



IDENTIFICATION OF CYTOCHROME P450 GENES AND DOWNREGULATED KNOCKOUT CANDIDATES FOR ENHANCING SHEATH BLIGHT RESISTANCE IN RICE (*ORYZA SATIVA L.*)

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Sheath blight (ShB), caused by *Rhizoctonia solani*, is a devastating fungal disease of rice (*Oryza sativa L.*) leading to substantial yield losses. Despite extensive breeding efforts, the development of complete resistance remains a major challenge. Cytochrome P450 monooxygenases (CYPs), a large and functionally diverse superfamily, are known to participate in hormone metabolism, secondary metabolite biosynthesis, and detoxification processes, playing vital roles in plant defense responses. In this study, a comparative transcriptomic, *R. solani* data and their phylogenetic analysis of CYP genes was conducted across resistant (Tetep, Pankaj) and susceptible (TN1, BPT5204) rice cultivars. Differentially expressed CYP genes (DEGs) were identified in RNA-seq data, followed by protein sequence retrieval and phylogenetic clustering to elucidate their evolutionary distribution and functional divergence. Several CYP families, including CYP71, CYP76, CYP94 and CYP716, exhibited significant differential regulation between resistant and susceptible cultivars. Resistant genotypes (e.g., Os03g30227700-01, Os02g07305600-01/-02, Os01t08840400-01, Os02g0666500-01, Os08g0262500-01), exhibited several large, tightly clustered clades, indicating gene expansion and diversification, neofunctionalization and potential involvement in secondary metabolite biosynthesis and detoxification pathways. In contrast, susceptible genotypes displayed fewer and less compact clades, reflecting limited diversification of defense-associated CYP genes. Among the identified CYP genes in resistant genotypes, four genes such as CYP71Z2 (OS07G0217600), CYP94D7 (OS01G0804400), CYP71T2 (OS01G0227500), and CYP90B2 (OS03G0227700) showed downregulation under infection conditions and CYP71Z2 (OS07G0217600) is the only gene that is consistently downregulated in both the susceptible and the resistant genotypes. These genes are promising targets for CRISPR/Cas9-mediated knockout or genome editing studies to evaluate their functional roles in sheath blight resistance. These findings provide insight into the evolutionary specialization of defense-related CYPs and highlight promising genetic targets for improving disease resistance in rice.

ABSTRACT

Sheath blight (ShB), caused by *Rhizoctonia solani*, is a devastating fungal disease of rice (*Oryza sativa L.*) leading to substantial yield losses. Despite extensive breeding efforts, the development of complete resistance remains a major challenge. Cytochrome P450 monooxygenases (CYPs), a large and functionally diverse superfamily, are known to participate in hormone metabolism, secondary metabolite biosynthesis, and detoxification processes, playing vital roles in plant defense responses. In this study, a comparative transcriptomic, *R. solani* data and their phylogenetic analysis of CYP genes was conducted across resistant (Tetep, Pankaj) and susceptible (TN1, BPT5204) rice cultivars. Differentially expressed CYP genes (DEGs) were identified in RNA-seq data, followed by protein sequence retrieval and phylogenetic clustering to elucidate their evolutionary distribution and functional divergence. Several CYP families, including CYP71, CYP76, CYP94 and CYP716, exhibited significant differential regulation between resistant and susceptible cultivars. Resistant genotypes (e.g., Os03g30227700-01, Os02g07305600-01/-02, Os01t08840400-01, Os02g0666500-01, Os08g0262500-01), exhibited several large, tightly clustered clades, indicating gene expansion and diversification, neofunctionalization and potential involvement in secondary metabolite biosynthesis and detoxification pathways. In contrast, susceptible genotypes displayed fewer and less compact clades, reflecting limited diversification of defense-associated CYP genes. Among the identified CYP genes in resistant genotypes, four genes such as CYP71Z2 (OS07G0217600), CYP94D7 (OS01G0804400), CYP71T2 (OS01G0227500), and CYP90B2 (OS03G0227700) showed downregulation under infection conditions and CYP71Z2 (OS07G0217600) is the only gene that is consistently downregulated in both the susceptible and the resistant genotypes. These genes are promising targets for CRISPR/Cas9-mediated knockout or genome editing studies to evaluate their functional roles in sheath blight resistance. These findings provide insight into the evolutionary specialization of defense-related CYPs and highlight promising genetic targets for improving disease resistance in rice.

