EgJUB1 and EgERF113 transcription factors as master regulators of defense response in Elaeis guineensis against the hemibiotrophic Ganoderma boninense

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Abstract

Background

Hemibiotrophic pathogen such as the fungal pathogen *Ganoderma boninense* that is destructive to oil palm, manipulates host defense mechanism by strategically switching from biotrophic to necrotrophic phase. Our previous study revealed two distinguishable expression profiles of oil palm genes that formed the basis in deducing biotrophic phase at early interaction which switched to necrotrophic phase at later stage of infection.

Results

The present report is a continuing study from our previous published transcriptomic profiling of oil palm seedlings against *G. boninense*. We focused on identifying differentially expressed genes (DEGs) encoding transcription factors (TFs) from the same RNA-seq data resulting in 106 upregulated and 108 downregulated TFs being identified. DEGs involved in four established defense-related pathways are presented which are responsible in cell wall modification, reactive oxygen species (ROS)-mediated signaling, programmed cell death (PCD) and plant innate immunity. We discovered upregulation of *JUNGBRUNNEN 1* (*EgJUB1*) during fungal biotrophic phase while *Ethylene Responsive Factor 113* (*EgERF113*) demonstrated prominent upregulation when the palm switches to defense against necrotrophic phase. *EgJUB1* was shown to have binding activity to a 19 bp palindromic SNBE1 element, WNNYBTNNNNNNAMGNHW found in the promoter region of co-expressing *EgHSFC-2b*. Further *in silico* analysis of promoter regions revealed co-expression of *EgJUB1* with TFs containing SNBE1 element with single nucleotide change at either the 5th or 18th position. Meanwhile, *EgERF113* binds to both GCC and DRE/CRT elements promoting plasticity in upregulating the downstream defense-related genes. Both TFs were proven to be nuclear localized based on subcellular localization experiment using onion epidermal cells.

Conclusion

Our findings demonstrated unprecedented transcriptional reprogramming of specific TFs potentially to enable regulation of specific set of genes during different infection phases of this hemibiotrophic fungal pathogen. The results propose *EgJUB1* and *EgERF113* as key TFs in orchestrating the defense mechanisms during biotrophic and during the subsequent transition to necrotrophic phase, respectively. Identification of these phase-specific oil palm TFs is important in designing strategies to tackle or attenuate the progress of infection.

Background
*Ganoderma boninense* is a pathogenic species which causes basal stem rot (BSR) disease in oil palm. Infected palms remain symptomless even though they are already physiologically impaired [1]. Half of the basal stem would have been destroyed by the fungus compromising intake of water and nutrients before the first symptom is observed [2,3]. The fungus *Ganoderma boninense* is recognized as a hemibiotroph, which established intermediate lifestyle of biotroph before switching to necrotroph [4,5]. Biotrophic phase involves colonization of host plant tissues and extraction of nutrients for survival by the pathogen, while keeping the host cells intact [3]. Biotrophs thrive in intracellular region between mesophyll cells, some produce haustoria to penetrate and successfully draw out water and nutrient [6,7]. The invasion requires minimal release of cell wall degrading enzymes (CWDEs) to allow loosening of plant cell wall [8,9].

Pathogen-associated molecular patterns (PAMPs) are elicitors such as bacterial flagellin and fungal chitin derived from the phytopathogens that are recognized by plant cell surface pattern recognition receptors (PRRs) leading to PAMP-triggered immunity (PTI) [10,11,12]. The susceptibility of plant host to microbes colonization relies on effectors secreted by pathogens to deceive PAMPs recognition, thus suppressing PTI signaling [12,13]. Breaching of PTI remarks activation of second line plant defense response termed as effector-triggered immunity (ETI). ETI comprises of resistance (R) proteins to counteract successful invasion of pathogens by recognizing virulence effectors (Avr) in host cells [14]. ETI, accompanied by increased signaling of phytohormones including lipid-based jasmonic acid (JA) and gaseous ethylene (ET) stimulate production of ROS [15]. The ROS induce hypersensitive response (HR) at sites of infection to limit the invasion of pathogens [16].

Unfortunately, the accumulation of ROS and induced programmed cell death (PCD) created favorable environment for necrotroph to intensify infection strategy [17]. It was postulated that the increasing pressure by plant defense responses result in switching of the pathogen infection mode from biotrophy to necrotrophy but the time taken differs between specific host-pathogen interaction [18]. Hemibiotrophs may require extended periods of biotrophic phase to establish colonization and once sufficed, transition to necrotrophic phase is rapid [19]. Necrotrophs secrete phytotoxic compounds and excessive CDWEs to induce host cell death [20]. The dynamic intermediate lifestyle of hemibiotroph enables manipulation of the host plant defense mechanisms which ultimately result in the plant succumbing to the infection. Salicylic acid (SA) signaling which often functions antagonistically to JA-ET signaling has been widely studied to determine the fine-tuning against biotrophic or necrotrophic infection [21,22, 23]. However, there is poor molecular information available explaining the defense regulation by transcription factors (TFs) during transitions of biotrophic to necrotrophic state. Our previous transcriptomic profiling via high-throughput RNA-seq analysis has pointed out the counter-act defense mechanisms executed by plant during transition of biotrophic to necrotrophic phase [5]. In the attempt of identifying key defense pathways that are transcriptional regulated, the next generation sequencing (NGS) data set was mined for TFs responsible in triggering the downstream responses.

TFs are the ‘master switches’, which regulate the expression of a large set of genes initiated by unique signaling networks in response to stresses [24]. Modulation of defense response genes expression may
vary depending on the intensity and intricacy of multiple stresses [25]. There are six major TF families involved in plant defense response including MYB, bHLH, AP2/ERF, NAC, bZIP and WRKY [26]. Regulation by TFs is crucial to mediate the transcriptional reprogramming which include induced expression of defense-related genes responsible in the production antifungal proteins or the antimicrobial secondary metabolites known as phytoalexins [27]. Intriguingly, plants also display cross-tolerance phenomena in which single type of stress may trigger multitude tolerant levels to different stresses [28]. For instance, heat stress transcription factors (HSFs) play important role as master regulator of defense response under multiple stresses [29,30]. Over-expressed HSFs confer resistance against dehydration, bacterial and oomycetes infections and improve yield under water-limited conditions [31].

TFs bind to cis-acting elements located in promoters of either other mediator TFs or downstream target genes which results in up- or down-regulation of their expression [32,33,34]. NAC TFs are recognized as master regulator for secondary cell wall biosynthesis mediated by MYB TF through formation of NAC-MYB-CESA signaling cascade [35]. In addition, bHLH-MYB association was involved in the biosynthesis of flavonoids secondary metabolites under phytohormones signaling, wounding and fungal interaction [36,37]. Interaction to specific DNA sequences (binding motifs) is dependent on the DNA binding domain (DBD) of TFs [38]. binding preferences of TF during biotic or abiotic stress such as ERFs have been suggested to correlate with the composition of amino acid sequences in DBD [38,39]. The presence of multiple cis-acting elements in the promoter region contribute to overlapping roles in development and/or defense against multiple stresses of the expressed proteins [26].

