ARTICLE

SNAP markers for pyramided yield and BLB resistance genes in rice

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Abstract: *Molecular markers are pivotal in rice breeding as they enable gene pyramiding to improve yield and disease resistance. This study reports the development and validation of Single Nucleotide Amplified Polymorphism (SNAP) markers targeting qTSN4 (yield), xa5, and Xa21 (bacterial leaf blight resistance) genes. An EF1-a primer was integrated as an internal control in multiplex PCR to enhance assay precision and distinguish allele absence from PCR failure. An annealing temperature of 60 °C and an internal control primer concentration of 0.5 µM were optimal. While the xa5-based SNAP marker failed to distinguish alleles in the tested lines, qTSN4- and Xa21-based markers successfully differentiated the parental genotypes, confirming their applicability in marker-assisted gene pyramiding. These markers were validated in 72 BC1F1 plants and showed consistent Mendelian 1:1 segregation for qTSN4 and Xa21. Markers were used to select 18 plants with positive alleles A for qTSN4 and C for Xa21.*

Keywords: *Marker-assisted selection (MAS), multiplex PCR, qTSN4 gene, Xa21 gene*

INTRODUCTION

Global rice production increased by 1 % from 2015 to 2024, while there was no increase in Indonesian rice production over the same period (USDA 2025). As rice is a global staple food, productivity improvement is a worldwide concern, especially as climate change diminishes productive land and increases susceptibility to diseases, such as bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). This disease has exacerbated significant global yield losses (Qi et al. 2025, Abdullahi et al. 2025). To bolster global food security, leveraging gene pyramiding is crucial to integrate genes that confer high productivity and robust BLB resistance. Genetic advancements depend on identifying key genes; for example, *qTSN4* boosts productivity by 13-36 % in IR64 and 20 % in the Code variety (Fujita et al. 2013, Tasliah et al. 2020). Numerous *Xa* and *xa* genes exist for BLB resistance, with the national rice variety Inpari 32 (derived from IRBB64) predicted to carry vital *Xa4*, *xa5*, *Xa7*, and *Xa21* genes (Suryadi et al. 2016, Thamrin et al. 2023, Cheng et al. 2024).

Molecular marker-assisted selection (MAS) is indispensable for efficient plant breeding, as it enables early and environmentally independent selection (Song et al. 2023). Simple sequence repeat (SSR) markers such as RM17483 and sequence tag site (STS) PTA248 have been previously used (Ashan et al. 2023, Sowmiya et al. 2024) to identify *qTSN4* and *Xa21*, respectively. However,

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these markers rely on differences in DNA fragment size and require high-resolution gel electrophoresis, such as PAGE (Kristamtini et al. 2015). Moreover, many traditional markers are often distant from target genes. Recombination events can reduce the selection accuracy of such markers, leading to false-positive results (Hasan et al. 2021).

Single nucleotide amplified polymorphism (SNAP) markers offer a promising solution for detecting single nucleotide polymorphisms (SNPs), which are more abundant in the genome and are often located within or very close to target genes (Drenkard et al. 2000, Choudhury et al. 2023, Reddy et al. 2024). The SNAP marker is an allele-specific marker that can be designed for the target gene (not only in proximity, but also in the gene itself). SNAP utilizes a straightforward gel electrophoresis system based on band presence or absence, which is achieved by designing SNP-targeting primers with a specificity-enhancing mismatch near the SNP site (Drenkard et al. 2000, Reddy et al. 2024). A multiplexing approach incorporating an internal control primer pair that produces a distinguishable amplicon will ensure robust detection, confirm successful PCR, and validate the true allele-specific absence.

Three SNP locations were identified in the *qTSN4* gene when comparing the IR64 and NIL-*qTSN4* genotypes (Fujita et al. 2013). Additionally, two consecutive SNPs were found in the *xa5* gene between the IR24, IRBB5, and Nipponbare (Iyer and McCouch 2004). To our best knowledge, while Nanayakkara et al. (2020) identified variation in the form of indels within the *Xa21* gene, SNPs in the respective gene observed in resistant and susceptible rice genotypes are not available. Therefore, in developing an *Xa21*-based SNAP marker, identifying SNPs within the *Xa21* gene from both the resistant and susceptible genotypes is a relevant step. Therefore, the objective of this study was to develop and optimize a multiplex SNAP-PCR system targeting the *qTSN4* and *Xa* genes, and to validate its application in selecting breeding populations derived from the parental lines Inpari 32 and Code-*qTSN4*.

