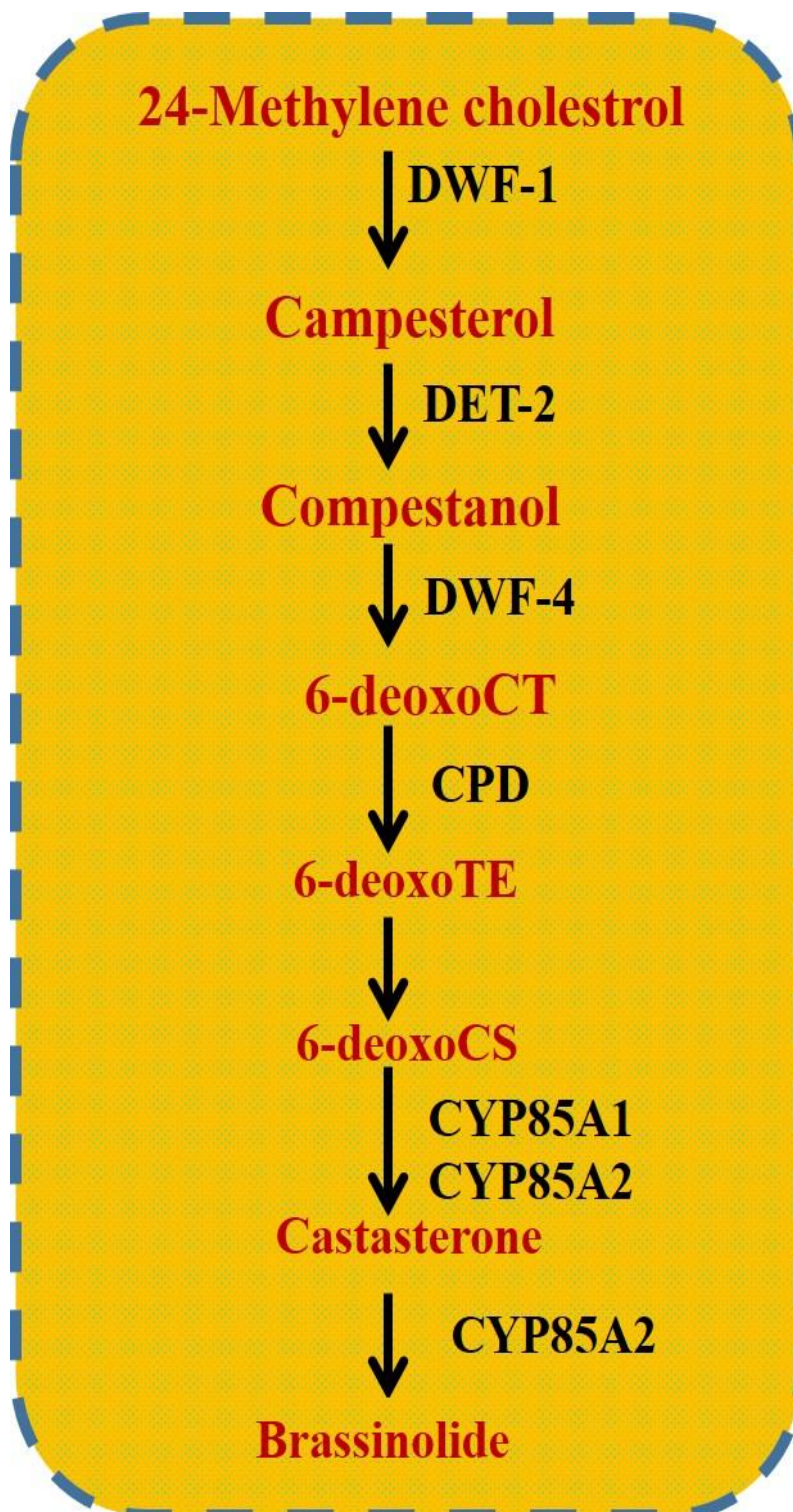
DWF-1: Dwarf-1; DWF-4: Dwarf-4; CYP-85A1: cytochrome-85A1 family; CYP-85A2: cytochrome-85A2 family; CPD: carboxypeptidase-D, DET-2: de-etiolated 2 homolog