In addition to biotic or abiotic stresses [40], in this study we are revealing that the regulation of TFs is dependent on modes of pathogen's infection (biotrophic and necrotrophic). Only EgUNE10 TF and a few TF families have been reported to regulate defense against later stage of G. boninense infection [4,41,42]. However, there is no comprehensive report on transcriptomic profiling of defense-related TFs during different infection phases of hemibiotroph in plants. Thus, this study is the first attempt to recognize specific TFs as ‘key’ biomarkers involved in transcriptional switching from biotrophic to necrotrophic infection phase based on early oil palm-G. boninense interaction. Their potential targeted defense response pathways that distinguished the two phases are discussed based on the identification of specific motifs interacting with the newly discovered TFs. The findings might allow a more effective disease management strategy to attenuate the progress of G. boninense infection of oil palm and prevent the spread of the disease.

Results

Elaeis guineensis defense-related transcription factors and biomarkers of biotrophy-necrotrophy switch

In order to identify the TF families involved in early defense of oil palm against. G. boninense, transcriptomics analysis of Ganoderma-treated root tissues at 3, 7 and 11 d.p.i was carried out. We identified 106 of upregulated and 108 of downregulated TFs upon early interaction with G. boninense (Fig. 1a and b). Two distinct phases can be observed having profound expression at 3 d.p.i (biotrophic)
and 11 d.p.i (early necrotrophic) with intermediate at 7 d.p.i. We have previously reported the transition of biotrophic to necrotrophic defense mechanism, based on preliminary screening using defense-related molecular biomarkers *EgPR1* (biotrophic) and *EgMYC2* (necrotrophic) [5]. The pairwise comparison was constructed between control against time course treatments using stringent cut-off values of log₂ fold change (FC) ≥ |1.0| and *P*-value < 0.01. We found that the expression patterns of the genes showed either decreasing or increasing over time. Major TF families upregulated during early interaction with *G. boninense* were mainly *bHLH > MYB > AP2/ERF*, followed by *bZIP > MADS > TCP > OFP > NAC > GATA > HSF > NFY > E2F > WRKY > EIN/EIL*. On the other hand, highest downregulated families of TF were found to be mainly *AP2/ERF > bHLH > MYB* followed by *MADS > CAMTA > NAC > TCP > GATA > bZIP > HSF > NFY > E2F > WRKY > OFP*. The families of oil palm's TFs involved during defense response against *G. boninense* were found to be the same but involving different members in both upregulated and downregulated DEGs. Distinctively, *EIN/EIL* and *CAMTA* TF family was only found in upregulated and downregulated DEGs, respectively. *AtCAMTA3* and *AtCAMTA4* were reported to negatively regulate plant defense response under SA-mediated signaling pathway against obligate biotroph [43,44].

To further understand plant response against *G. boninense* interaction, common pathways regulating defense mechanisms were selected from the RNA-seq data (Fig. 1c). Four main defense-related pathways are presented. Reported genes involved in cell wall modification, ROS-mediated signaling, PCD and plant innate immunity were all differentially expressed indicating active regulation of defense response in *E. guineensis* against hemibiotroph *G. boninense*. The expression patterns of selected biotic stress-related genes were important to distinguish the defense mechanisms executed by plant during two different phases of biotrophic and necrotrophic infections.

Six genes of *Cellulose synthase* (*CESA*) and *Cellulose synthase-like D* (*CSL*) which were reported in cell wall modification including *EgCESA2, EgCESA4, EgCESA5, EgCESA9, EgCSLD2* and *EgCSLD5* demonstrated similar expression patterns with high upregulation at 3 d.p.i that successively decreased across time. Three out of six genes involved in ROS-mediated signaling including *Superoxide dismutase* (*EgSOD*), *Glutathione S-transferase* (*EgGSTF12*) and *Respiratory burst oxidase homolog* (*EgRBOHA*) were increasingly expressed from 3 d.p.i to 11 d.p.i, whilst *EgGSTU17, EgGST3* and *EgRBOHB* demonstrated decreasing expression patterns. Two genes reported in PCD, *Metacaspase 9* (*EgMC9*) and *Bifunctional nuclease* (*EgBFN*) showed antagonistic expression patterns of increasing and decreasing regulation, respectively. Five genes involved in plant innate immunity of PTI/ETI signaling were found to be differentially expressed. Three of the genes including *NONEXPRESSOR OF PATHOGENESIS-RELATED GENE 1* (*EgNPR1*), *Calmodulin-like 3* (*EgCML3*) and *Calcium dependent protein kinase 7* (*EgCDPK7*) were upregulated highest at 3 d.p.i and 7 d.p.i before subsequently declined. The other genes which were *EgCML7* and *EgCDPK28* were successively upregulated with highest expression at 11 d.p.i. The reliability of RNA-seq data has been validated in our previous report [5] using representative upregulated and downregulated DEGs with different levels of fold change compared to untreated seedlings (control).

**Expression patterns of transcription factors regulating defense response against** Ganoderma boninense
qPCR analysis was able to distinguish TFs regulated under biotic and/or abiotic stresses by comparing *Ganoderma*-treated (GT) with mock-treated (MT) samples, respectively at designated time points (Fig. 2). Biotrophic-associated TFs, *EgJUB1* and *EgTCP15* were highly upregulated at 3 and 7 d.p.i in GT samples. However, *EgTCP15* also exhibited significant upregulation in MT samples at 11 d.p.i. TCP15 was reported in both developmental and stress response pathways under SA-mediated signaling, enhanced by interaction with NPR1 [45,46]. The expression levels in MT samples at all time points were significant for all analyzed genes except for *EgJUB1*, which indicates its specific role during biotic stress.

Meanwhile, the qPCR analysis on candidates of necrotrophic-associated TFs revealed that *EgERF113*, *EgEIN3* and *EgMYC2* were highly upregulated at 11 d.p.i on GT samples. Both *EIN3* and *MYC2* are JA-dependent which were upregulated and downregulated in the RNA-seq data, respectively. The expression of these genes is known to be mutually exclusive whereby MYC2 relies on co-actions of JA-abscisic acid (ABA) while *EIN3* regulates plant defense response through JA-ET signaling [47,48]. *EgERF113* which demonstrated non-significant expression on MT samples (abiotic stress) at all time points were selected for further characterization as novel necrotrophic-specific TF.