MATERIAL AND METHODS

Plant material and DNA isolation

The rice variety IR64 (IR5657-33-2-1 × IR2061-465 1-5-5) (Mackill and Khush 2018) served as a negative control for optimizing SNAP markers for *qTSN4* and *Xa21*, whereas IR24 (IR8 × {(Century Patna 231 × SLO 17) × Sigadis)}) (IRRI 1972) served as the negative control for *xa5*. Code-*qTSN4* (Code × IR64-NILs *qTSN4*[YP9]) (Tasliah et al. 2020) served as a positive allele control for *qTSN4*, IRBB21 (IR24 + *Xa21*) for *Xa21,* and IRBB5 (IR24 + *xa5*) for *xa5* (Deng et al. 2016). The BC1F1 population (72 plants) used for SNAP marker validation was derived from Inpari 32 (male) and F1 (female) hybrids developed by Ashan et al. (2023). DNA was isolated from 100 mg of rice leaf tissue using a modified 2 % CTAB method, excluding the use of β-Mercaptoethanol and polyvinylpyrrolidone (PVP) (Aboul-Maaty and Oraby 2019). The extracted DNA quantity and quality were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and 1 % agarose gel electrophoresis.

SNAP primer design

To confirm the SNP locations in the *qTSN4* gene, alignments were performed using the *qTSN4* sequences from IR64 (Tanaka et al. 2020) and the Japonica group (NM_001419864) (Qi et al. 2008). Similarly, SNP confirmation for the *xa5* gene involved the alignment of the IR24 (AF532975), IRBB5 (AY643716), and Nipponbare (AY643717) genotypes (Iyer and McCouch 2004). For the *Xa21* gene, SNPs were identified by aligning the IR64 (Tanaka et al. 2020) and IRBB21 (U37133) genotypes (Song et al. 1995). All alignments were performed using the MUSCLE method with default parameters in the Ugene program (Okonechnikov et al. 2012). The SNP positions identified within *qTSN4*, *xa5*, and *Xa21* were subsequently used to design SNAP markers.

Primers were designed to target 500 bp regions flanking each SNP using WebSNAPPER (Drenkard et al. 2000). While most parameters remained at default, the optimal amplicon length was precisely set to 325–450 bp. A critical selection criterion prioritized primers with a mismatch at the third base from the 3'-OH end. Subsequently, an OligoAnalyzer (Owczarzy et al. 2008) was employed for stringent quality control and to check for hairpin structures, self-dimers, and heterodimers. Macrogen (South Korea) was used to synthesize the primers. An additional primer, specific to the *elongation factor 1 alpha* (*EF-1α*) gene (NM_001402055.1) (Jantasuriyarat et al. 2005), was designed for PCR control in a multiplex PCR. This control primer could be used to amplify other SNAP regions of the rice genome.

PCR optimization and genotyping

Optimization of the PCR protocol focused on determining optimal initial concentrations of control primers (0.5 µM and 1 µM) and annealing temperatures (55, 58, 60, 65, and 68 °C). Each PCR reaction was performed in duplicate, with differences in the specific primers used for each replicate, and each reaction had a final volume of 10 µL. For each 10 µL PCR reaction, 5 ng μL^{-1} DNA template, 1 µM specific and universal SNP primers, and the control primer were added, along with 1× GoTaq Green Master Mix (Promega, USA). The thermal cycling protocol, consisting of 30 cycles, was structured as follows: an initial denaturation at 95 °C for 3 min; subsequent denaturation at 95 °C for 30 sec; annealing for 30 sec; elongation at 72 °C for 45 sec; and a final elongation step at 72 °C for 5 min. Amplified products were resolved by electrophoresis on a 1 % agarose gel for 30 min at 100 volts, and band sizes were estimated using a 100 bp Plus DNA Ladder (VC, Malaysia). Genotyping results in the BC1F1 population obtained using the optimization program were analyzed using the chi-square test.

