

RESEARCH ARTICLE

## ***In silico* Analysis of *qBFR4* and *qLBL5* in Conferring Quantitative Resistance Against Rice Blast**

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### **Abstract**

The devastating yield losses caused by rice blast can be mitigated by breeding resistant cultivars with QTLs enriched with resistance (R-gene) and defence related genes. These genes are the arsenal of the plant's immune system against pathogen invasion. *qBFR4* and *qLBL5*, are two stable QTLs known to contribute moderate level of resistance against blast disease. In this study we aim to characterize and understand the interconnectivity between the genes within these QTLs by producing a defence model based on the interplay of defence and resistance genes found within these QTLs. To achieve this end we identified defence and R-genes in *qBFR4* and *qLBL5* into functional groups and classes of R-genes, and classified their roles in mounting a defence against pathogens. Blast2GO analysis retrieved the description, gene ontology annotations and domains for 361 genes in *qBFR4* and 617 genes in *qLBL5*. With this, it is concluded that *qBFR4* (2.38 Mbps) has 27 R-genes (7.33%) and 14 (0.04%) defence- genes whereas *qLBL5* (4.1 Mbps) has 25 R-genes (3.88%) and 17 defence genes (0.03%). R-genes and defence genes were classified into domains and functional groups respectively and directed acyclic graphs (DAG) were constructed for both QTLs explaining the role of these QTLs in quantitative resistance against rice blast. In conclusion, *qBFR4* is was found to be more beneficial than *qLBL5* based on the defence and resistance gene composition where SA/JA mediated signalling plays a crucial role in signal transduction between R genes and the defence system. Apart from QTL pyramiding using the QTLs in this study, the major R-genes found within these QTLs can be subjected to cloning to develop resistant cultivars.

**Keywords:** QTL, rice blast, *Magnaporthe oryzae* (*M. oryzae*), R-gene, defence-related genes.

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## INTRODUCTION

Rice has been an essential component of daily dietary intake as it serves as a primary source of nutrients. With almost 90% of rice being consumed by Asians, the increasing demand for rice is inevitable. However, the sustainable production of rice is often unachievable due to yield loss prompted by various factors such as poor management practices, diseases, drought, and flood. In Malaysia, disease like rice blast, sheath blight and bacterial blight cause devastating yield losses. Rice blast, caused by a pathogenic fungus known as *M. oryzae*<sup>1,2</sup>, is the number one disease that causes up to 60% yield loss worldwide. This disease has caused serious constraints in cereal crop production globally and due to its high genetic variability, poses a major challenge to rice breeders and pathologists<sup>3</sup>. Being a hemibiotrophic organism, *M. oryzae* affects the crop at different growth stages and at different parts including leaf, stem, nodes, panicle and root<sup>4</sup>, leading to leaf blast, neck and panicle rot, collar rot and node blast<sup>5</sup>. Common symptoms for this disease are the formation of diamond shaped lesions on the leaves, white to grey-green lesions or spots, with dark green borders on leaf and collar.

Apart from good management practices and application of fungicides, the development of resistant cultivars can curb this issue effectively. Two types of resistance known as qualitative and quantitative resistance are known to exist in resistant cultivars. Qualitative resistance is controlled by a single resistance gene known as R-gene and are race specific. Plant R-genes can detect the presence of foreign entity like pathogens by interacting with pathogen avirulent gene (*Avr*) upon attack and thence initiating an appropriate signalling cascades to activate the defence mechanism in the plant<sup>6,7</sup>. These R-genes are classified into 8 distinct groups and differ in terms of the domain and repeats found in the N-terminal and C-terminal in the R-gene. Most of the R-genes are found with a nucleotide binding site domains (NBS)<sup>8</sup> and leucine rich repeats (LRR) at the C-terminal and with either a toll-interleukin-1-receptor (TIR) domain or putative coiled coil domain at the N-terminal. These R-genes are being increasingly investigated in the rice genome over the past few years and it is evident that most of it are found in rice based on past studies Several

major R genes such as *Pib*, *Pita*, *Pia*, *Pi1*, *Pikh*, *Pi2* and *Pi4* have been successfully introgressed into rice lines through breeding programs<sup>9,10</sup>. Some of these genes *Pi-1*, *Pi2*, *Pi9*, *Pi20*, *Pi27*, *Pi39*, *Pi40* and *Pikh* are reported to have conferred broad-spectrum resistance (BSR) and some of them including *Pia*, *Pib*, *Pij*, *Pi-km*, *Pi-t*, *Pi12* and *Pi19* confer race specific resistance<sup>7</sup>. Unfortunately, as the pathogen constantly evolves into a new race, the existing R-genes become obsolete and may not be so reliable which coerce the breeders to continuously mine for new R-genes to breed for durable and resistant cultivars corresponding to the evolution of the pathogen<sup>11</sup>. Besides, the identification of R-genes with broad spectrum resistance against wide array of pathogen will be an effective measure to eradicate this disease<sup>12</sup>.

Apart from that, disease resistance is also conferred through quantitative resistance which is controlled by a region known as quantitative trait loci (QTL) controlled by several major and minor genes that work together to provide resistance and defence in response to the attack imposed by the pathogen. For the past few years, several QTLs have been characterized based on experimental study on mapping population<sup>13</sup>. These QTLs may be enriched with genes involved in signalling, pathways and processes that interconnected to safeguard the plant from the attacks imposed by pathogens.

Two stable QTLs, namely qBFR4 and qLBLE5 identified from previous studies were selected for analysis. QTL qBFR4 was identified in a F3 mapping population resulting from a cross between Inggoppor-tinawon (IT) (resistant variety) and Koshikari (susceptible variety) with a phenotypic variation of 73.5%. The mapped position of qBFR4 shares the similar position to the *Pi39*-gene. This QTL is situated in a 2.38 Mbps region in chromosome 4 between the marker interval ID04\_15 and RM3843<sup>14</sup>. On the other hand, qLBLE5 was identified from the F2 population of a cross between Akhanaphou (resistant varieties) and Leimaphou (susceptible varieties) for two consecutive years in two different conditions Rajendranagar and Manipur. with a phenotypic variation of 26.23%<sup>15</sup>. The mapping of this QTL revealed that it colocalizes with a meta-QTL reported in another study<sup>16</sup>. This QTL is flanked by the marker interval RM18408 and

RM18882<sup>15</sup> with an approximate size of 4.1 Mbps in chromosome 5. qLBL5 has demonstrated to confer resistance to leaf blast and neck blast in both types of conditions.