**EgJUB1 binds to novel SNBE motif during biotrophic infection**

Characterization of *EgJUB1* TF against *G. boninense* infection was carried out via Y1H assay and EMSA. Three potential binding motifs; one NAC binding site (NACBS) and two secondary wall NAC binding elements (SNBEs) with respective mutants were tested on *EgJUB1* (Fig. 3). NACBS is the established binding motif for JUB1 TF during abiotic stress while SNBEs are the novel target motifs tested in the present study. The SNBE consensus sequence was identified as WNNYBNNNNNNAMGNHW, whilst NACBS was RRYGCCGT. Y1H assay demonstrated positive interaction with SNBE1 motif but not to NACBS and SNBE2. The results indicate regulation of alternative pathway by JUB1 in enhancing resistance during biotic stress. The positive binding with one of SNBE1 motif indicates that the binding affinity is dependent on the core motifs of SNBE consensus sequence, whereby four nucleotides change on the core motif of SNBE1 resulted in no interaction with SNBE2 (Fig. 3a). Colony PCR was carried out on five positive clones and sequencing was performed using T7 promoter primer of pGADT7-Rec vector to confirm genuine positive interaction. p53-AbAi yeast reporter vector was used as positive control in the Y1H assay. We further confirmed the protein-DNA interaction via EMSA by testing the binding of nuclear protein extracted from positive clone of Y1H assay on biotinylated DNA probes. Shifted band was observed upon testing with biotinylated DNA probe. Unlabeled probe (molar excess 200-fold) was able to compete effectively for binding with biotinylated target DNA probe. The inability of mutated fragment to compete with biotinylated target DNA probe revealed specific binding of *EgJUB1* to SNBE1 (Fig. 3b). SNBE consensus sequence is the predicted DNA binding motif of *EgJUB1* (Fig. 3c), based on Plant Transcription Factor Database (PlantTFDB), but thus far there is no reported evidence to support this.

Here, we listed SNBE motifs identified in the 1.5 kb promoter regions of TFs co-expressing with *EgJUB1* during biotrophic phase (Fig. 3d). The core motif of SNBE1 sequence was identified in the promoter region of *EgHSFC-2b*, suggesting direct regulation of *EgJUB1* with the *EgHSFC-2b*. We highlighted the
promoter regions of a few TFs co-expressing with *EgJUB1* with single nucleotide change in the SNBE1 core motif tested in the present study at the 5th or 18th position, including *EgHSFB-4b*, *EgGAMYB-X2*, *EgERF003*, *EgKAN1-like-X3*, *EgILI-5-like*, *EgERF086-like*, *EgPIF3-X1* and -X2. It is plausible that changes of single nucleotide on SNBE1 core motif, still maintain the ability of *EgJUB1* to activate these TFs. Changes of two and more nucleotides in the core motif of SNBE1 is expected to result in significant decline in binding affinity [49], which merit analysis in more detail.

**EgERF113 binds to both GCC and DRE/CRT motifs during necrotrophic infection**

*EgERF113* TF was tested via Y1H assay and EMSA (Fig. 4) on two AP2/ERF DNA binding preferences which were GCC-box also known as Ethylene-Response Element (ERE) and Dehydration Response Element/C-Repeat (DRE/CRT). The GCC-box and DRE/CRT motifs in the study share 6-bp core sequence of GCCGMC. Our findings revealed recognition of *EgERF113* by both motifs in the Y1H assay. The binding affinity of *EgERF113* with both binding motifs was supported by the EMSA results. Unlabelled probes of GCC-box and DRE/CRT (molar excess 200-fold) were able to compete to respective biotinylated target DNA probes. No shifted band was observed on mutated fragments which proved binding specificity of *EgERF113* with both GCC-box and DRE/CRT motifs. The findings suggest that *EgERF113* can regulate stress-related genes harboring GCC-box and/or DRE/CRT in their promoter region.

**EgJUB1 And EgERF113 Are Localized In The Nucleus**

Analysis of the deduced amino acid sequence revealed the NAC binding domain of *EgJUB1* with five highly conserved subdomains A to E at the N-terminal region. The C-terminal region showed highly conserved domain between different species which suggested its possible role as a transcriptional activator, repressor or in binding to other protein. Putative nuclear localization signals (NLS), KKSLVYYLRGSAGTKT was identified in subdomain D (Fig. 5a).

The DBD of *EgERF113* TF consists of three-stranded β-sheets and one α-helix running almost in parallel. Analysis of the deduced amino acid sequence of *EgERF113* proved the presence of tryptophan (W) amino acid at 14th position of the DBD which explained the binding specificities to both GCC-box and DRE/CRT motifs. The extra amino acid at 24th position of DBD implies GCC-box binding specificity of ERF TF is 61 amino acids long [39]. However, AP2/ERF DBD of *EgERF113* lacks this additional amino acid. Putative NLS PWGKWAAEIRDPRKAI RV was identified in β-sheets (Fig. 5b).

To determine the subcellular localization of *EgJUB1* and *EgERF113* proteins, we utilized the *Agrobacterium*-mediated transformation for transient expression of green fluorescent protein (GFP) in onion epidermal cells. As shown in Fig. 5c, both GFP-labelled *EgJUB1* and *EgERF113* could co-accumulate in the nucleus. The observation is consistent with the putative role of these proteins acting as TFs.

**Discussion**
Upon assaulted by pathogens, plants respond by activation of intricate defense systems. Depending on the nature of pathogens, biotrophic and necrotrophic infections are fundamentally different in terms of their infection approach, effector proteins, and the host defense response [50]. Thus, tackling the disease based on infection stage, hypothetically should be able to save or at least prolong the life span of Ganoderma-infected palms. Identifying the infection at biotrophic phase may help planters to take suitable disease management strategy to prevent the disease from transition to the more chronic necrotrophic phase. Meanwhile, infected palms at necrotrophic phase may be treated with more intensified practice such as using chemical fungicide. Differentiation of biotrophic and necrotrophic TFs were based on established defense-related biomarkers in known defense mechanisms such as plant innate immunity and HR that can distinguish these two phases. In this study, we demonstrated the key roles of EgJUB1 as a biotrophic-specific while EgERF113 as a necrotrophic-specific transcriptional regulator, respectively during early oil palm-G. boninense interaction.

**EgJUB1 mediates defense response during biotrophic phase through SNBE motif**

Expression profile of EgJUB1 observed in RNA-seq was validated via qPCR which confirms its role in host defense regulation during biotrophic phase of G. boninense infection independent of abiotic stress. JUB1 was first linked to homeostasis of oxidative stress particularly related to hydrogen peroxide (H$_2$O$_2$) signaling. It binds to cis-element that serves as the NACBS in the promoter region of DREB2A TF for tolerance to abiotic stresses [51]. DREB2A TF binds directly to DRE sequence of drought-stress responsive genes, including HSFs but the mechanisms are still unclear [52]. AtJUB1 (ANAC042) was also reported as a key TF that induces camalexin expression, a major phytoalexin of Arabidopsis against bacterial pathogen [53]. A more recent study revealed induced expression of NAC042_5, an orthologue of AtJUB1 in response to biotrophic fungus Erysiphe necator [54]. The JUB1 which acts independent of SA was induced specifically during pathogen colonization. Intriguingly, oil palm EgJUB1 was found to co-express with two SA-dependent genes of EgTGA1 and EgNPR1. Our results were more in line with the SA-dependent master regulator of NPR1, a cofactor of TGA1 reported by [55], which induces Pathogenesis related (PR) genes [56] during biotrophic phase.

We are reporting for the first time induced expression of EgJUB1 under pathogen challenge, regulating defense-related gene(s) harbouring SNBE binding motif. SNBE motif is composed of an imperfect palindromic 19-bp sequence which can be present in various targets’ promoters including TFs and downstream genes involved in secondary cell wall biosynthesis, cell wall modification as well as PCD [57]. Binding to the 19 bp SNBE (A/T)NN(C/T)NC(G/T)NNNNNNNA(A/C)GN(A/C/T)(A/T) consensus sequence is critical at the 9 core nucleotides, regardless of mutation on the other nucleotides [49]. They reported that mutation(s) on these 9 core nucleotides causes reduced and/or elimination of the transcriptional activation, on the contrary changes in the other non-critical nucleotides enhance the binding affinity.