Keywords: *Oryza sativa*, *Rhizoctonia solani*, Cytochrome P450, Sheath blight, Differential gene expression, Phylogeny, CRISPR.

Introduction

Rice (*Oryza sativa L.*) is one of the most important staple food crops worldwide, feeding more than half of the global population and serving as a major source of calories in Asia (Khush, 2013).

Sustainable rice production is critical to ensuring global food security; however, various biotic and abiotic stresses severely constrain yield potential. Among rice diseases, sheath blight (RSB), caused by the necrotrophic, soil-borne basidiomycete *Rhizoctonia*

solani Kühn, is considered one of the most destructive, resulting in yield losses of up to 50% under favorable environmental conditions across major rice-growing regions of China, India, and Southeast Asia (Naveenkumar *et al.*, 2023; Zheng *et al.*, 2019). Due to the complex quantitative nature of sheath blight resistance and the lack of highly resistant germplasm, breeding for durable resistance remains a major challenge.

Traditionally, sheath blight management has relied heavily on chemical fungicides; however, their prolonged use leads to environmental contamination, increased input costs, and the emergence of fungicide-resistant pathogen populations (Kumar *et al.*, 2020). Consequently, enhancing genetic resistance has become a central objective in modern rice improvement programs. To achieve effective and durable disease control, the pyramidization of multiple resistance (R) genes and the exploration of novel candidate defense genes have gained increasing attention (Leng *et al.*, 2023).

Cytochrome P450 monooxygenases (CYP450s or CYPs) represent one of the largest gene superfamilies in plants, comprising approximately 1% of the total plant genome (Maeda *et al.*, 2019). These heme-thiolate enzymes catalyze a broad spectrum of oxidation-reduction reactions involved in the biosynthesis and metabolism of primary and secondary metabolites, including lignin, flavonoids, terpenoids, alkaloids, and phytohormones. Consequently, CYPs play critical roles in plant development, stress tolerance, and defense modulation. Several CYPs have been implicated in the biosynthesis of defense-related secondary metabolites and phytohormones such as jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA), which coordinate plant immune responses against pathogen attack (Mizutani and Ohta, 2010; Wang *et al.*, 2022). In rice, specific CYP members including *CYP701A8*, *CYP71Z7*, and *CYP716A16* have been reported to participate in defense-related metabolic pathways, contributing to enhanced resistance against *R. solani* infection (Wang *et al.*, 2022).

The interplay between phytohormone signaling and pathogen response forms a well-orchestrated component of plant defense networks. Upon infection, induction of JA, ethylene (ET), and indole-3-acetic acid (IAA) pathways reinforces resistance against necrotrophic pathogens such as *R. solani* (Poonguzhali *et al.*, 2022; Rao *et al.*, 2020). These hormones modulate gene expression cascades that activate downstream defense responses, often mediated by CYP enzymes involved in hormone biosynthetic routes and

detoxification processes. Thus, investigating CYP gene expression under pathogen infection offers significant insights into the metabolic reprogramming and defense adaptation of rice.

Recent advances in high-throughput transcriptomic technologies have facilitated the identification of differentially expressed genes (DEGs) associated with disease resistance. Comparative transcriptome profiling between resistant (e.g., Tetep, Pankaj) and susceptible (e.g., TN1, BPT5204) cultivars under sheath blight infection enables the elucidation of CYP genes potentially contributing to varying resistance levels (Naveenkumar *et al.*, 2023). Moreover, phylogenetic characterization of rice CYP families aids in tracing their evolutionary diversification and functional specialization, providing a framework for identifying lineage-specific defense-related genes. Integrating transcriptomic expression patterns with phylogenetic insights can therefore accelerate the functional validation of key CYP candidates and support molecular breeding strategies aimed at improving sheath blight resistance in rice.