RESULT AND DISCUSSION

Sequence analysis of target gene and relevant SNPs for marker development

SNP positions in *qTSN4* and *xa5* were confirmed by comparative sequence analysis, and their flanking regions were used for primer design. A comparative analysis between the *qTSN4* gene sequences of IR64 and the Japonica group revealed three distinct SNP loci within the *qTSN4* gene (*Spike* 1, *Spike* 2, and *Spike* 3) that led to amino acid substitutions (Figure 1A). Similar findings have been observed when comparing IR64 and NIL-*qTSN4* (Fujita et al. 2013). *Spike* 1, located in exon 2, contains a histidine residue that is crucial for serine protease catalytic activity and gene expression (Li et al. 2023). *Spike* 2 and 3 are located in exon 4. The SNP positions in *xa5* identified by Iyer and McCouch (2004) were confirmed to be located in the first exon. This exon contained three SNP loci (Figure 1B), two of which (TC/AG) differentiated the susceptible (IR24, Nipponbare) and resistant (IRBB5) varieties.

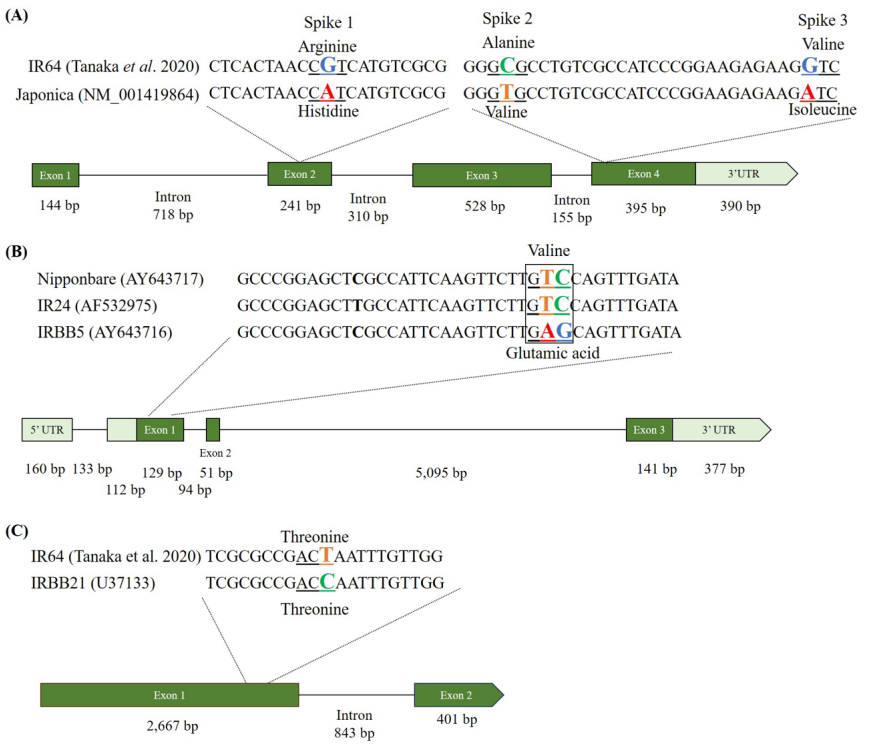

Figure 1. Structure of the *qTSN4* (LOC_Os04g52479) (A), *xa5* (LOC_Os05g01710) (B), and *Xa21* (LOC_Os11g36180) (C) genes, illustrating their exon-intron organization with corresponding lengths (bp), and highlighting the specific single nucleotide polymorphisms (SNPs) used for SNAP primer design.

Sequence comparison between IR64 and IRBB21 identified numerous SNPs and indels. The SNP selected for primer design was located at position 18,644,636 bp on chromosome 11 (T/C) (Figure 1C). Although this nucleotide substitution does not result in an amino acid change, the marker resides within the gene-coding region, minimizing the likelihood of recombination. Additionally, the flanking sequence was highly conserved, facilitating the design of universal primers.