With the establishment of rice genomic map (Rice Genome Browser), it is much easier now to locate individual genes that may be involved in blast resistance based on the markers flanking the QTL region. Although a number of R genes and QTLs have been characterized for the past few years, the underlying mechanism on how these major R-genes work together with other defence-related genes is yet to be well elucidated. The intricate characterization of these genes may help in understanding the defence processes in plants. It also may provide an idea on the signalling network within the plant that initiates the defence processes. These may help in deciphering how defence and resistance mechanism work altogether. Apart from that, discovery of more putative R-genes will be a starting point in mining and characterizing novel R-genes. Hence this study aims (1) to identify and classify defence genes and R-genes in qBFR4 and qLBL5 according to functional groups and classes of R-genes, and (2) to explain the role of each defence gene and R-gene in the plant defence system by producing a defence mechanism model. With the characterization of genes along these QTLs and the generation of sufficient information on the defence processes, breeders can assess the suitability of the QTLs in this study to be selected for appropriate rice breeding programs. In addition, key genes found within these QTLs may be useful in marker assisted selection<sup>17</sup>.

## METHODS

### The identification of the physical position of qBFR4 and qLBL-5

Bioinformatics based analysis are employed in retrieving the results for this study. Two QTLs, qBFR4 and qLBL-5 conferring resistance against rice blast were selected from published papers for analysis as these QTLs were found stable across different years and different environment. The forward and reverse primer sequences of SSR flanking markers of qBFR4 (ID04\_15 and RM3843), and qLBL-5 (RM18638-RM18894) were retrieved from the Gramene database ([www.gramene.org/](http://www.gramene.org/)) and subjected to BLASTn in ([\[ncbi.nlm.nih.gov/BLAST/\]\(https://www.ncbi.nlm.nih.gov/BLAST/\)\) with the specification of search set against \*Oryza sativa\* \(taxid:4530\) nucleotide database. Hits with 100% match were selected and the physical positions of the QTLs were determined. The physical positions of qBFR4 \(29308303-31683794\) and qLBL5 \(19724422-23842688\) were viewed in Rice Genome Browser \(\[rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/\]\(http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/\)\) and the first and last locus IDs of qBFR4 \(LOC\\_Os04g49160-LOC\\_Os53210\) and qLBL5 \(LOC\\_Os05g33580- LOC\\_Os05g40650\) were determined. The cDNA sequence in the FASTA format starting from the first locus ID until the last locus ID of the QTLs were downloaded from Rice Genome Annotation Project \(\[rice.plantbiology.msu.edu/\]\(http://rice.plantbiology.msu.edu/\)\).](https://www.</a></p></div><div data-bbox=)

### Proximity analysis of Transposable Elements

To explore the possible influence of transposable elements on both QTLs in conferring resistance against pathogen, the genes in proximity with transposable elements were investigated. The transposable element and genes found in the QTL were used as queries to generate an output file with calculated distance. The output is then filtered to retrieve the distances within 5kb. The visualization of transposable elements distribution with genes in proximity is viewed and captured using Rice Annotation Project Database (RAP-DB) Genome Browser ([rapdb.dna.affrc.go.jp/](http://rapdb.dna.affrc.go.jp/))

### Bioinformatic analysis using Blast2GO

The files with the FASTA sequences of the qBFR4 and qLBL-5 are then uploaded onto Blast2GO software (<https://www.blast2go.com/>) separately for further analysis. BLAST was performed on the uploaded sequences to get the descriptive annotations for a total of 453 genes in qBFR4 and 804 genes in qLBL-5 (Blast2GO > blast). These genes are then subjected to mapping and annotation analysis to obtain the gene ontologies in an attempt to identify genes associated to processes or functions that are associated to defence against fungal pathogens (Blast2GO > mapping > annotation). A function known as enzyme coding was utilized to retrieve the enzyme annotation for the genes (Blast2GO > Analysis > Enzyme Code and KEGG > Run GO-EnzymeCode Mapping). Then, domain analysis was conducted for each annotated gene to identify the presence of domains that are prevalent in R-genes (Blast2GO > Run InterProScan). Finally, a directed

acyclic graph that explains the interconnecting pathways between the defence processes was mapped (Blast2GO > Graphs > Make GO Graph)

## RESULTS AND DISCUSSION

### Overview of the gene distribution in qBFR4 and qLBL-5

A total of 368 genes and 644 genes were represented in the 2.38Mbp region of qBR4 and 4.12Mbp region of qLBL5 respectively. These genes are distributed at an average of 155/Mbp for qBFR4 and 156/Mbp for qLBL5 which shows that the density of genes in both QTLs are more or less the same regardless of the size of QTL region. The BLAST analysis via Blast2GO successfully retrieved the description annotations of 361 genes out of 368 genes in qBFR4 (Table S1) and 617 genes out of 644 in qLBL-5 (Table S2) (Supplementary data Table S1 and S2: [https://drive.google.com/drive/folders/1ZLdMkzWpUKPffZZ\\_Y-msl9ZXD\\_mSFN9M?usp=sharing](https://drive.google.com/drive/folders/1ZLdMkzWpUKPffZZ_Y-msl9ZXD_mSFN9M?usp=sharing)).

Based on the results, transposable elements marks the highest percentage in both QTLs with a composition of 54 genes (14.67%) in qBFR4 and 114 genes (17.7%) in qLBL-5. The abundance of TE in these QTLs suggest the possible impact of TE insertion in driving the evolution of genes and possibly even R-gene or defence-related genes to counteract against the evolution of pathogen. Transposable elements (TE) also known as jumping genes, are mobile cluster of genes that move from one location of the genome to another<sup>18</sup>.

The transposable elements found in the current study were further dissected and two distinct classes of TE were observed. Class I TE known as retrotransposons were found abundantly in both QTLs, with a composition of 42 genes in qBFR4 and 78 genes in qLBL5. Retrotransposons are transposed in a “copy and paste” fashion where the DNA sequences are transcribed to an RNA intermediate which is then subjected to reverse transcription to create an identical DNA sequence and inserted back into the genome at a preferential site<sup>19</sup>. In contrast to retrotransposons, fewer DNA transposons were found in both QTLs. Having said that, a total of 12 DNA transposons were found in qBFR4 and 36 DNA transposon were found in qLBL5. DNA transposon are from class II TE and they are transposed using a

“cut and paste” mechanism by a transposase that cut out the DNA segment from a site and insert it elsewhere in the genome.