Materials and Methods

Plant Material and Data Source

Four rice (*Oryza sativa* L.) cultivars differing in resistance to *Rhizoctonia solani* were selected for this study *ie.*, TN1 and BPT5204 (susceptible) and Tetep and Pankaj (resistant/tolerant). These cultivars have been widely characterized for their contrasting responses to sheath blight, making them suitable models for comparative transcriptomic analyses (Marchetti and Bollich, 1991; Prasad and Eizenga, 2008). RNA-Seq data corresponding to both infected and uninfected control samples were retrieved from publicly available repositories such as the NCBI Sequence Read Archive (SRA) and Rice Expression Database (RED). High-quality reads were aligned to the *Oryza sativa* reference genome (MSU Rice Genome Database, Release 7) using HISAT2 v2.2.1 (Kim *et al.*, 2019).

Identification of Differentially Expressed CYP Genes

Differential gene expression analysis was performed using the DESeq2 package (Love *et al.*, 2014) in R (v4.3.1). Genes exhibiting an absolute \log_2 fold change (\log_2FC) ≥ 1 and an adjusted p -value ≤ 0.05 (Benjamini-Hochberg FDR correction) were considered differentially expressed. Cytochrome P450 genes were annotated using the MSU Rice Genome Annotation Project (Release 7) and validated using qRT-PCR. The expression of CYP genes showing consistent up- or downregulation patterns in resistant

(Tetep and Pankaj) compared to susceptible (TN1 and BPT5204) cultivars are considered as significant DEGs potentially associated with sheath blight resistance mechanisms.

Sequence Retrieval

Coding sequences (CDS) and corresponding protein sequences of the identified CYP genes were retrieved from both the MSU Rice Genome Database and Ensembl Plants (Howe *et al.*, 2020). The physicochemical properties of the encoded proteins, including molecular weight, theoretical isoelectric point (pI), instability index, aliphatic index, and GRAVY score, were computed using the ExPASy ProtParam tool (Gasteiger *et al.*, 2005).

Phylogenetic Analysis

Multiple sequence alignments of CYP protein sequences were carried out using MAFFT v7.505 (Katoh and Standley, 2013) with the E-INS-i strategy optimized for sequences with conserved motifs and variable-length regions. Phylogenetic trees were constructed using the IQ-TREE v2.2.0 software (Nguyen *et al.*, 2015) based on the Maximum Likelihood (ML) method with 1000 bootstrap replications to assess the reliability of clades. The best-fitting substitution model (JTT+G) was automatically determined through the ModelFinder algorithm implemented in IQ-TREE. Evolutionary relationships among CYP proteins were visualized and annotated using the Interactive Tree of Life (iTOL v6) platform (Letunic and Bork, 2021).

This combined computational workflow provided a robust characterization of CYP gene family members potentially involved in the rice defense response to *R. solani*.

Results and Discussions

Identification of Differentially Expressed CYP Genes

By comparing *R. solani* data in rice and transcriptomic data, a total of more than 20 CYP genes were expressed in both susceptible (TN1 and BPT5204) and resistant (Tetep and Pankaj) genotypes. These genes were differentially expressed among themselves and in between resistant and susceptible cultivars under *R. solani* infection. Among them, four genes (OS07G0217600, OS01G0804400, OS01G0227500, OS03G0227700) were commonly downregulated in resistant cultivars (Tetep, Pankaj) (Table 2) (Figure 4), while 8 genes (OS07G0217600, OS09G0530300, OS06G0294600, OS03G0760000, OS12G0268000, OS10G0525200, OS02G0703600) were commonly showed downregulation in susceptible

cultivars (TN1, BPT5204) (Table 1) (Figure 3). Major families in resistant lines included CYP71 (secondary metabolism, defense compounds), CYP76 (oxidative reactions, JA metabolism), CYP94 (JA-Ile catabolism), CYP99 and CYP701 (diterpenoid phytoalexin synthesis), CYP716 (triterpene biosynthesis). Most DEGs belonged to the CYP71 clan, followed by CYP70, CYP94, and CYP87 clans.