SNAP primer design

WebSNAPPER generates a variety of candidate SNAP primers, classifying them as either forward or reverse, and performs experiments with different additional mismatch positions. A key selection criterion involves an additional mismatch at the second position upstream of the SNP, which significantly boosts primer specificity. This particular placement improves specificity by 31 % compared to the first position and 13 % over the third position (Liu et al. 2012). Beyond this, further selection relies on standard primer parameters: a GC content between 40-60 %, avoiding an abundance of A and T bases at the primer ends, and ensuring that the ΔG for potential secondary structures like hairpins, self-dimers, and heterodimers is not less than -10 kcal mol^{-1} (Yuan et al. 2021, Fulghum et al. 2024).

For the *qTSN4* gene, the SNAP primers designed for the *Spike* 1 and *Spike* 3 loci worked effectively without any modifications (Table 1). Conversely, the *Spike* 2 primer required five extra bases at its 5'end due to high GC content, which could hinder template binding and require additives like DMSO (Yang et al. 2023). Meanwhile, the *xa5* gene's SNAP primers underwent several modifications. Because this gene has two consecutive SNPs, both were incorporated into specific primer ends to enhance allele differentiation. The *Xa21*-specific primers had 10 bases added to their 5' ends to address their low GC content, aiming to increase their Tm value and specificity (Alvarez-Fernandez 2013).

Optimization of multiplex PCR conditions for SNAP marker detection

Multiplex PCR was performed using SNAP primers and *EF1-α* internal control primers. Multiplex PCR can reduce time and cost by avoiding the need for separate runs (Mendez-Alvarez et al. 2025). Although Almeida et al. (2024) successfully implemented allele-specific PCR, the methodology could be strengthened by including internal controls to confirm band absence. Owing to the involvement of two primer pairs, the annealing temperature was optimized. Our results showed that the SNAP gene *qTSN4* loci *Spike* 1 and 2, were amplified successfully and consistently across all five annealing temperatures tested (Figure 2A, Figure 2B). Each tested sample produced a 716 bp PCR product from a pair of control primers. Each sample was tested using two specific primers. Sample IR64 did not produce an amplicon

Table 1. Characteristics of SNAP primers targeting the *qTSN4*, *xa5*, *Xa21* genes and internal control primers (*EF1-α*) for multiplex PCR

Name	**Sequences** (5'-3')	**ΔG** (kcal mol^{-1})			**Amplicon size** (bp)
		Hairpin	**Selfdimer**	**Heterodimer**	
Spike1-U-F	GGATTTTACACAATCCTCCTACCGTTTACCAGT	-2.51	-8.06	-	-
Spike1-(+)-A-R	GGTAGTCCAAGTCAACCGCGACATCAT	-2.10	-10.36	-6.97	386
Spike1-(-)-G-R	GGTAGTCCAAGTCAACCGCGACATCAC	-2.10	-10.36	-6.97	386
Spike2/3-U-F	TGGCCCTGAAAACTGGACTAGTGGG	-1.17	-9.28	-	-
Spike2-(+)-T-R	TTCTCTTCCGGGATGGCGACAGTCA	-1.13	-9.75	-6,21	341
Spike2-(-)-C-R	TTCTCTTCCGGGATGGCGACAGACG	-1.17	-9.75	-6,21	341
Spike3-(+)-A-R	CCCAATGGCTCGAAGATCTCTTCCAT	-2.43	-7.82	-8,09	367
Spike3-(-)-G-R	CCCAATGGCTCGAAGATCTCTTCCAC	-2.43	-7.82	-8,09	367
Xa5-(+)-G-F	CGGAGCTCGCCATTCAAGTTCTTCAG	-0.31	-9.49	-7.07	386
Xa5-(-)-C-F	CGGAGCTCGCCATTCAAGTTCTTATC	-0.31	-9.49	-6.95	386
Xa5-U-R	GACAACGCTGAATCCACAACCAAG	0.49	-3.61	-	-
Xa21-U-F	GGAAGTGCCAACCATTGGTGCTTTCGCAGATG	-4.97	-6.97	-	-
Xa21-(+)-C-R	GTATACTGAGCCAAATGATCCAGAACCCAACAAAGTG	-1.41	-6.08	-6.97	349
Xa21-(-)-T-R	GTATACTGAGCCAAATGATCCAGAACCCAACAAAGTA	-1.41	-6.08	-6.97	349
EF1-F	GGATGCCACCACTCCCAAGTACTCCAAG	-0.71	-6.84	-8.14	716
EF1-R	GAATCATCTTAACCATACCAGCATCACCG	0.54	-4.85	-8.14	-