Fig.3 BR's signaling pathway genes in healthy and malformed tissue of
Mango cultivar Amrapalli
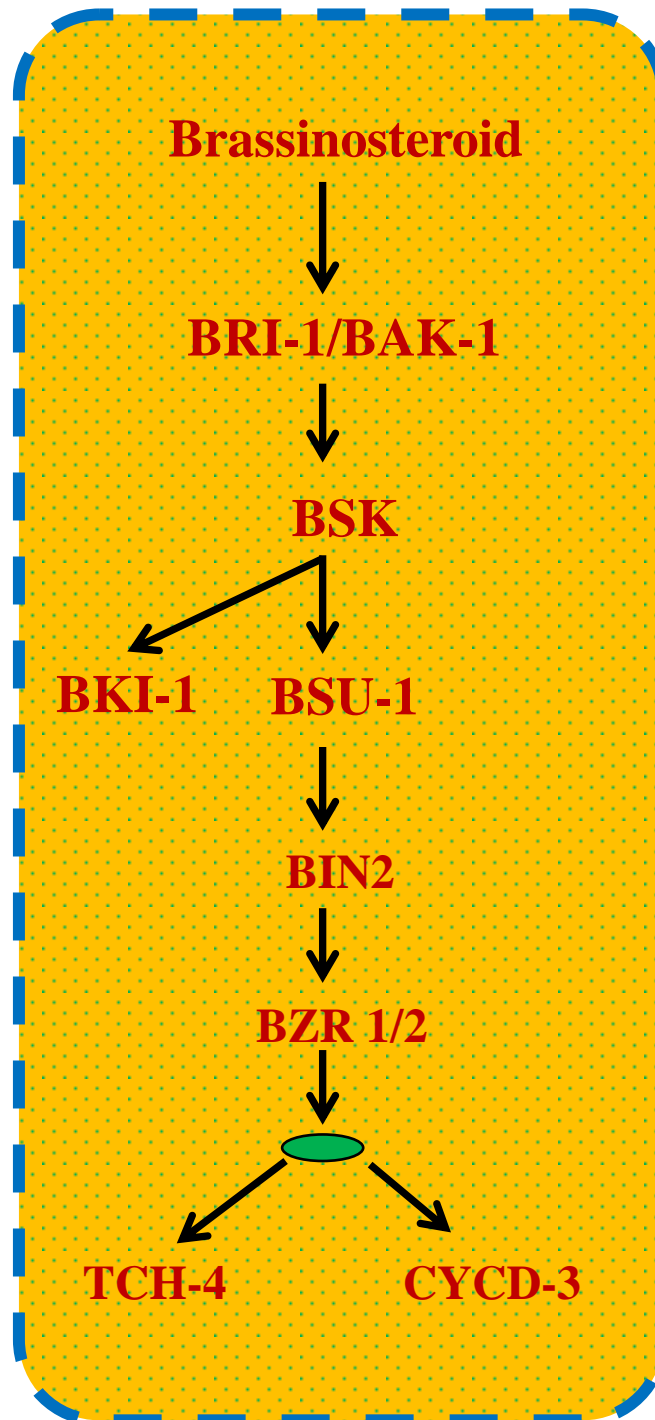
BRI-1: Brassinosteroid Insensitive1; BAK1: BRI1-Associated Receptor Kinase1; BKI1: BRI1 Kinase Inhibitor1, BSK1: Brassinosteroid Signaling Kinase1; BZR1: Brassinazole-Resistant1; BZR 2: Brassinazole-Resistant 2; TCH-4: for touch; CYCD-3: Cyclin Delta-3 (D-type cyclins); BIN2: Brassinosteroid Insensitive 2, BSK: Brassinosteroid Signaling Kinase and BSU1:BRI1-Suppressor1