It was discovered from our study that EgJUB1 directly regulates EgHSFC-2b to promote resistance against biotrophic phase through SNBE1 motif. The SNBE1 motif tested in this study consists of the
nucleotides G and T at the 5th and 18th position of the core motif, respectively. Based on the report by Zhong et al. [49], the binding affinity can still be maintained if a single nucleotide in the core motif of SNBE consensus sequence is changed, however, changes of two and more nucleotides may reduce the binding affinity significantly. Thus, promoters of the listed oil palm TFs (Fig. 3D), *EgHSFB-4b, EgGAMYB-X2, EgERF003, EgKAN1-like-X3, EgILI-5-like, EgERF086-like, EgPIF3-X1* and -X2, which harbour single nucleotide change at either the 5th or the 18th position of the SNBE1 core motif most likely are still able to bind to SNBE1.

Our findings are in line with a recent study which observed high up-regulation of *HSF* and *heat shock proteins* (*HSPs*) against biotrophic fungus [58,59]. HSF was also reported during bacterial infection which directly regulated *Enhanced Disease Susceptibility 1 (EDS1)* and *PR4* under SA-mediated signaling [60].

**Proposed defense-related pathways regulated by *EgJUB1* co-expressing genes**

Here, we report that high expression of *EgCESA4, EgCESA9* and *EgCSLD2* correlates with the expression of *EgGAMYB-X2* TF during biotrophic phase (3 and 7 d.p.i) before a subsequent decline in expression. GAMYB TF interacts with GAMYB binding motif to activate downstream genes [61]. The GAMYB motif was found in the promoter region of *CESA* responsible in secondary cell wall cellulose biosynthesis [35,62]. Consistently, *CESA4, CESA7* and *CESA9* were reported as regulators of secondary cell wall cellulose synthesis [63]. In addition, local cell wall reinforcement by *CSLD2* has been proven under biotroph challenge of powdery mildew fungus (Douchkov et al., 2016). Thus, it is strongly postulated that *EgJUB1* binding to SNBE1 motifs in the promoter regions of *EgGAMYB-X2* activates oil palm defense response through regulation of secondary cell wall biosynthesis.

Increased production of ROS accompanied with PCD [7], provide evidence on the occurrence of HR. In the current study, genes regulating antioxidant enzymes *EgSOD, EgGSTU17, EgGST3* and *EgGSTF12* were highly upregulated during early interaction with *G. boninense*. We also observed the expression of *EgNPR1* and *EgTGA1* which have been recognized to be overexpressed exclusively during biotrophic attack under SA-mediated signaling pathway [65,66]. The co-actions of *EgNPR1* and *EgTGA1* results in upregulation of *EgPR1* which has been proven as a biotrophic marker in our previous report [5].

**EgERF113 mediates defense response during necrotrophic phase through GCC-box and DRE/CRT motifs**

MYC2 relies exclusively on co-actions of JA-ABA branch response to regulate resistance against insects and wounding by repressing the JA-ET branch [48]. Although *EgMYC2* was preliminarily screened as a necrotrophic biomarker [5], the TF is categorized as a downregulated DEG in our RNA-seq data. This can be explained by elevated expression of ethylene insensitive 3 (*EgEIN3*) which then activates *EgERF113* through ET regulation. Binding of ET to its receptors inactivates CONSTITUTIVE TRIPLE RESPONSE 1 (*CTR1*) which in turn release repression on EIN2 activity and stabilize EIN3 and EIN3-LIKE1 (*EIL1*) within the nucleus [67]. As a result, *ERFs* are activated and the ERF TFs modulate transcriptional activity of developmental as well as stress-induced responsive genes [68]. Master regulator of ethylene signaling pathways, *EIN3* and *EIN3-like* (*EIL*) have been proven to modulate multitude cascades of downstream
transcriptional responses [47,69]. De-repression of EIN3/EIL from JA-ZIM domain (JAZ) activates JA-ET signaling that positively regulate transcriptional activations of development as well as defense response against necrotrophic pathogens [47,70]. The findings demonstrated that oil palm establishes resistance against early necrotrophic through co-actions of JA-ET, rather than JA-ABA.

Here, we report regulation of defense response against necrotrophic phase of G. boninense through multi-cascades activation of JA-ET branch leading to overexpression of EgERF113. ERF113, also recognized as RELATED TO APETALA2.6-LIKE (RAP2.6L) which is closely related to ERF108 (RAP2.6) was found to be responsive to abiotic stresses (salinity, heat and drought) as well as stress hormones, particularly JA and/or ET [71]. ERF108 has been reported in JA-induced defense response against wounding and pathogens [72,73]. Similarly, ERF113 was shown to be induced upon wounding [74,75], as well as pathogens infection [76,77].

We are the first reporting on EgERFF113 with binding preferences for GCC-box and DRE/CRT motifs. AP2/ERFs are known to have multiple conserved DNA binding preferences [78]. However, DREBs are typically known to recognize DRE/CRT conferring resistance against abiotic stresses whilst ERFs bind to GCC-box promoting defense against biotic stresses. ERF113 was reported to bind to the GCC-box [77], but not tested on DRE/CRT motif. To date, only a few ERFs were reported with binding preferences on both GCC-box and DRE/CRT conferring resistance against pathogens attack [79,80,81,82]. Binding to GCC-box and DRE/CRT motifs that are present in plant defensin (e.g PDF1.2) as well as PR genes, activates defense-related genes [83,84]. Kaur et al. [84], also reported DRE/CRT element in the promoter region of calcium-responsive genes. Calcium ion (Ca\(^{2+}\)) signaling plays paramount importance in defense mechanisms of plant in perceiving invading pathogens [85].

Phukan et al. [39] has studied the divergent of AP2/ERF TF DNA-binding specificities based on sequence characterization. Their findings suggested glutamic acid (E) at the 20th and alanine (A) at the 48th positions as identified in EgERF113 in the 60 amino acids long DBD denote binding specificity to DRE/CRT motif. EgERF113 also showed conservation of amino acids for GCC-box binding at the 10th, 18th, 20th, 37th and 59th positions. Although the specific amino acid in DBD that regulates binding of TFs to both GCC-box and DRE/CRT motifs has not yet been confirmed, replacing phenylalanine (F) at the 14th position to tryptophan (W) in EgERF113 changes the binding specificity from GCC-box only to binding both GCC-box and DRE/CRT motifs, as suggested by Phukan et al. [39]. An additional amino acid (basic polar) at the 24th position of DBD which is lacking in EgERF113 is essential for specific binding to GCC-box only. Thus, EgERF113 with 60 amino acids DBD opposes the classification of specific GCC-box binding DBD of 61 amino acids in length.

**Proposed defense-related pathways regulated by** EgERF113 **co-expressing genes**

Two RBOH genes, EgRBOHA and EgRBOHB have been previously reported in our study [5], and reduced EgRBOHB expression during early necrotrophic phase may suggest plant’s response in delaying the progression of disease. This is in line with the report on the response against hemibiotroph
Macrophomina phaseolina [86], wherein increase expression of RBOH leads to increase susceptibility of plants to necrotrophic infection.