Description: U = universal; F = forward; R = reverse; bp = base pair; base with underline = extra mismatch location.

with primers A and T, whereas primers G and C produced an amplicon based on specific *Spike* 1 and 2 primers. IR64 has genotypes AA and TT for *Spikes* 1 and 2, and vice versa for Code-*qTSN4.*

Optimizing the annealing temperature is crucial for successful PCR amplification of both *qTSN4 Spike* 3 and *xa5* SNAP genes. Excessively high temperatures above 60 °C could hinder primer binding and lead to no PCR product on the *qTSN4* marker (Figure 2C). For *xa5* SNAP, temperatures (55–63 °C) successfully differentiated between IR24 (CC genotype) and IRBB21 (GG genotype) (Figure 2D). Conversely, overly low temperatures led to non-specific primers preventing differentiation, as seen with *Xa21* SNAP at 55 °C (Figure 2E). One strategy for obtaining a more specific

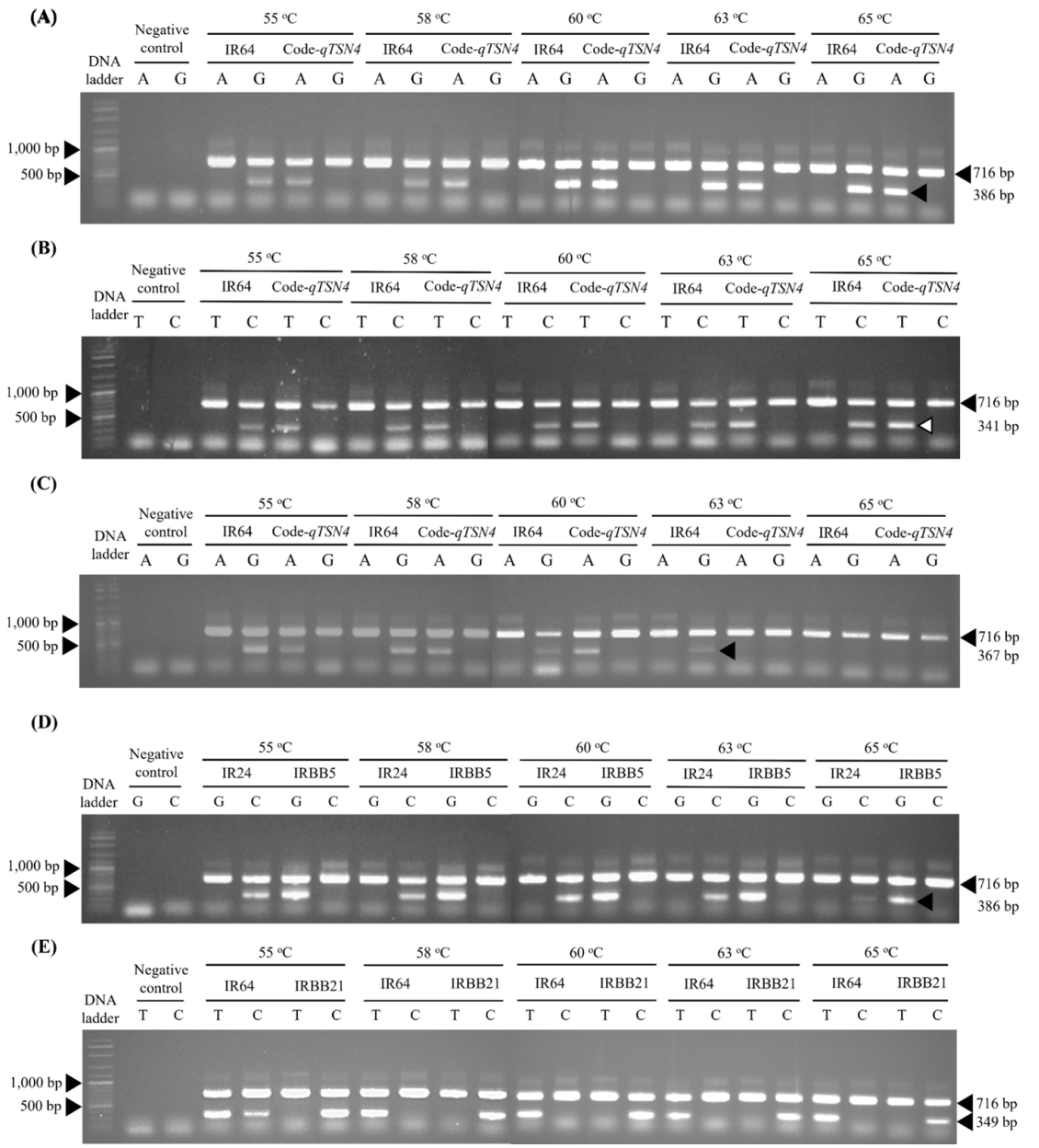

Figure 2. Agarose gel electrophoresis results for annealing temperature optimization of SNAP markers. A. *qTSN4 Spike* 1 (IR64: GG, Code-*qTSN4*: AA), B. *qTSN4 Spike* 2 (IR64: CC, Code-*qTSN4*: TT), C. *qTSN4 Spike* 3 (IR64: GG, Code-*qTSN4*: AA), D. *xa5* (IR24: CC, IRBB5: GG), and E. *Xa21* (IR64: TT, IRBB21: CC).