Fig.4 Target of BR signaling genes in healthy and malformed tissues of
Mango cultivar Amrapalli
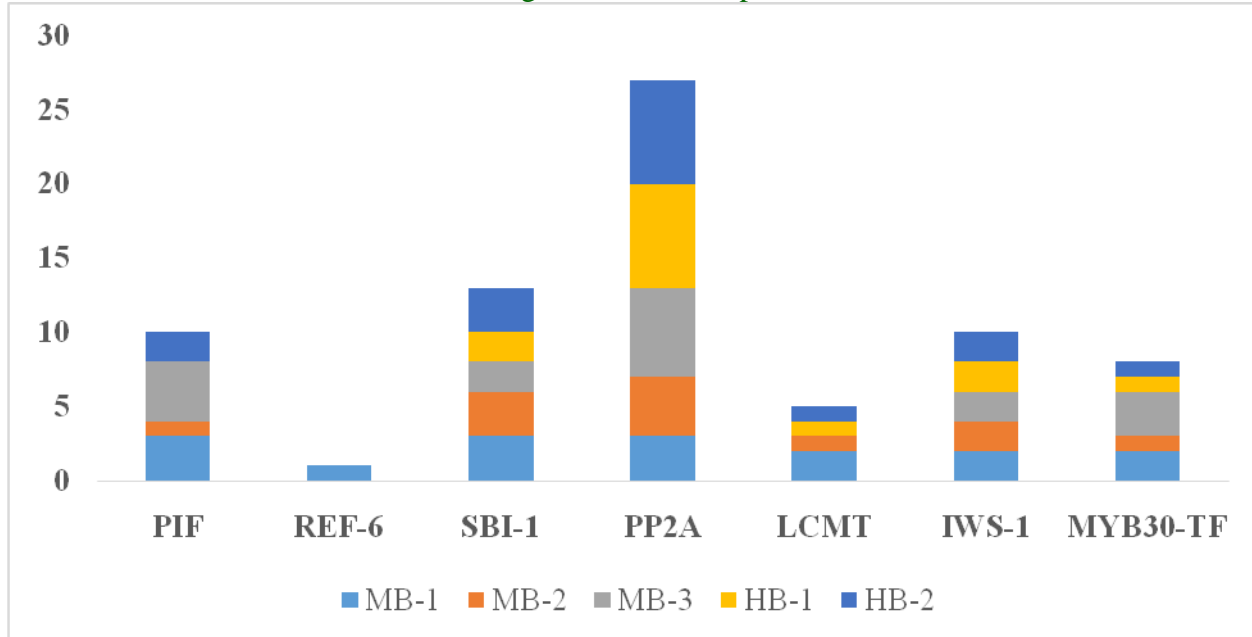
Brassinosteroids are the most important naturally occurring plant growth-promoting steroidal hormones which not only play a pivotal role in inducing the disease resistance or plant innate immunity but also regulates several growth and development processes like photo-morphogenesis, hormonal regulation, dwarfism, seed germination, change in the distribution pattern of stomata, flowering, senescence, pollen tube elongation, male sterility, xylem differentiation and root growth, confer tolerance/resistance to a broad range of biotic and abiotic stresses (thermal, salinity, oxidative, heavy metal stresses) in several plant species (Krishna, 2003; Kagale *et al.,* 2007; Bajguzand Hayat, 2009; Divi and Krishna, 2010; Coll, *et al.,* 2015).We observed that number of brassinosteroids synthesis genes in healthy (HB-1) and malformed buds (MB-1) were similar. In malformed tissues, the biosynthesis genes increased as the stage of malformation proceeds from MB-1 to MB-3; whereas in healthy tissues, from bud to panicle development stage, it remained unchanged. This probably could be due to the reason that

in malformed panicles, the numbers of flowers are more and all flowers are male. Brassinosteroids are mainly synthesized in pollens resulting in more number of brassinosteroids biosynthesis genes in malformed tissues when compared to healthy tissues. Similar reports were also made in other crops by several workers (Yasuta *et al.,* 1995; Zullo *et al.,* 2002; Zhu *et al.,* 2013).

Brassinosteroids signaling pathway genes in malformed tissue did not increase from bud to panicle development stages and are also less in number compared to healthy tissues. The brassinosteroids signaling pathway genes in healthy tissues increased from bud to panicle development stages. The BZR-1 is a positive regulator of BR signaling pathways (Lee *et al.,* 2015) and its fold change value is more in healthy tissues compared with malformed tissues and could be reason for observed more number of signaling genes in healthy tissues. The more number of signaling genes in healthy tissue might be the reason of resistance to malformation because it has been reported that the up-regulation of

pathogen resistance is mainly due to BR signaling (Miyaji *et al.,* 2014). We observed that the BR's signaling pathway is less active in malformed tissues compared to healthy tissues. Plant defective in BR's signaling results in dwarfism, leaves curling, and male sterility (Li *et al.,* 2001).

BZR-1 gene was up-regulated in healthy tissues (HB-2) when compared to malformed tissues at all growth stages. The fold change value in HB-2$_{VS}$MB-1, HB-2 $_{VS}$MB-2, HB-2 $_{VS}$MB-3 were 45.36, 24.59 and 42.11. In our results we found that BZR-1 expression is low in malformed tissues due to which biosynthetic pathway gene of BR's are slightly more in malformed tissues. When BZR-1 expression is low, it leads to increase in the number of BR's biosynthesis genes that lead to formation of more BR's (He *et al.,* 2005). Therefore BRs play crucial roles in bud growth and responses to *Fusarium magiferae* induced biotic stress in infected mango buds that lead to development of malformed panicles.

Brassinosteroids are a novel group of phytohormones having a broad spectrum of physiological activity and play crucial roles in plant growth and responses to stresses due to their growth promoting nature. BRs are having pleiotropic effect due to their diverse role in plant life cycle. We found that total numbers of biosynthesis pathway genes were more in mal formed tissues compared to healthy tissues; whereas the numbers of signaling genes were more in healthy tissues compared to malformed tissues. DEG's analysis showed that BZR-1 signaling gene was significantly up regulated in healthy panicle development stage compared to mal formed tissues at all growth stages. Among the target genes of BR's signaling PIF, MYB-30 TF were higher in number in malformed tissues compared to healthy tissues. BR's role in regulating many different physiological processes appears to culminate in producing multiple beneficial effects in mango buds preventing infection by *Fusarium mangiferae*. BR-related genes could serve as breeding targets for mango crop improvement.

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