Another less studied TF, AtMYB122 was reported to regulate genes involved in camalexin biosynthesis [87]. Increased accumulation of indolic glucosinolates during induction of glucose signaling was evidently regulated by AtMYB122 [88]. Glucosinates [89,90]. Based on the expression patterns of EgMYB122, the TF channeled its regulation from responding to abiotic stress at 3 d.p.i. into defensing against Ganoderma attack and it is expected that the gene regulation will be more extensive at later stage of the necrotrophic phase.

Rapid and transient increase of cytosolic Ca$^{2+}$ particularly during pathogens interaction results in activation of both PTI and ETI signaling [85]. CMLs and CDPKs are known as Ca$^{2+}$ sensor proteins which are responsible in perceiving the transduction during plant innate immunity [91,92]. Based on our RNA-seq data analysis, both EgCML7 and EgCDPK28 were upregulated during necrotrophic phase. Consistent with the analysis, EgERF113 is suggested to orchestrate defense mechanisms through regulation of PR and calcium-responsive genes.

**Other transcription factors differentially regulated under biotrophic or necrotrophic phase**

The Calmodulin-binding transcription activator (CAMTA) gene was first discovered in Nicotiana tabacum, regulating senescence and cell death [93], and was recently comprehensively studied by Kakar et al. [94]. We discovered members of the novel CAMTA TF family, namely EgCAMTA3 and EgCAMTA4 which were downregulated at 3 d.p.i. The suppression of both genes was later reduced across time, which might be the result of infection phase transition from biotrophic to early necrotrophic phase. CAMTA was proven to regulate responses under Ca$^{2+}$ signaling during both abiotic and biotic stresses [95,96].

In general, most downregulated DEGs of TFs showed de-repression across time. It is reasonable to postulate that changes of expression patterns might be the results of plant immunity interplay against biotrophic and necrotrophic infection phases. For instance, downregulation of EgMYB108 was highest at 7 d.p.i before reducing at 11 d.p.i. Coherently, MYB108 was proven to positively regulate defense mechanism against hemibiotroph Verticillium dahliae in the presence of calmodulin and Ca$^{2+}$, antagonistic to the regulation of CAMTA3 [97,98]. In contrast, EgERF9 was found to be downregulated exclusively during necrotrophic phase at 11 d.p.i. The result provides agreement with other study which reported repression activity of AtERF9 in enhanced resistance against necrotrophic Botrytis cinerea [83]. Likewise, expression patterns of TFs in upregulated DEGs were higher at early interaction against G. boninense before decreasing over time. The plant-specific EgTCP15 demonstrated upregulation during biotrophic phase before declining but showing an opposite expression pattern under abiotic stress.

**Conclusions**

Together from the results presented above, we were able to recognize TF genes that were regulated during switching of the fungal mode of infection. With this first analysis of oil palm RNA-seq data encoding TFs,
Six common major families of TFs were identified responsible in promoting oil palm defense response against *G. boninense* attack which include MYB, bHLH, AP2/ERF, NAC, bZIP and WRKY. Reported genes involved in cell wall modification, ROS-mediated signaling, PCD and plant innate immunity were all differentially expressed indicating active regulation of oil palm defense response against the hemibiotrophic *G. boninense*. The biotrophic and necrotrophic infection phases of *G. boninense* was further confirmed through gene expression of biotrophy-specific, *EgJUB1* and necrotrophy-specific, *EgERF113*. Our finding is the first reporting *EgJUB1* as a potent master regulator based on its positive interaction with the imperfect palindromic SNBE consensus sequence which may promote branches of biotrophy-associated defense mechanisms including cell wall strengthening and HR-mediated defense responses. In addition, *EgERF113* is the first AP2/ERF TF reported to modulate multifaceted defense mechanisms through binding to GCC-box and DRE/CRT motifs during necrotrophic phase. Binding to these motifs result in transcriptional upregulation of *PR* and calcium-responsive genes. Based on our findings, a proposed defense mechanism inferring oil palm against hemibiotroph *G. boninense* during biotrophic and necrotrophic infection phases is illustrated as in Fig. 6. The information presents promising first step in recognizing the downstream target defense-related genes regulated by the infection phase-specific TFs.

**Methods**

**Plant materials and fungal treatment**

Four-months old of oil palm seedlings (*Elaeis guineensis* Jacq. *Dura x Pisifera*) were purchased from Sime Darby Plantations, Banting, Selangor, Malaysia. Pathogenic *Ganoderma boninense* strain PER71 was obtained from GanoDROP Unit, Biology Division, Malaysian Palm Oil Board (MPOB). Artificial infection of oil palm seedlings with *G. boninense* using rubber wood blocks (RWBs) was carried out following previous study [5]. Control (C) was set as seedlings without treatment. Two different treatments were carried out; mock treatment (MT) consisted of oil palm seedlings with bare RWBs while *Ganoderma* treatment (GT) involved oil palm seedling treated with *Ganoderma*-inoculated RWBs. Destructive sampling was performed on two pooled biological replicates of oil palm seedlings at different days post inoculation (3, 7 and 11 d.p.i). Each biological replicate consisted of pooled RNA provided equally from six constituent seedlings. Instead of mathematical averaging of individual sample, biological averaging is more cost efficient and commonly practiced in the attempt of reducing high biological variability among samples in RNA-seq studies [99]. Pooling bias can be reduced by using three to eight biological individual samples per pool with two pools per treatment group [100].

**RNA Extraction And DEGs Analysis Of TFs**

Total RNA of all samples was extracted following the method reported in Bahari et al. [5]. The extracted RNA was used in all subsequent experiments. A high-throughput NGS data analysis was performed as described in Bahari et al. [5]. Sequences that are reproducible in both pooled biological replicates were
chosen to eliminate biased profiling of transcripts due to manipulations stages during library construction [101]. Alteration of gene expression profile was analyzed by comparing genes expressed from control with GT samples. DEGs were further evaluated following stringent cut-off values of log$_2$ FC $\geq |1.0|$ (corresponding to 2-fold or more upregulation/downregulation) and $P$-value < 0.01 [5,102]. Comparative analysis was conducted between two biological replicates of control and GT samples at all time points. DEGs of TFs that met the cut-off values were clustered according to upregulated and downregulated genes. Transcripts that were identified in both pooled biological replicates were further analyzed for identification of DEGs. DEGs of a few stress-related genes involving cell wall modification, ROS production and PCD were mined from NGS data analysis.