TE insertion has a role in altering the regulation of nearby genes that results in differential expression of the genes<sup>20</sup>. Recently, it has been discovered that TE has a role in altering the genome expression that corresponds to the stress exerted on plants<sup>21</sup>. Hence, we are interested in investigating the pattern of insertion of this group of genes and how it may impact the genes in proximity specifically to provide resistance against pathogen. It is known that some R-genes constantly evolve to adapt to the rapidly evolving pathogen and perhaps the insertion of TE in a given position near to R-genes, may drive the alteration in the existing R-genes in accordance to the biotic stress posed.

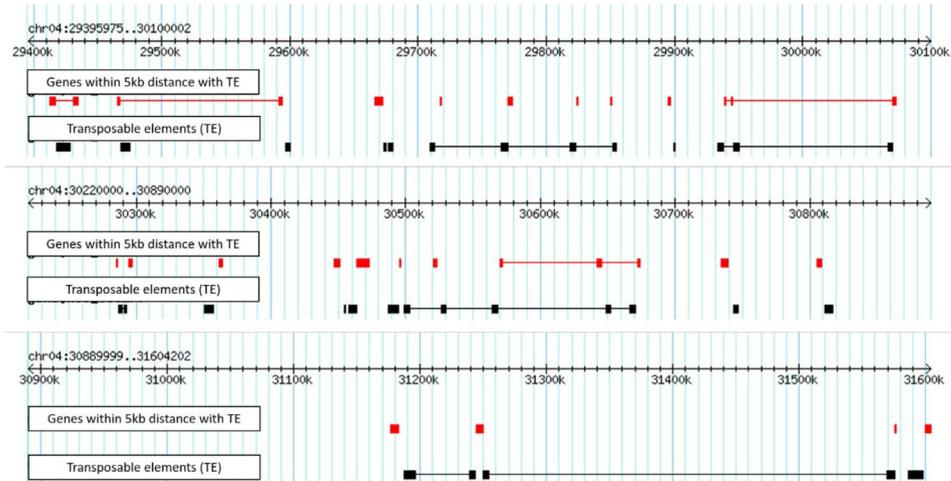
### Identification of genes in proximity with transposable elements

The proximity analysis on qBFR4 and qLBL5 retrieved all the genes within 5kb distance from transposable elements (see Supplementary Table S3 for qBFR4 and Supplementary Table S4 for qLBL5: [https://drive.google.com/drive/folders/1ZLdMkzWpUKPffZZ\\_Y-msl9ZXD\\_mSFN9M?usp=sharing](https://drive.google.com/drive/folders/1ZLdMkzWpUKPffZZ_Y-msl9ZXD_mSFN9M?usp=sharing)). The visualization of TE distribution in qBFR4 and qLBL5 is depicted in Figure 1 and Figure 2 respectively. Out of 29 genes that were situated in proximity with TE in qBFR4, two genes (0.07%) were important in plant defence and resistance against pathogen. These genes were NBS-LRR-like and cathepsin B. NBS-LRR is a domain that is often associated to R-gene whereas, cathepsin B were shown to take part in hypersensitive response following the gene-gene interaction between the pathogen *Cladosporium fulvum Avr4* and the R-gene in tomato known as *Cf-4*<sup>22</sup>.

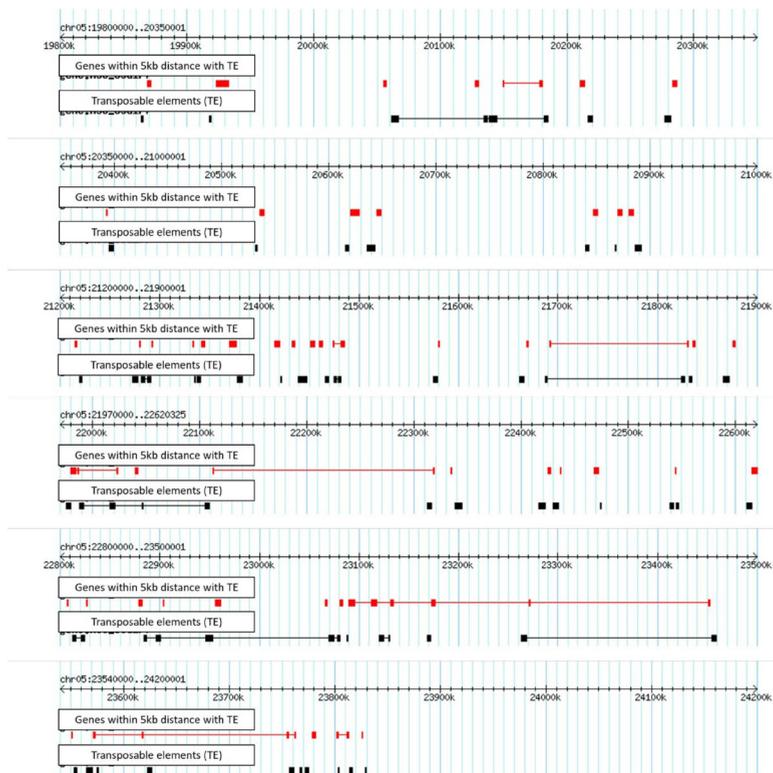
A total of 68 genes in qLBL5 were found to be in proximity with TE and out of these only six genes (0.08%) were associated to defence and resistance against disease. This includes inactive receptor kinase, LRR-receptor kinase, serine/threonine-protein kinase, LRR-F-box containing protein and powdery mildew resistance protein 5 (PMR 5). LRRs are ubiquitously known as an essential R-gene domain which is important in interaction between proteins especially during host-pathogen interaction<sup>6</sup>. Serine threonine

kinase and receptor kinase on the other hand acts as receptors in transducing the signals after pathogen elicitor recognition for downstream defence mechanism<sup>23</sup>. Apart from that, F-box

proteins in Arabidopsis were demonstrated in regulating a novel defence response that is independent of both salicylic acid and systemic acquired resistance<sup>24</sup>. Two PMR 5 genes found



**Fig. 1.** Overview of TE distribution in qBFR4 (red box represent genes within 5kb distance with TE, black box represent TE, line between genes represent tandem repeats of the genes)



**Fig. 2.** Overview of TE distribution in qBFR4 (red box represent genes within 5kb distance with TE, black box represent TE, line between genes represent tandem repeats of the genes)

in proximity with TE were shown to demonstrate resistance against *Erysiphe cichoracearum* through the activation of a novel form of defence<sup>25</sup>.