Identified CYP genes showed diversification between resistant and susceptible rice genotypes. Resistant lines (Tetep and Pankaj), known for strong responses to *Rhizoctonia solani*, displayed large and well-defined clades, reflecting selective pressure for diversification of cytochrome P450-mediated defense functions (Wang *et al.*, 2022).

Phylogenetic Clustering analysis of CYP Genes in Resistant and Susceptible Genotypes

A comparative phylogenetic analysis of CYP gene families was performed using resistant genotypes Tetep and Pankaj, and susceptible genotypes TN1 and BPT5204. Clear differences were observed in the clustering patterns of CYP genes between the two groups.

Clade Structure in Resistant Genotypes (Tetep & Pankaj)

The resistant genotypes exhibited several large, tightly clustered clades, suggesting the presence of lineage-specific gene expansion. Prominent clusters included a major upper clade containing Os03g30227700-01, Os03g3225900-01, Os03g365688 00-01, Os11t0289700-01, Os02g07305600-01/-02, Os01t08840400-01, Os02g0666500-01, Os01t0525000-01, Os08g0262500-01. A mid-level clade consisting of Os01t0513900-01, Os10t0139700-01, Os06g0680 700-01. Additional clusters including Os03g30570100-01, Os07g0217600-01, Os02g0570500-01, Os01t0144 700-00, and a lower clade comprising Os01t0227500-01, Os12t0268000-01, Os05g0424300-01, Os06g0102 100-01. A small but conserved clade with Os04g0178400-01 and Os06g0294600-02 (Figure 2). These clusters indicate functional specialization and potential roles in defense-associated metabolic pathways.

Previous results were observed by Waseem *et al.* 2021, that Resistant rice lines show unique clusters of CYP genes, such as Os02g07305600, Os01t08840400, and Os02g0666500, which are absent in susceptible varieties, indicating gene duplication followed by neofunctionalization. Zheng *et al.*, 2025 observed that overexpression of rice CYP genes (e.g., CYP716A16 and cyt02) enhances resistance to sheath blight and

other pathogens, supporting roles in detoxification and secondary metabolite biosynthesis pathways.

Clade Structure in Susceptible Genotypes (TN1 & BPT5204)

Susceptible genotypes showed fewer and smaller clades, with reduced clustering compactness. Key groups included are an upper paired clade of Os03g0568800-01 and Os12t0139300-01. A conserved cluster of Os03g3225900-02 and Os07g0217600-01. A large central clade containing Os02g0185200-01, Os09t06530300-01, Os12t0268000-01, Os03g307600-01, Os02g06030900-01, Os06g0294600-02. A lower functional pair of Os10t0513900-01 and Os10t0525200-01 (Figure 1). The reduced cluster expansion in susceptible varieties suggests diminished diversification of defense-related CYP genes.

Conserved Clades Shared Between Resistant and Susceptible Genotypes

Across both trees, the genes consistently clustered together are Os06g0294600-02, Os12t0268000-01, Os07g0217600-01, Os10t0513900-01, Os03g3225900 (paralogous forms). These represent evolutionarily stable and likely essential CYP genes. Certain genes formed unique clades only in resistant genotypes such as Os02g07305600-01 / -02, Os01t08840400-01, Os08g0262500-01, Os02g0666500-01. These genes are absent or weakly clustered in susceptible genotypes, indicating expansion or specialization of defense pathways in resistant varieties.

Similar results were observed previously by Rai *et al.*, 2015 showed that genes that cluster similarly in both resistant and susceptible trees, like Os12t0268000-01 and Os06g0294600-02, tend to perform core biochemical tasks vital to plant health, not directly linked to disease resistance. Wang *et al.*, 2022 observed that disruption of conserved clades may result in pleiotropic effects, as these CYP genes are necessary for fundamental metabolism and not ideal candidates for knockout experiments.