**Validation By Quantitative Real Time Pcr (qPCR)**

qPCR was performed using qPCR Green Master Mix LRox (2X) (Biotechrabbit GmbH, Germany). Stability of five endogenous controls; *EgGAPDH2, EgNADH5, EgMSD, EgUBQ*, and *Egß-actin* were tested across treated and control samples. The qPCR analysis was performed using Bio-Rad CFX Manager™ Software version 3.1. Expression levels of all target genes were normalized with the expression level of three most stable reference genes which were *EgGAPDH2, EgNADH5* and *Egß-actin*. Real-time PCR was carried out on control (C), mock-treated (MT) and *Ganoderma*-treated (GT) oil palm root samples at 3, 7 and 11 d.p.i. The data included three biological replicates of root samples of oil palm seedlings. Comparative analysis of expression levels was expressed as fold change ± standard error of mean (SEM) of three individual technical replicates at $P$-value < 0.01. Significant differences of expression levels between test groups were determined using one-way analysis of variance (ANOVA) analysis followed by Tukey's test. The primers used for the qPCR are listed in Table 1.
### Table 1
List of primers for quantitative Real-Time PCR (qPCR).

| Target Gene | Sense sequence (5'-3') | Anti-sense sequence (5'-3') |
|-------------|-----------------------|---------------------------|
| EgJUB1      | AATGGAAACTCAGTACCTCAGGC | ATTATCCTTCAAGCTCATCCC    |
| EgERF113    | AGCAGCATAAGTCCAAGG     | GAATAAGGTCTGGGTAGGAGG    |
| EgTCP15     | GACAAAACCCTAACAGGCAATGA | AAATGTAGCCCACAGCATGGG    |
| EgEIN3      | GGAAGGAGAAGGTGAAGTTTGAT | CCATAAGGCTATGCTAATTTG    |
| EgMYB122    | AGTACCAGACAAGCTTGAAGG  | TTACATCCTTAGCTAAACGCGG   |
| Egβ-actin   | GAGAGAGCGTGCTACACTCTCT | CGGAAGTGCCTTGAGATCC      |
| EgNADH5     | GCTCCCTTTATTTGAATACC   | AATAGTTAGAGAGCCGCAAGC    |
| EgGAPDH2    | GAAGGTCATCATATCTGCTCCC | CATCAACAGTCTCTGAGTGGC    |

### Sequence Characterization Of EgUB1 And EgERF113

Sequence analysis was carried out using Basic Local Alignment Search Tool (BLAST 2.9) accessed from [http://www.ncbi.nlm.nih.gov.BLAST](http://www.ncbi.nlm.nih.gov.BLAST). A homology search was carried out using BLASTX algorithm by comparing the translated protein sequence of interest (EgJUB1 and EgERF113) with other protein sequences available in National Centre of Biotechnology Information (NCBI) database. The nucleotide sequences were translated into protein sequences through accessible website, ExPASy Molecular Biology Server ([http://web.expasy.org/translate/](http://web.expasy.org/translate/)). Multiple sequence alignment of protein sequences from different species were carried out using T-Coffee software version 11.0 ([http://tcoffee.crg.cat/apps/tcoffee/do:expresso](http://tcoffee.crg.cat/apps/tcoffee/do:expresso)). NLS was predicted using accessible website of NLS Mapper ([http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi](http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi)).

### Yeast One-hybrid (Y1H) Assay

Coding regions of *EgJUB1* and *EgERF113* (500 ng total RNA) flanking SMART sequences were amplified from GT samples at 3 and 11 d.p.i, respectively, using Q5® Hot Start High-Fidelity 2x Master Mix (New England Biolabs). Purified SMART-EgJUB1 and -EgERF113 were further amplified by long-distance PCR using Advantage® 2 PCR Mix (Takara Bio). Putative tandem repeats of target baits fragments (NACBS, SNBE1, SNBE2, GCC-box and DRE/CRT) as well as the respective mutated fragments were cloned into
pAbAi vector and integrated into yeast genome. Minimal inhibitory concentration (MIC) of Aureobasidin A (AbA) for each bait- and mutant-reporter yeast strain was determined.

Yeast One-Hybrid screening was conducted using Yeastmaker™ Yeast Transformation System 2 (Takara Bio). Components for yeast co-transformation reaction were added in given orders as follows; 2 µg of SMART-EgJUB1 or -EgERF113, 1 µg of pGAD7-Rec AD (SmaI-linearized), 50 µg of denatured yeastmaker carrier DNA, 50 µL of competent yeast cells Y1HGld [pAbAi-baits] or Y1HGld [pAbAi-mutants] and 500 µL of PEG/LiAc solution. Transformations were plated on selective plates SD/-Leu and SD/-Leu/AbA. Transformation control, p53 was plated on SD/-Leu/AbA\textsuperscript{200}. Plates were incubated at 30 °C for 3 days. Conformation of positive clones was performed by colony PCR using Matchmaker Insert Check PCR Mix 2 (Takara Bio) and sent for sequencing using T7 promoter primer. Single colony of positive clone was cultured in synthetic dropout (SD) medium lacking leucine (SD/-Leu) broth to an optical density (OD\textsubscript{600}) of 0.1 and diluted in ten-fold dilution series. From each dilution, 10 µL of yeast culture was spotted on SD/-Leu and SD/-Leu/AbA plates. Oligonucleotides and primers used in Y1H assay are listed in Table 2.

| Target Motifs | DNA element sequences (5' − 3') |
|---------------|---------------------------------|
| NACBS         | GATGCCGTGATGCCGTGATGCCGTGATGCCGT |
| mNACBS        | GATGACGTGATGACGTGATGACGTGATGACGT |
| SNBE1         | TTGTGCTGTGGAAGTTTTTGTGCTGTGGAAGTTTT |
| mSNBE1        | TTGTGGGCTGTGGAAGTTTTTGTGGCTGTGGAAGTTTT |
| SNBE2         | TGGCTTGTGCAAAAAAGTAATGCTTTGTGCAAAAAGTAA |
| mSNBE2        | TGGCTCGTGCAAAAAAGTAATGGCTCGTGCAAAAAGTAA |
| GCC-box       | TAAGAGCCGCCTAAGAGCCGCCTAAGAGCCGCCTAAGAGCCGCCTAAGAGCCGCCT |
| mGCC-box      | TAAGATCCTCCTAAGATCCTCCTAAGATCCTCCTAAGATCCTCCTAAGATCCTCCTAAGATCCTCCTAAGATCCTCCT |
| DRE/CRT       | TGCCGACATGCGACATGCGACATGCGACATGCGACATGCGACATGCGACATGCGACATGCGACAT |
| mDRE/CRT      | TATTTACATTTTACATTTTACATTTTACATTTTACATTTTACATTTTACATTTTACATTTTACATTTTACATTT |

Notes: The tandem repeats of DNA elements of each target are bold and underlined. Point mutations are bold and underlined with red.
Isolation Of EgJUB1 And EgERF113 Nuclear Protein

Single colony of positive clones from Y1H assay was incubated in 5 mL SD/-Leu broth with overnight shaking at 30 °C. Yeast culture was transferred into 45 mL SD/-Leu broth and further grown with overnight shaking (18–20 hrs) at 30 °C until OD<sub>600</sub> reached 0.8-1.0. Yeast cells were pelleted by centrifugation at 1,000 g for 5 min at 4 °C. Pellet was washed with 30 mL ice-cold sterile ultrapure water and centrifuged at 1,000 g for 5 min at 4 °C. Pellet was immediately flash-frozen in liquid nitrogen and ground into fine powder. Extraction of nuclear protein was carried out using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). EgJUB1 and EgERF113 nuclear extracts were stored at -80 °C.