From past studies, the existence of TE have been indicated in the cluster of disease genes in several plants including rice and shown to be induced by plant pathogens. Wang 2010 have successfully demonstrated the decreased level of resistance against the downy mildew disease after conducting the gene-knockout procedure on a retrotransposon found in Arabidopsis known as AtCOPIA4<sup>26</sup>. In addition, RTE Tnt1A inserted in a tobacco resistance gene cluster has shown to drive partial transcription of the neighbouring disease resistance gene TNLL1<sup>27</sup>. Hence, it can be hypothesized that the insertion of TE in qBR4 and qLBL5 may affect the neighbouring defence related genes or R-genes. Although the impact of TE insertion on the neighbouring defence/R-genes in the current study remains unknown, the findings may provide a basis to conduct a loss of function or gene knockout/silencing studies in future to validate whether the existence of TE among the putative R-genes or defence-related genes affects the resistance against pathogens in plants.

#### Identification and classification of disease resistance genes

The interaction between host plant and pathogen involves the recognition of pathogen effectors known as avirulence genes (*Avr*) by R-genes in host plants which in turn activates the weaponry of the plant defence mechanism<sup>28</sup>. This interaction is well represented in rice, where the *Avr-Pita* gene of *M. grisea* binds to the *Pita* gene, a well-characterised R-gene in rice for the subsequent activation of defence processes<sup>29</sup>. Fundamentally, R-genes are classified into 8 major classes based on the arrangement of domains mainly consisting NBS, LRR and kinases<sup>28</sup>. The results retrieved from InterProScan is analysed to identify genes with possible domains or motifs found in the different classes of R-genes. The results and discussion for this is provided in the following subsection.

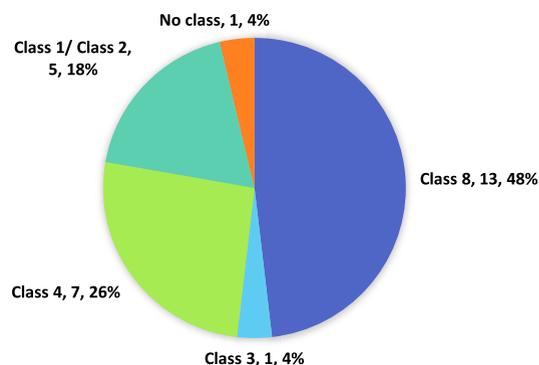
#### Disease resistance genes in qBFR4

The output of the analysis unveiled 27 (7.33%) genes in qBFR4 with domains related to R-genes which has been classified according to relevant classes (Figure 3). Table 1 shows the

locus ID and domains of all the putative R-genes in qBFR4. Based on our results, five genes in qBFR4 have NBS-LRR domains. However, it is unclear if these genes have an additional CC or TIR domain to be classified under class 1 or class 2 R-gene, hence these genes are classed as Class 1/Class 2 in Figure 3. One of the gene with LRR domain maybe a class 3 R-gene (Figure 3). Seven genes with LRR receptor-like serine/threonine kinase that encode protein kinase & LRR domain were classified into Class 4 and out of these seven genes, six have an additional malectin domain and based on a study, a malectin-like leucine-rich repeat receptor-like kinase in Arabidopsis contributes to downy mildew disease resistance<sup>30</sup>. R-genes from class 5-7 were not found in qBFR4. Ser/Thr Kinase and protein kinase domain with Ser/Thr activity were vastly found in qBFR4. Since protein kinase domain is an umbrella term for Ser/Thr Kinase, there is a strong possibility that the protein kinase domain with Ser/Thr Kinase activity is Ser/Thr Kinase domain. Thirteen (13) genes with these domains were classified under Class 8 R-gene. Three genes associated to kinase family did not return any InterProScan results and these gene were classified as putative R-genes without any class.

#### Disease resistance genes in qLBL5

qLBL5 has 25 putative R-genes that makes up about 3.88% of the genes in the QTL (Figure 4). Table 2 shows the locus ID and domains of all the putative R-genes in qLBL5. Four genes with NBS

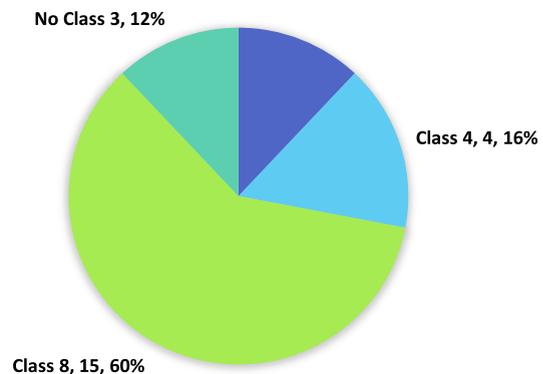


**Fig. 3.** Classification of putative R-genes in qBFR4 according to relevant classes

**Table 1.** List of disease resistance genes in qBFR4

Name/ Locus ID	Description Annotation	Domain
LOC_Os04g49220	Probably inactive receptor kinase At2g46850	No IPR
LOC_Os04g49460	Probable L-type lectin-domain containing receptor kinase	Protein Kinase & Ser/Thr Kin
LOC_Os04g49480	Probable L-type lectin-domain containing receptor kinase	Legume-lectine domain
LOC_Os04g49510	Calcium-dependent kinase	Protein Kinase
LOC_Os04g49690	Receptor kinase FERONIA	Malectin
LOC_Os04g51009	Wall-associated receptor kinase	Protein Kinase
LOC_Os04g51030	Wall-associated receptor kinase	Protein Kinase
LOC_Os04g51040	Wall-associated receptor kinase 5	Protein Kinase
LOC_Os04g51050	Wall-associated receptor kinase 5	Protein Kinase
LOC_Os04g51370	Serine threonine- kinase minibrain	Protein Kinase
LOC_Os04g51580	Plant intracellular Ras-group-related LRR 1	LRR
LOC_Os04g51950	Kinase superfamily	Protein Kinase & Ser/Thr Kin
LOC_Os04g52140	Serine threonine- kinase CTR1 isoform X1	Protein Kinase & Ser/Thr Kin
LOC_Os04g52590	Probable LRR receptor-like serine threonine- kinase At1g56130	Protein Kinase & Malektin & LRR
LOC_Os04g52600	Probable LRR receptor-like serine threonine- kinase At1g56130	Protein Kinase & Malektin & LRR
LOC_Os04g52606	Probable LRR receptor-like serine threonine- kinase At1g56140	Protein Kinase & Malektin & LRR
LOC_Os04g52614	Probable LRR receptor-like serine threonine- kinase At1g56140	Protein Kinase & Malektin & LRR
LOC_Os04g52630	Probable LRR receptor-like serine threonine- kinase At1g56140	Protein Kinase & Malektin & LRR
LOC_Os04g52640	Probable LRR receptor-like serine threonine- kinase At1g56140	Protein Kinase & Malektin & LRR
LOC_Os04g52780	LRR receptor-like serine threonine- kinase FLS2	Protein Kinase & LRR
LOC_Os04g52840	Serine threonine- kinase	Protein Kinase
LOC_Os04g52860	Probable receptor kinase At1g30570	Protein Kinase & Ser/Thr Kin
LOC_Os04g52970	Disease resistance RGA2-like	NB-ARC & LRR
LOC_Os04g53000	Disease resistance RGA2	LRR
LOC_Os04g53030	Disease resistance RGA2-like	NB-ARC & LRR
LOC_Os04g53050	NBS-LRR-like resistance	NB-ARC & LRR
LOC_Os04g53120	NBS-LRR type resistance for bacteria	NB-ARC & LRR