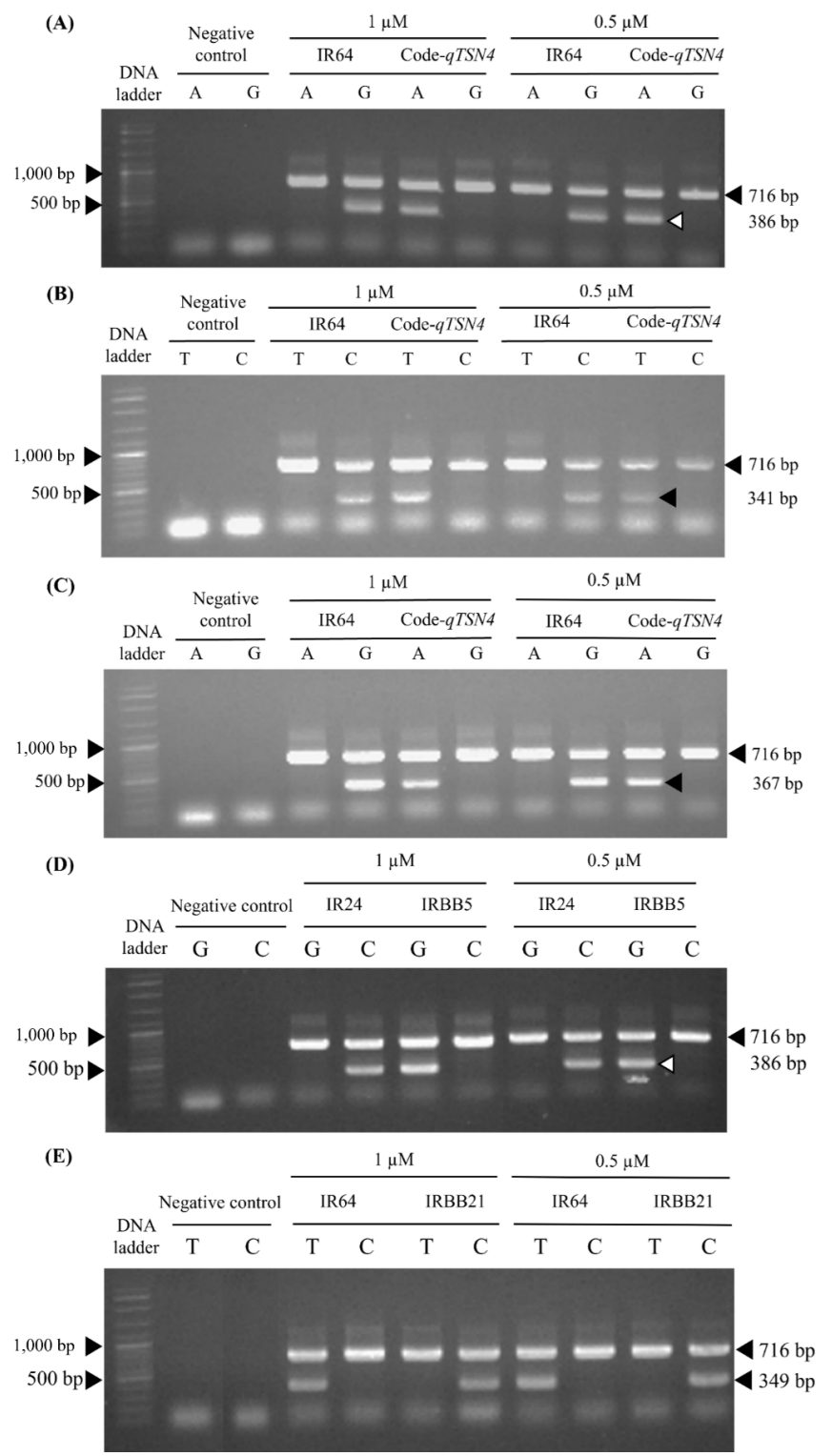

Figure 3. Agarose gel electrophoresis results for primer internal control concentration optimization of SNAP markers. A. *qTSN4 Spike* 1 (IR64: GG, Code-*qTSN4*: AA), B. *qTSN4 Spike* 2 (IR64: CC, Code-*qTSN4*: TT), C. *qTSN4 Spike* 3 (IR64: GG, Code-*qTSN4*: AA), D. *xa5* (IR24: CC, IRBB5: GG), and E. *Xa21* (IR64: TT, IRBB21: CC).