Electrophoretic Mobility Shift Assay (EMSA)

Double-stranded of sense and anti-sense oligonucleotides were biotin-labelled using Biotin 3’ End DNA Labeling kit (Thermo Scientific). Oligonucleotides used are listed in Table 2. Binding reaction system of EMSA was prepared using LightShift EMSA Optimization and Control kit (Thermo Scientific). The binding mixture was resolved in 6% non-denaturing polyacrylamide gel in 0.5X Tris-borate-EDTA (TBE) buffer and transferred to a positively charged nylon membrane. The membrane was cross-linked at 120 mJ/cm<sup>2</sup> for 1 min. The protein-DNA complexes were visualized by LightShift® Chemiluminescent EMSA kit (Thermo Scientific) according to manufacturer’s protocol.

Subcellular Localization

The open reading frames of <i>EgJUB1</i> and <i>EgERF113</i> lacking stop codon were amplified using KAPA HiFi HotStart ReadyMix PCR kit (Thermo Scientific). The gene-specific primers with CACC flanking at 5’end of forward primer are listed in Table 3. The PCR products were transferred into the gateway pENTR/D-TOPO entry vector using the pENTR™ Directional Topo Cloning Kit (Thermo Fisher Scientific). TOPO Cloning Reaction was transformed into One Shot® TOP10 Chemically Competent cells (Thermo Fisher Scientific) to generate entry clone and sequence verified. The expression clones for EgJUB1 and EgERF113 were generated using Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific) by recombination of entry clones into Gateway-compatible destination vector consisting double CaMV 35S promoter (2 × 35S) of pMDC85, respectively. The construction of pMDC85 vector without <i>ccd</i>B gene and insert was used as a negative control. The LR reaction was transformed into One Shot™ OmniMAX™ 2 T1® Chemically Competent cells (Thermo Fisher Scientific).
Table 3
List of primers for vector construction of subcellular localization

| Target Gene | Sense sequence | Anti-sense sequence |
|-------------|----------------|---------------------|
|             | (5’-3’)        | (5’-3’)             |
| EgJUB1      | CACCATGGAGGAGAAGATGGA CAA | AGCGTATCTACATTGACCG TG |
| EgERF113    | CACCATGGAGACCGAGATTAGA ATCC | CTCTTTGGGTGCTAGTTTC TG |

The resulting plasmids of 2 × 35S:GFP (negative control), 2 × 35S::EgJUB1-GFP and 2 × 35S::EgERF113-GFP were sequence verified and transformed into competent Agrobacterium tumefaciens strain LBA4404 [103]. Agrobacterium-mediated transformation of onion epidermal cells was carried out according to Azzeme et al. [104]. Fresh onion scales (1.5 × 1 cm) were immersed into 20 mL Agrobacterium suspension harbouring control and GFP constructs, respectively for 16 h at 28 °C. The onion scales were transferred to a Murashige and Skoog (MS) medium pH 5.8 and further co-cultivated with Agrobacterium for 2 days. The peeled onion epidermal cells were rinsed with sterile water and transferred to glass slides. Fluorescence images were captured using 20X lens of confocal laser scanning microscope (LSM 5 PASCAL EXCITER, Zeiss, Germany) with excitation at 488 nm and analyzed by LSM 5 Image Browser software (Ver. 4.1).

**Statistical Analysis**

For RNA-seq data analysis, DEGs were determined following cut off-values of log₂ FC ≥ |1.0| and P-value < 0.01. Expression levels of each gene from qPCR analysis were normalized by three reference genes; EgGAPDH2, EgNADH5 and Egβ-actin. Data was presented as mean ± standard error of mean (SEM) of three independent technical replicates. Differences of expression level between samples at different time points to control and between group of treatments (MT and GT) were determined by using one-way ANOVA analysis followed by Tukey’s test. Significantly different expression levels as compared to the control were measured according to **P < 0.01, ***P < 0.001 and ****P < 0.0001. ns is defined as not significant. All graphs were generated and analyzed using GraphPad Prism version 5.0 (GraphPad software Inc., USA).

**Abbreviations**
| **A** | **Alanine** |
|-------|-------------|
| AbA   | Aureobasidin A |
| ABA   | Abscisic acid |
| ANOVA | Analysis of variance |
| AP2/ERF | APETALA2/ethylene responsive factor |
| Avr   | Virulence effectors |
| bHLH  | basic helix-loop-helix |
| BLAST | Basic Local Alignment Search Tool |
| bZIP  | basic leucine zipper |
| Ca²⁺  | Calcium ion |
| CAMTA | Calmodulin-binding transcription activator |
| BSR   | Basal stem rot |
| CWDE  | Cell wall degrading enzyme |
| DAMP  | Damage-associated molecular pattern |
| DBD   | DNA-binding domain |
| DEG   | Differentially expressed gene |
| d.p.i | Days post inoculation |
| DREB  | Dehydration responsive element-binding |
| DRE/CRT | Dehydration-Responsive Element/C-Repeat |
| EDTA  | Ethylenediaminetetraacetic acid |
| EIN3  | Ethylene-insensitive 3 |
| EIL   | EIN3-like |
| EMSA  | Electrophoretic Mobility Shift Assay |
| ERE   | Ethylene-Response Element |
| ERF   | Ethylene Responsive Factor |
| ET    | Ethylene |
| ETI   | Effector-triggered immunity |
| F     | Phenylalanine |
| G     | Glutamic acid |
| A                     | Alanine                                                                 |
|----------------------|-------------------------------------------------------------------------|
| GAPDH                | Glyceraldehyde 3-phosphate dehydrogenase                                |
| GT                   | *Ganoderma*-treated                                                     |
| HR                   | Hypersensitive response                                                 |
| HSF                  | Heat stress transcription factor                                         |
| HSP                  | Heat shock protein                                                      |
| Ile                  | Isoleucine                                                              |
| JA                   | Jasmonic acid                                                           |
| JAZ                  | Jasmonic acid-ZIM domain                                                |
| JUB1                 | JUNGBRUNNEN 1                                                           |
| Leu                  | Leucine                                                                 |
| LiAc                 | Lithium acetate                                                         |
| MIC                  | Minimal Inhibitory Concentration                                        |
| MPOB                 | Malaysian Palm Oil Board                                                |
| MT                   | Mock-treated                                                            |
| MYB                  | Myeloblastosis                                                          |
| MYC                  | Myelocytomatosis                                                        |
| NADH                 | Nicotinamide adenine dinucleotide dehydrogenase                         |
| NACBS                | NAC binding site                                                        |
| NCBI                 | National Centre of Biotechnology Information                            |
| NGS                  | Next Generation Sequencing                                              |
| NLS                  | Nuclear localization signals                                            |
| NPR1                 | NONEXPRESSOR OF PATHOGENESIS-RELATED GENE 1                             |
| ns                   | Not significant                                                         |
| OD                   | Optical density                                                         |
| PAMP                 | Pathogen-associated molecular pattern                                   |
| PlantTFDB            | Plant Transcription Factor Database                                     |
| PCD                  | Programmed cell death                                                   |
### Declarations

#### Ethics approval and consent to participate

Not applicable

**Consent to publish**
Availability of data and materials

The sequenced mRNA data was deposited at European Nucleotide Archive with accession number PRJEB27915. https://www.ebi.ac.uk/ena/data/view/PRJEB27915

Competing interests

All authors declare no competing interests.