LRR domains were classified into Class 1/Class 2 R-gene. These genes also lack CC or TIR domain which makes it impossible to differentiate these genes into Class 1 and 2. Besides, four genes annotated as LRR receptor-like serine threonine-kinase have been validated for the presence of LRR and protein kinase domain via InterProScan and these group of genes are classified into Class 4. Class 8 R-genes in qLBL5 consists of 16 kinase associated genes that are largely annotated as receptor like kinase, serine threonine kinase, calcium dependent kinase and CBL-interacting kinase. Three unclassified genes annotated as serine threonine receptor-like kinase, serine threonine- kinase and CBL-interacting serine threonine- kinase could be putative R-genes as



**Fig. 4.** Classification of putative R-genes in qLBL5 according to relevant classes

**Table 2.** List of disease resistance genes in qLBL5

Name / ID Locus	Gene Description	Domain
LOC_Os05g33690	LRR receptor-like serine threonine- kinase ERL1	Protein-kinase & LRR
LOC_Os05g34220	Disease resistance RGA3	NB-ARC & LRR
LOC_Os05g34230	Disease resistance RGA3	NB-ARC & LRR
LOC_Os05g34270	Probably inactive leucine-rich repeat receptor kinase At5g48380	Ser-Thr Kin & LRR
LOC_Os05g34390	G-type lectin S-receptor-like serine threonine-kinase At2g19130	Protein Kinase
LOC_Os05g34950	Probable receptor kinase At1g11050	Ser-Thr Kin
LOC_Os05g35760	Serine threonine kinase SAPK5	Protein kinase
LOC_Os05g35770	Serine threonine kinase SAPK4	Protein kinase
LOC_Os05g36050	Probable serine threonine- kinase At1g01540	Protein Kinase
LOC_Os05g36960	Serine threonine- kinase Nek5	Protein kinase
LOC_Os05g38020	Serine threonine receptor-like kinase NFP	No IPR
LOC_Os05g38070	CBL-interacting kinase 27	Protein kinase
LOC_Os05g38770	Receptor-like kinase LIP2	Protein Kinase
LOC_Os05g38800	Serine threonine- kinase ATM	PWWP domain
LOC_Os05g39080	Kinase superfamily	Protein Kinase
LOC_Os05g39090	Calcium-dependent kinase 13	Protein Kinase
LOC_Os05g39860	CBL-interacting serine threonine- kinase 10	No IPR
LOC_Os05g39870	CBL-interacting kinase 11	Protein Kinase
LOC_Os05g39890	CBL-interacting kinase 28	Protein Kinase
LOC_Os05g39900	CBL-interacting kinase 28	Protein Kinase
LOC_Os05g40050	Probable LRR receptor-like serine threonine- kinase IRK	Protein Kinase & LRR
LOC_Os05g40150	Disease resistance RPP13	NB-ARC & LRR
LOC_Os05g40180	Serine threonine- kinase chloroplastic	Protein Kinase
LOC_Os05g40270	Probable LRR receptor-like serine threonine-kinase At1g06840 isoform X1	Protein Kinase & LRR
LOC_Os05g40540	Cyclin-dependent kinase B2-1	Protein Kinase

well. Based on the annotation of these genes, it is suspected that they may be associated to R-gene although it didn't return any results associated to R-gene domains through InterProScan.

Class 8 R-genes that comprise of kinases are prevalent in both QTLs. Receptor-like kinases (RLK) are particularly abundant in this group of kinases. RLK is a pathogen recognition receptor (PRR) that recognizes chitin, a type of elicitor present in especially fungal pathogen<sup>31,32</sup>. It is noteworthy that major diseases in rice like blast and sheath blight are caused by fungal pathogen and perhaps a myriad of RLKs is required in rice to detect the fungal pathogen for efficient activation of defence mechanism to halt the pathogen infection.

#### The identification and classification of defence-related genes

##### Defence-related genes in qBFR4

The mapping and GO annotation via Blast2GO provided the processes and functions carried out by individual genes and relevant genes that carry out defence-related processes

and functions are handpicked for classification into respective functional groups. A total of 14 defence-related genes makes up about 0.04% of genes in qBFR4 (Table 3).

Two subtilisin-like proteases in qBFR4 are involved in signal transduction. Signal perception and transduction is probably the most crucial step to apprise the plant system to flick the switch to defence mode. The significance of subtilisin-like protease in transmitting signalling cascades has been indicated in past studies and were also shown to be expressed upon pathogen inoculation<sup>33</sup>. The pathogen recognition incident happens at the plant extracellular or cell surface and the accumulation of subtilisin-like protease in this location suggest that they may play a pivotal role in transducing the signal pertaining the pathogen recognition<sup>34</sup>. Besides, a nuclear pore complex NUP88 in qBFR4 is associated to immune response. In past studies, NUP88 is indicated in systemic acquired resistance (SAR) mediated by multiple R-genes, thus permitting resistance against wide range of pathogens<sup>35</sup>. The establishment of SAR