Best Genes for Functional Knockout or knock-in Testing

The only genes consistently downregulated (and thus most suitable for knockout in both genotypes) are CYP71Z2 (OS07G0217600), CYP94D7 (OS01G0804400), CYP71T2 (OS01G0227500), and CYP90B2 (OS03G0227700) based on combined phylogenetic clustering and transcriptomic studies. These genes show negative fold change and downregulation in both Tetep and Pankaj rice varieties, making them prime candidates for functional knockout experiments. CYP71Z2 (OS07G0217600) is clustered

distinctly, forming its own branch, indicating it belongs to a separate clade. CYP94D7 (OS01G0804400) and CYP71T2 (OS01G0227500) are close together on the tree, suggesting they are in the same clade or closely related clades. CYP90B2 (OS03G0227700) branches off separately, indicating a different phylogenetic group. Cross-genotype analysis identified CYP71Z2 (OS07G0217600), CYP94D7 (OS01G0804400), CYP71T2 (OS01G0227500), and CYP90B2 (OS03G0227700) as reliably downregulated candidates belonging to three distinct phylogenetic clades, thus narrowing targets for gene knockout experiments focusing on core cytochrome P450-related defense pathways in rice.

Interestingly, CYP71Z2 (OS07G0217600) is the only gene that is consistently downregulated in both the susceptible genotypes (TN1 and BPT-5204) and the resistant genotypes (Tetep and Pankaj). This gene is therefore the effective candidate for knockout studies targeting core defense pathways since its function is suppressed in every tested genotype and condition. CYP71Z2 appears as a distinct branch in both phylogenetic trees, indicating that it forms a separate clade or lineage among the cytochrome P450 genes in rice. This spectral separation supports its evolutionary and functional uniqueness, further justifying its selection for knockout experiments focused on dissecting cytochrome P450-mediated defense responses.

Similar results were observed by Zheng *et al.*, 2025 says that resistant-specific clades are excellent candidates for CRISPR knockout studies to validate their involvement in sheath blight resistance. Knockout approaches can clarify the functional role of these genes in resistance mechanisms, while overexpression in susceptible lines could offer improved defense traits.

Conserved genes present across both genotype groups should be excluded from knockout testing due to their fundamental metabolic functions (Rai *et al.*, 2015) due to essential roles and lack of involvement in differential resistance.

Conclusion

Comparative expression and phylogenetic analyses revealed that specific CYP families are key contributors to sheath blight resistance in rice. The comparative phylogenetic analysis clearly demonstrates that resistant rice genotypes (Tetep and Pankaj) possess unique, expanded, and tightly clustered CYP gene families that are absent in susceptible genotypes (TN1 and BPT5204). These resistant-specific clades suggest functional specialization driven by evolutionary adaptation to

pathogen pressure. The genes CYP71Z2 (OS07G0217600), CYP94D7 (OS01G0804400), CYP71T2 (OS01G0227500), and CYP90B2 (OS03G0227700) emerge as strong candidates for functional validation using genome editing. Their targeted knockout in resistant genotypes or over expression in susceptible genotypes can help determine

their specific roles in sheath blight resistance. Conserved CYP genes, in contrast, are not suitable for knockout due to their essential metabolic functions. Overall, the phylogeny supports the hypothesis that diversification of CYPs contributes significantly to the enhanced disease resistance in Tetep and Pankaj.