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Authors’ contributions

SNA conceived and designed the research plan. NMS performed most of the experiments and prepared the manuscript. MNB contributed in RNA-seq data analysis. AMA and NAS provided technical assistance in analyzing assays, Y1H and EMSA. IAS provided fungal material and assisted in artificial inoculation. SNA complemented in the writing of the manuscript. All authors have read and approved the final version of the submitted manuscript.

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Figures
Figure 1

Time course of differentially expressed genes (DEGs) during early interaction with Ganoderma boninense. (a) Heat map clustering of upregulated transcription factors. Colored blocks indicate ascending expression level from green (0) to red (6). (b) Heat map clustering of downregulated transcription factors. Colored blocks indicate descending expression level from green (0) to red (-8). (c) Heat map clustering of upregulated DEGs commonly expressed during biotic interaction. Colored blocks indicate ascending expression level from white (0) to blue (4). For RNA-seq data analysis, two biological replicates which each consisted of pooled RNA provided equally from six constituent seedlings were used. Each heatmap
data was constructed using average of pooled biological replicates. Pairwise comparison of RNA-seq data was evaluated according to cut-off values of log2 fold change (FC) $\geq |1.0|$ and P-value < 0.01.

**Figure 2**

Expression patterns of transcription factors in response to Ganoderma boninense infection at different time points. The expression patterns of each gene was normalized by three most stable reference genes; EgGAPDH2, EgNADH5 and Egß-actin expression levels. Real-time PCR was carried out on control (C), mock-treated (MT) and Ganoderma¬-treated (GT) oil palm root samples at 3, 7 and 11 days post inoculation (d.p.i). The data included three biological replicates of root samples of oil palm seedlings. Error bars represent the mean ± SEM of three technical replicates of each sample. The statistical analyses were performed by comparing expression levels of different treatments at all time points to control using one-way ANOVA analysis followed by Tukey's test. Significantly different expression level as compared to control are measured according to **P < 0.01, ***P < 0.001 and ****P < 0.0001. ns is defined as not significant.
Figure 3

EgJUB1 interacts with secondary wall NAC binding element (SNBE) during defense response against biotrophic infection. (a) Yeast One-Hybrid analysis reveals interaction of EgJUB1 with tandem repeats of
SNBE1 motif. Negative interaction was observed with both NAC binding site (NACBS) and SNBE2 motifs. Transformed yeast cells were cultured on SD/-Leu/AbA†, wherein † denotes optimized concentration of antibiotic Aureobasidin A (AbA) for each motif. (b) EMSA shows direct binding of EgJUB1 to SNBE1 motif. (c) Prediction of binding motifs for EgJUB1 derived from Plant Transcription Factor Database (PlantTFDB) version 5.0. http://planttfdb.cbi.pku.edu.cn/. (d) Putative SNBE sequences identified from 1.5-kb promoters of EgJUB1 direct targets. The number shown on the left of each sequence is the position of first nucleotide relative to start codon. The plus or minus symbol on the right of each sequence indicates first nucleotide of SNBE sequences from forward or reverse strand of the targets DNA. ‡ indicates SNBE1 core motif and § indicates SNBE2 core motif tested in the study. ¶ represents SNBE1 core motif used in the present study, with single nucleotide change. X is defined as isoform.

Figure 4

EgERF113 interacts with both GCC-box and DRE/CRT elements during defense response against necrotrophic infection. (a) Yeast One-Hybrid analysis reveals positive interactions with both GCC-box and DRE/CRT motifs. Transformed yeast cells were cultured on SD/-Leu/AbA†, wherein † denotes optimized concentration of antibiotic Aureobasidin A (AbA) for each motif. EMSA shows specific binding of EgERF113 to (b) GCC-box and (c) DRE/CRT motifs.
Figure 5

Sequence characterization and nuclear localization of EgJUB1 and EgERF113. (a) Multiple sequence alignment of the deduced amino acid sequence of EgJUB1 protein with other JUB1 in different species. Highly conserved NAC subdomains (A to E) are indicated by black lines. (b) Multiple sequence alignment of the deduced amino acid sequence of EgERF113 protein with other ERF113 in different species. Pink shadings indicate specific amino acid for binding to GCC-box and DRE/CRT motifs. Structure based alignment was constructed using mode Expresso of T-Coffee software http://tcoffee.crg.cat/apps/tcoffee/do:expresso. The asterisks indicate fully conserved residues. The colons indicate conservation of strong group. The full stops indicate the conservation of weak group. The dashes indicate no consensus. The red shadings indicate reliable and consistent alignment. Yellow and green shadings indicate average reliability while blue shadings indicate very poor alignment. The putative nuclear localization signal is shown by double-headed arrow below the sequences. The deduced amino acids sequences of JUB1 and ERF113 are derived from different species; Ananas comosus (Ac), Arabidopsis thaliana (At), Asparagus officinalis (Ao), Elaeis guineensis (Eg), Hevea brasiliensis (Hb), Musa acuminate (Ma), Nicotiana tabacum (Nt), Panicum hallii (Ph), Phalaenopsis equestris (Pe), Phoenix dactylifera (Pd), Populus trichocarpa (Pt), Setaria italic (Si), Theobroma cacao (Tc) and Ziziphus jujube (Zj). (c) Subcellular localization of EgJUB1 and EgERF113 in onion epidermal cells. EgJUB1-GFP and EgERF113-GFP were expressed in the nuclei of cells and fluorescence signals were visualized with confocal microscope. Scale bars = 100 µm.
Figure 6

Proposed defense mechanisms of oil palm seedlings against hemibiotroph Ganoderma boninense during biotrophic and necrotrophic infection phases. Based on the NGS data on differentially expressed genes encoding TFs, EgJUB1 and EgERF113 were discovered to regulate specifically under biotic stress during biotrophic and necrotrophic phases, respectively. The EgJUB1 TF binds to SNBE motif and directly regulates EgGAMYB and EgHSFs. The EgERF113 TF binds to GCC-box and DRE/CRT motifs which promotes PR and calcium responsive genes. The oil palm defense pathways are identified based on established defense mechanisms and new findings from this study. Black arrows represent direct regulation by encoded proteins/genes; blue arrows with broken line suggest highly probable defense regulatory pathways; brown arrows indicate activation of downstream targets through binding to respective motifs. The question marks highlight the gaps in oil palm defense mechanism that need to be further elucidated. Ca: calcium; CESA: cellulose synthase; CSLD: cellulose synthase-like D; DAMPs: damage-associated molecular patterns; DRE/CRT: dehydration response element/C-repeat; EIN3: ethylene insensitive 3; ET: ethylene; ETI: effector-triggered immunity; HR: hypersensitive response; HSFs: heat stress transcription factors; HSP: heat shock protein; JA: jasmonic acid; NPR1: NONEXPRESSOR OF PATHOGENESIS-RELATED GENE 1; PR: pathogenesis-related; PRRs: pattern recognition receptors; PTI: PAMP-triggered immunity; secondary wall NAC binding element (SNBE); SA: salicylic acid; TSS: transcription start site.

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