**Table 3.** List of defence related genes in qBFR4 with descriptonal and GO annotation

Name/Locus ID	Descriptonal Annotation	GO Annotation	Enzyme Annotation	Functional group
LOC_Os04g49194	DOWNY MILDEW RESISTANCE 6-like	Defence response to fungi	Transferring DMR phosphorus-containing groups	
LOC_Os04g49210	DMR6-LIKE OXYGENASE 1	Defence response to fungi	Transferring DMR phosphorus-containing groups	
LOC_Os04g50750	Subtilisin-like protease	Signal transduction	No	Protease
LOC_Os04g52310	Zinc transporter 3	Response to stimulus	No	Zinc transporter
LOC_Os04g50184	Cathepsin B	Defence response	No	Protease
LOC_Os04g50700	S-norcochlorogenic acid synthase	Defence response	No	PR protein
LOC_Os04g50710	S-norcochlorogenic acid synthase	Defence response	No	PR protein
LOC_Os04g52880	BAG family molecular chaperone regulator 1-like	Cell death	No	Chaperone
LOC_Os04g52890	BAG family molecular chaperone regulator 1	Cell death	No	Chaperone
LOC_Os04g51300	Thylakoid lumenal 29 kDa chloroplastic	Peroxidase activity	Peroxidase	PR protein
LOC_Os04g51900	Nuclear pore complex NUP88	Immune system process	No	Nucleoporin
LOC_Os04g51130	Myb family transcription factor PHL11	Defence response	No	MYB TF
LOC_Os04g52670	Auxin-responsive SAUR71	Response to auxin	No	SAUR
LOC_Os04g52684	Auxin-responsive SAUR71-like	Response to auxin	No	SAUR

is attributable to salicylic acid (SA) mediated signalling, a crucial part of signal transduction for defence responses.

A thylakoid lumenal 29 kDa chloroplastic gene in qBFR4 is enzyme coded as peroxidase and is involved in peroxidase activity. Peroxidases (PR-9) is essential in generating reactive oxygen species<sup>36</sup>. Rapid accumulation of ROS mediated by SA signals observed as one of the earliest event upon pathogen recognition. The involvement of ROS is not only restricted to direct antimicrobial activity but also has been implicated in signalling to establish hypersensitive response and activate other defence-related genes<sup>37</sup>. Two BAG family molecular chaperone regulator 1 in qBFR4 are involved in cell death and response to stress. Rapid cell death in plant cell is a type of hypersensitive response to halt the progression of infection in host plant<sup>38</sup>.

A zinc transporter that responds to stimuli was identified in qBFR4. The occupation of pathogen on host plant triggers some genes to exert response to stimuli. It is proposed that zinc transporter may transport zinc in response to this

type of stimulus to trigger defence responses. A previous study suggested that zinc may play a role as regulatory factor in defence response as zinc is proven to induce JA/ETH signalling pathway which leads to enhanced PAD3 expression to provide resistance against *Alternaria brassicicola* in *Arabidopsis thaliana*<sup>39</sup>.

Apart from that, two auxin-responsive SAUR71 that responds to auxin were also present in qBFR4. Small Auxin Up RNAs (SAURs) is the largest family of auxin response genes. Auxin, an important plant hormone associated to biotic stress, regulates the expression of these genes in response to infection. Ghanashyam & Jain (2009) have shown that several auxin responsive genes which includes SAUR have responded to the attack of *M. oryzae*<sup>40</sup>. Two S-norcochlorogenic acid synthase also regarded as pathogenesis-related proteins (PR proteins) classed as PR-10 protein<sup>41</sup> were identified in current study. PR proteins are widely regarded as antifungal agents and directly associated to defence response which accumulates in host cell in response to SA-mediated signalling upon disease transmission by pathogen.

**Table 4.** List of defence related genes in qLBL5 with annotations and functional groups

Descriptive Annotation	GO Annotation	Enzyme Annotation	Functional group
Autophagy-related 18g	Response to stress	No	Autophagy-related
Glutathione transferase GST 23-like	Signal transduction	Glutathione transferase	GST
MLO 1	Defence response	No	GPCR
Subtilisin-like protease	Signal transduction	Peptidase	Protease
Subtilisin-like protease	Signal transduction	Peptidase	Protease
Glucan endo-1,3-beta-glucosidase 14	Defence response	No	Pathogenesis related protein
Pathogenesis-related 1-like	Defence response	No	Pathogenesis related protein
Zinc finger ZAT8	Response to chitin	No	Zinc finger
Respiratory burst oxidase homolog H	Defence response	Peroxidase	Pathogenesis related protein
Probable WRKY transcription factor 70	Transcription factor activity	No	WRKY TF
Probable WRKY transcription factor 71	Transcription factor activity	No	WRKY TF
ACCELERATED CELL DEATH 6	Signal transduction	No	Plasma-membrane-localized protein
Hydroxyproline-rich glyco-like	Cell wall component	No	HPRG
GPCR	Signal transduction	No	GPCR
Phosphatidylinositol:ceramide inositolphosphotransferase	Response to stress	No	Transferase
Coronatine-insensitive 1	Response to stress	No	F-box protein
Abscisic acid receptor PYL4-like	Signal transduction	No	Receptor

In contrary to the above, a few genes were found to negatively regulate the defence response and increase the susceptibility of host plant to pathogen. On that premise, two genes annotated as downy mildew resistance 6 (DMR 6) were identified in qBRF-4. In a study, DMR 6 were upregulated when it was inoculated with the causative pathogen and only the mutant *dmr6* were shown to increase the salicylic acid (SA) level<sup>42</sup>. This could mean that the defence pathway activated may have been SA independent and not one requiring SA.

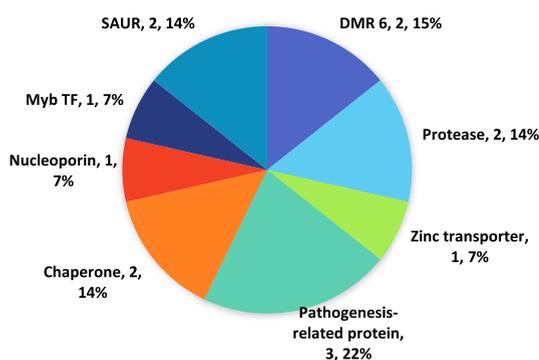
To sum up the above discussion, after pathogen recognition by R-genes in qBFR4, signalling cascades will be relayed and mediated through SA-mediated pathway to induce defence responses such as rapid ROS production, hypersensitive response and systemic acquired resistance (SAR). SAR involves the accumulation of PR proteins, and in the case of qBFR4, where PR-10 protein is involved. Aside from salicylic acid, plant hormone such as auxin may serve as an accessory to regulate the defence response through genes that are responsive to it. As a response to stimuli (pathogen invasion), zinc

transporter may transport zinc to regulate other signalling pathways such as JA/ETH signalling pathway for enhanced resistance. However, the negative regulation of defence response by DMR6 may reduce the resistance against pathogen in the host plant<sup>42</sup>.