Table 1: Expression data of different CYP genes in susceptible genotypes (TN1 and BPT-5204)

Gene	Gene ID	Fold Change (TN1)	Padj (TN1)	Expression (TN1)	Fold Change (BPT-5204)	Padj (BPT-5204)	Expression (BPT-5204)	Function
CYP87C2	OS03G0658800	-9.0235	1.74E-18	Down	8.1018	1.24E-10	Up	Cytochrome P450 family protein
CYP71Z2	OS07G0217600	-4.0952	0.000287	Down	-6.202	1.05E-09	Down	Putative Cytochrome P450 71D7
CYP71AK2	OS09G0530300	-3.1738	0.00539	Down	-2.3633	0.01407	Down	Putative Cytochrome P450
CYP90A4	OS12G0139300	-2.1727	0.0152	Down	2.3716	0.01025	Up	Cytochrome P450 family protein
—	OS06G0294600	-6.5642	0.01768	Down	-6.1175	1.74E-06	Down	Putative cytochrome P450 monooxygenase
CYP81A5	OS03G0760000	-6.4401	0.02431	Down	-7.1371	0.0067	Down	Cytochrome P450 81E1
CYP71P1	OS12G0268000	-2.4058	0.03276	Down	-2.8254	0.00181	Down	Cytochrome P450 family protein
CPQ10	OS10G0525200	-6.1542	0.0476	Down	-9.6818	7.51E-07	Down	Cytochrome P450 family protein
—	OS02G0503900	5.5209	1.72E-08	Up	2.0056	0.03574	Up	Putative cytochrome P450
CYP709C9	OS07G0418500	8.1296	8.19E-05	Up	-3.7517	0.02965	Down	Putative cytochrome P450
—	OS02G0185200	2.7127	0.00205	Up	-7.9311	0.0019	Down	Putative cytochrome P450
CYP450	OS10G0513900	2.1567	0.02877	Up	-4.4429	4.44E-05	Down	Putative cytochrome P450; Transposon protein
CYP74A2	OS03G0225900	-3.5731	0.000672	Down	-12.1715	5.64E-13	Down	Allene oxide synthase 2
CYP707A5	OS02G0703600	-2.6246	0.0051	Down	-2.5699	0.04116	Down	Abscisic acid 8'-hydroxylase 1

Table 2: Expression data of different CYP genes in resistant genotypes (Tetep & Pankaj)

Gene	Gene ID	Fold Change (Tetep)	Padj (Tetep)	Expression (Tetep)	Fold Change (Pankaj)	Padj (Pankaj)	Expression (Pankaj)	Function
—	OS03G0570100	-9.0108	2.23E-13	Down	10.0462	1.02E-07	Up	Cytochrome P450 79A1
CYP71Z2	OS07G0217600	-5.8491	1.02E-10	Down	-2.8697	0.001769	Down	Putative cytochrome P450 71D7
CYP71AD	OS05G0424300	-5.7434	9.27E-07	Down	4.5161	0.00027	Up	Putative cytochrome P450
CYP450	OS10G0513900	-8.3963	2.65E-05	Down	6.7033	0.015683	Up	Putative cytochrome P450
CYP94D7	OS01G0804400	-3.4812	5.64E-05	Down	-2.4679	0.003219	Down	Fatty acid hydroxylase
CYP71T2	OS01G0227500	-3.3101	0.000102	Down	-2.0249	0.018734	Down	Putative Cytochrome P450 71A1
CYP704A5	OS10G0525000	-3.2148	0.000279	Down	3.2574	0.000484	Up	Cytochrome P450 family
CYP71P1	OS12G0268000	-2.5581	0.002021	Down	3.3879	0.008811	Up	Cytochrome P450 family
—	OS06G0294600	-3.7325	0.002904	Down	7.0721	0.006599	Up	Putative cytochrome P450 monooxygenase
CYP90B2	OS03G0227700	-2.3565	0.033618	Down	-2.162	0.030419	Down	Cytochrome P450 family protein
—	OS10G0139700	-2.0939	0.037938	Down	5.3778	5.24E-05	Up	Putative cytochrome P450
CYP87C2	OS03G0658800	12.4121	8.62E-25	Up	7.4015	3.71E-12	Up	Cytochrome P450 family
CYP96B8	OS08G0262500	13.9642	2.61E-19	Up	10.8315	1.51E-09	Up	Putative cytochrome P450
—	OS06G0680700	7.3641	3.33E-12	Up	10.1881	4.81E-08	Up	Putative cytochrome P450
—	OS11G0289700	5.5714	6.15E-09	Up	3.4541	0.000425	Up	Cytochrome P450 family protein
CYP93G2	OS06G0102100	8.0355	0.000127	Up	-2.9606	0.001998	Down	Putative cytochrome P450
—	OS02G0666500	3.8306	0.00074	Up	6.1625	1.25E-06	Up	Putative cytochrome P450
CYP76-14	OS10G0144700	4.2286	0.00521	Up	-8.3634	8.13E-05	Down	Putative cytochrome P450
CYP74A2	OS03G0225900	-6.0566	2.26E-10	Down	9.163	3.74E-13	Up	Allene oxide synthase 2
CYP99A3	OS04G0178400	-7.6254	8.74E-10	Down	2.9907	0.014012	Up	9-beta-pimara-7,15-diene oxidase
CYP707A5	OS02G0703600	-2.064	0.04446	Down	4.3463	3.35E-06	Up	Abscisic acid 8'-hydroxylase 1
CYP71Z6	OS02G0570500	-7.117	0.004836	Down	7.6749	0.00159	Up	Ent-isokaurene C2-hydroxylase

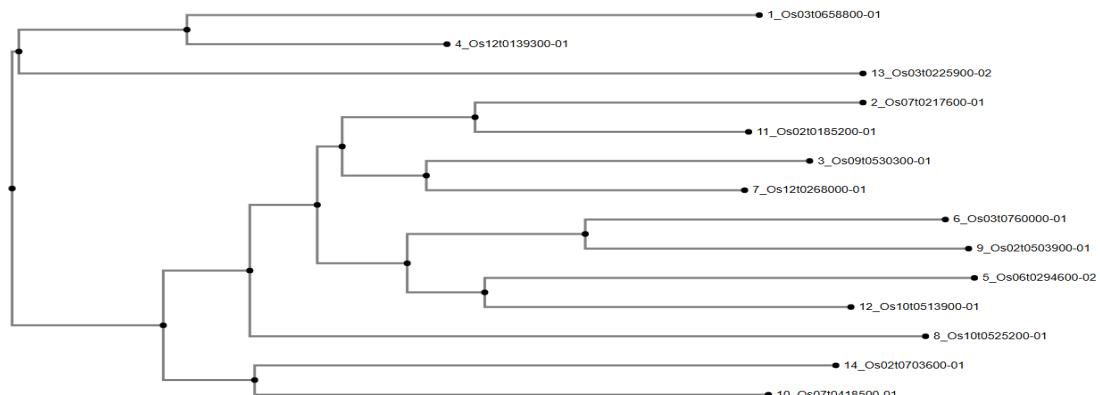


Fig. 1: Phylogeney of Susceptible (TN1&BPT 5204) genotypes

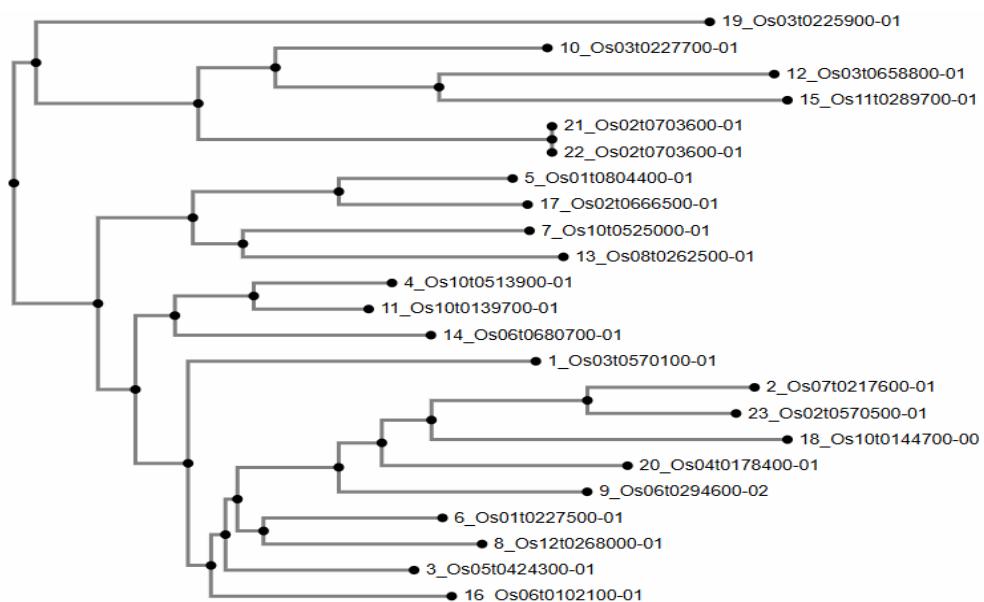


Fig. 2 : Phylogeney tree of Resistant (Tetep and Pankaj) genotypes



Fig. 3: Fold change expression of cytochrome