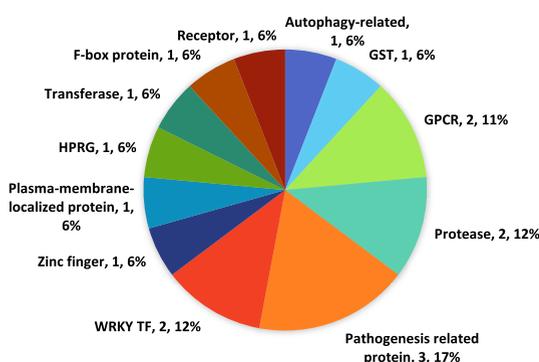
#### Defence-related genes in qLBL5

A total 17 (0.03%) defence related genes were found in qLBL5 and this sum comprises of a few transcription factors, PR proteins, autophagy related genes, and some enzymes.

A G-protein coupled receptor (GPCR) in qLBL5, is an important signal perceiving receptor<sup>43</sup>. As indicated earlier, signal transduction upon pathogen recognition is a prerequisite to actuate the responses from defence-related genes. G-protein coupled receptors (GPCRs) were implicated to mediate the extracellular signal to the intracellular environment for various physiological process including plant defence against pathogen. The signal transmitted by GPCR probably leads to SA mediated signalling pathway. Apart from that, a plasma membrane localized protein known as accelerated cell death 6 in qLBL5 regulates SA signalling pathway preceding



**Fig. 5.** Classification of defence related genes in qBFR4 according to functional groups



**Fig. 6.** Classification of defence related genes in qLBL5 according to functional groups

cell death<sup>44</sup>. By acting in a positive feedback loop with SA signal, it is most definitely involved in the activation of other defence-related genes<sup>45</sup>.

Two subtilisin-like proteases in this QTL may be involved in signal transduction and they are proposed to function as a receptor for pathogen recognition to activate downstream signalling cascades<sup>46</sup> as it was expressed rapidly right before the activation of SA responsive genes<sup>47</sup>. An isozyme in qLBL5 is known as glutathione transferase GST 23-like is also important in eliciting signalling cascades for defence responses<sup>48</sup>. Upon infection from *Colletotrichum destructivum* and *C. orbiculare*, GST genes were induced for transcription in *Nicotiana benthamiana*<sup>49</sup>.

One abscisic acid receptor, PYL4 was discovered in qLBL5 and is implicated in the crosstalk between abscisic acid and jasmonic acid (JA) signalling which elicits the biosynthesis of secondary metabolites when plant's face biotic stress including pathogen invasion<sup>50</sup>. Defence signalling against biotrophic pathogen usually

involve JA-mediated signalling whereas defence against necrotrophic pathogens involves SA-mediated signalling. Being a hemibiotroph, *M. oryzae* sustains its biotrophic lifestyle in living tissue and necrotrophic lifestyle in dead plant tissue. Hence, it is possible that both SA and JA mediated signalling utilized interchangeably by the host plant may be able to protect itself against a hemibiotroph like *M. oryzae*<sup>51</sup>.

The current study identified one peroxidase, annotated as respiratory burst oxidase homolog H suggesting the involvement of ROS in the defence system against fungal pathogen in qLBL5. These ROS species are often associated to hypersensitive response and also suggested to enhance other SA mediated defence response<sup>52</sup>. The production of the ROS species is largely contributed by peroxidase<sup>36</sup>. In a study, the formation of lignin to protect the cell wall of reed canary grass following the fungal penetration spiked the peroxidase activity level<sup>53</sup>.

Two WRKY transcription factor namely WRKY 70 and WRKY 71 in qLBL5 may carry out transcription activity to regulate expression of the defence-related signal and process. As a matter of fact, an increased expression of 15 WRKY genes in *Oryza sativa japonica* were observed upon the incompatible reaction between *M. grisea* and the host. Besides, WRKY 70 was reported to serve as a cross talk component in plant defence signalling network by activating SA mediated signals and repressing JA mediated signals<sup>54</sup>. The overexpression of WRKY71 in rice has upregulated the expression of defence-related genes induced by elicitors<sup>55</sup>.

Aside from that, one MLO1 gene in qLBL5 is directly associated to defence response against pathogen by preventing the penetration of fungus into the epidermal cell wall, and subsequently governs several processes for cell wall fortification. It may be regulated by Ca<sup>2+</sup>-dependent calmodulin binding and does not require heterotrimeric G proteins for signal transduction. Defence response of MLO1 varies between different pathogens where a homozygous mutant *mlo* in barley showed resistance towards powdery mildew but found susceptible in a very severe manner to *M. oryzae*, the causative pathogen of rice blast and vice versa for wild type MLO<sup>56</sup>. This shows that the wild type MLO1 is resistance or less susceptible to *M. oryzae*.

Three genes in qLBL5 responds to stress posed as pathogen infection by various means. An autophagy-related gene in qLBL5 executes cellular self-digestion to restrict the overspreading of pathogen-induced cell death to uninfected area. In the rice blast fungus *M. oryzae*, autophagic cell death is required for degradation of conidia and thus fungal pathogenicity<sup>57</sup>. Along with that, coronatine-insensitive protein 1 regulates the expression of plant genes during plant-pathogen interactions against *Pseudomonas syringae* and *Alternaria brassicicola* and also required for JA-mediated defence processes<sup>58, 59</sup>. One gene annotated as phosphatidylinositol:ceramide inositolphospho-transferase modulates plant programmed cell death (PCD) which has been indicated in Arabidopsis in conferring defence against *Golovinomyces cichoracearum*<sup>60</sup>.

Two PR proteins known as PR-1 and PR-2 were also found in qLBL5. PR-1 genes (*OsPR1a* and *OsPR1b*) were reported to be expressed in rice after blast infection<sup>8</sup> as a consequence of ROS activation<sup>61</sup>. Apart from that, (PR-2 protein) comprises of beta-1,3-glucanase carries out anti-fungal activity by hydrolysing the 1,3-β-D-glucosidic linkages in β-1,3-glucans, an important fungal cell wall structural component<sup>62</sup>.

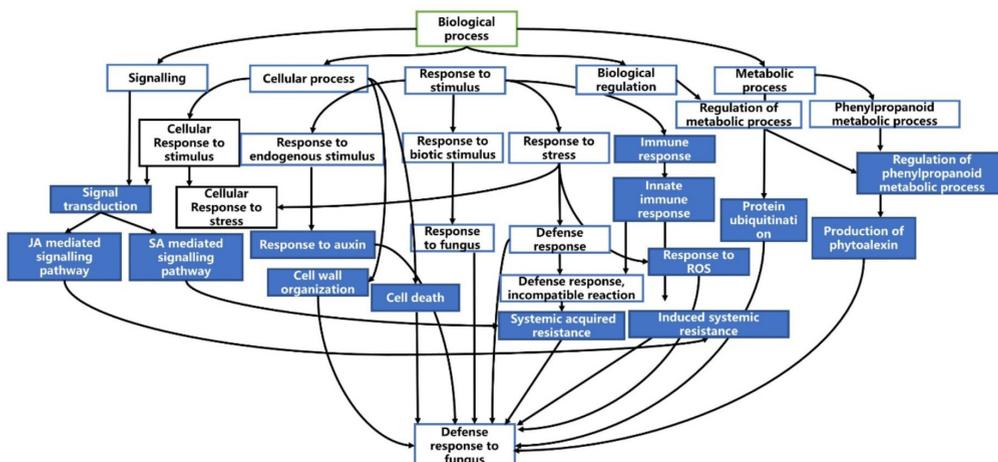
Briefly, the defence mechanism in qLBL5 is akin to qBFR4 to some extent. In addition to the ROS production, hypersensitive response and accumulation of PR proteins initiated by SA. In addition to the above strategies, response to

stress is also triggered after pathogen invasion which is exhibited through cellular digestion, cell death and regulation of defence-gene expression. The defence process ends with the regulation of defence gene-expression which is modulated through activity of transcription factor activity.

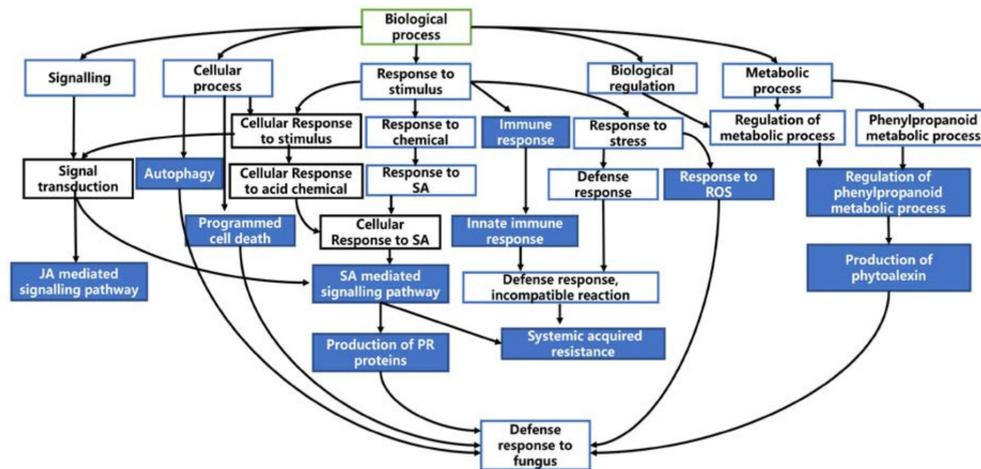
**The defence mechanism model using directed acyclic graph**

Directed acyclic graph (DAG) was produced using Blast2GO to visualize the gene ontologies associated with defence response. This graph portrays hierarchical structure of functional annotation related to defence processes.

Figure 7 and 8 shows the summarized version of DAG for biological processes related to defence for qBFR4 and qLBL5 respectively. Based on the DAG constructed, when the pathogen instigates infection, the host will mount the first line of defence through pathogen-associated molecular patterns (PAMP) triggered immunity (PTI), which happens at the cell surface. To vanquish the host PTI, the pathogen will produce effectors/elicitors and this will be recognized by R-gene in host which in turn activates the effector-triggered immunity<sup>30</sup> or also known as gene-gene resistance. ETI is mediated by R genes, such as NBS-LRR, receptor-like kinases (RLK) and serine/threonine kinase. After pathogen recognition and the incompatible reaction of R-gene with *avr* gene in pathogen, these R-genes will initiate and transduce signal to alarm the defence system in the host plant.



**Fig. 7.** Directed acyclic graph for biological processes related to defence in qBFR4



**Fig. 8.** Directed acyclic graph for biological processes related to defence in qLBL5

The signal transduction from R-genes will be relayed to other signal transmitting molecules for the activation of other defence responses. SA and JA mediated signalling pathway may be activated as a consequent of pathogen recognition. SA is a plant immune signal that induces systemic acquired resistance (SAR) whereas JA induces induced systemic resistance (ISR). SA-mediated signalling induces ROS production probably via peroxidase that carries out peroxidase activity. This will cause oxidative burst and some genes may respond to this oxidative stress for subsequent defence process. Thereafter, hypersensitive response (HR) will take place and this event is manifested as rapid cell death. Along with that, SAR activated through SA signal will lead to the production of pathogenesis-related (PR) proteins such as S-norcochlorogenic acid synthase (PR-10) in qBFR4, PR-1 and beta-1,3-glucanase (PR-2) in qLBL5 which carry out anti-fungal activity in response to stress.

With the signal transmission, responses to stimuli, stress and plant hormone like auxin will be activated for the regulation of defence responses. Response to stress is also represented through cell death and cellular self-digestion (autophagy). The transcription activity conducted by the transcription factors like WRKY and MYB may regulate the expression of other defence-related genes<sup>63</sup>.

**CONCLUSION**

Overall, it is evident that both QTLs exhibit a fairly low proportion of genes related to defence

and resistance which may explain why they confer moderate levels of resistance to plants. However, qBFR4 (0.11%) has relatively higher percentage of R-genes and defence-related genes as compared to qLBL5 (0.07%). This suggests that qBFR4 is more beneficial and efficient than qLBL5 as it gives a better coverage of defence related genes for a small QTL region. In concordance with this, it is proposed that qLBL5 has to be fine-mapped to eliminate genes that are not of interest to reduce unnecessary drag effect during breeding. In terms of R-genes, RLKs from Class 8 R-genes were vastly found in both QTLs which is attributable to ability of RLK to recognize chitin in fungal pathogen which is the causative agent for the major diseases in rice. The characterization of genes in both QTLs gave a rough outline on resistance mechanism in both QTLs which sets off with pathogen recognition by R-genes and subsequent signalling mediated through several pathways such as SA and JA for the activation of downstream defence processes. Major R-genes found along the QTLs such as disease resistance RGA2 and RGA4 in qBFR4 and disease resistance RGA3 and RPP13 may be utilized in various molecular techniques such as cloning to develop resistant cultivar. The broad spectrum resistance given by qBFR4 and qLBL5 can be used to maximum potential through QTL pyramiding technique with other well characterized QTLs such as qSBR11-1<sup>64</sup> and qShb9-2<sup>63</sup> to confer multiple disease resistance (MDR) towards rice blast and sheath blight.

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