P450 genes in susceptible rice genotypes TN1 and BPT-5204

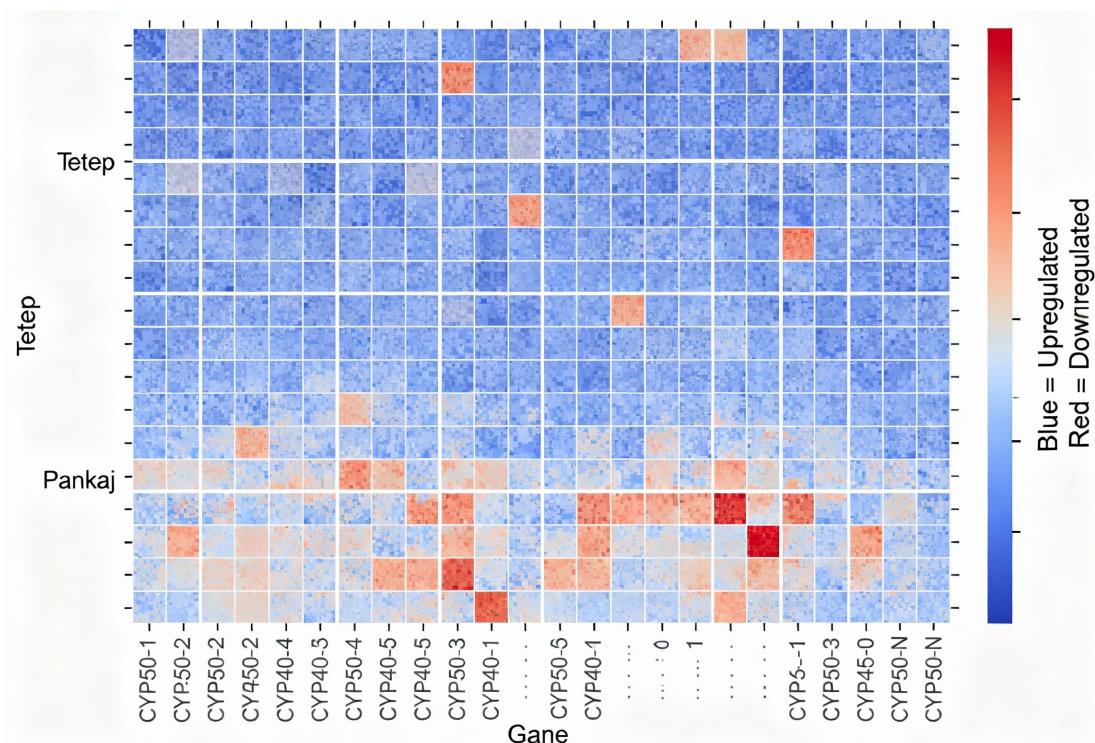


Fig. 4: Fold change expression of cytochrome P450 genes in resistant/tolerant rice genotypes Tetep and Pankaj

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