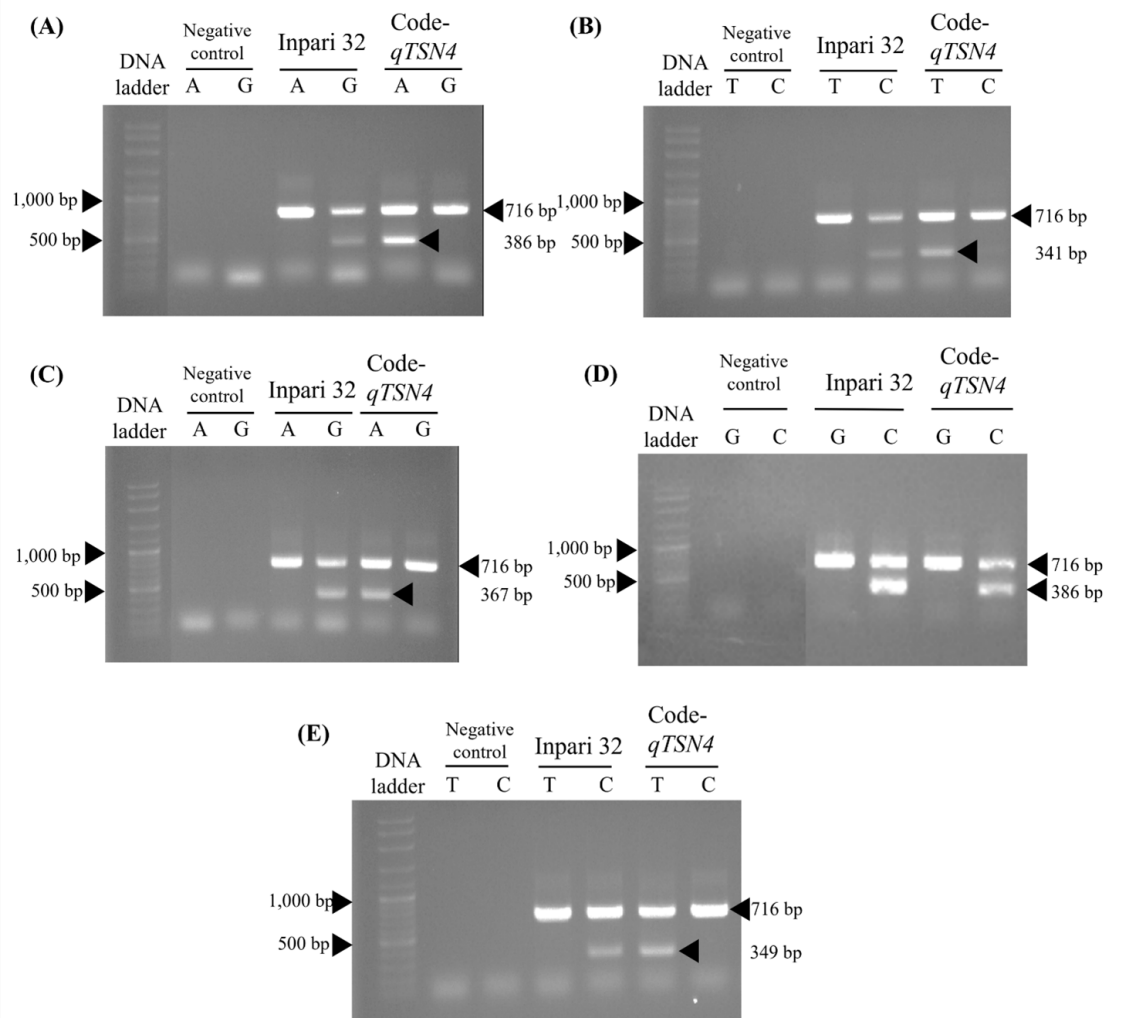

Figure 4. Agarose gel electrophoresis results of genotyping in parental lines of the breeding program using SNAP markers. A. *qTSN4 Spike* 1 (Inpari 32: GG, Code-*qTSN4*: AA), B. *qTSN4 Spike* 2 (Inpari 32: CC, Code-*qTSN4*: TT), C. *qTSN4 Spike* 3 (Inpari 32: CC, Code-*qTSN4*: TT), D. *xa5* (Inpari 32: CC, Code-*qTSN4*: CC), and E. *Xa21* (Inpari 32: CC, Code-*qTSN4*: TT).

product is to increase the annealing temperature during PCR (Rahmi et al. 2024). This has been demonstrated with the *Xa21* gene, where the optimal annealing temperature ranges from 58–65 °C and allows for differentiation between IR64 (TT genotype) and IRBB21 (CC genotype). For mass-scale genotyping, an annealing temperature of 60 °C can be used for all SNAP primers (*qTSN4*, *xa5*, and *Xa21*).

In addition to the annealing temperature, the concentration of the internal control primer is also critical for preventing competition with specific primers. Our study demonstrated that reducing the concentration of the internal control to 0.5 µM did not impact the results across all SNAP assays (Figure 3). Reducing the concentration of the control primers is beneficial if they interfere with specific primers. The *xa5* gene was successfully analyzed using multiplex PCR with the tetra-amplification refractory mutation system-polymerase chain (ARMS) method, which employs internal primers near the SNP and uniform primer concentrations (Liu et al. 2021). This finding aligns with our observation that a consistent 1 µM concentration for all primers had no adverse effect in our assays. Notably, the specific control employed for *xa5* in the tetra-ARMS method was not applicable to other SNAP analyses of rice. In contrast, our study provided a universal primer control applicable to all rice SNAP assays, regardless of the target gene or marker used.

Application of developed SNAP markers in parental line genotyping for pyramiding breeding

A plant breeding scheme to pyramid the *Xa* and *qTSN4* genes was recently developed by crossing Inpari 32 with Code-*qTSN4* (Ashan et al. 2023). F1 hybrid seeds were identified using SSR markers. SNAP markers, designed and optimized for *qTSN4*, *xa5*, and *Xa21*, were used to genotype the parental lines and assess their potential as selection tools. The SNAP *qTSN4* markers for *Spike* 1, *Spike* 2, and *Spike* 3 confirmed that Code-*qTSN4* was the donor of the *qTSN4* gene, exhibiting the genotypes AA, TT, and AA, respectively (Figure 4A, 4B, and 4C). In contrast, Inpari 32 carried negative alleles for genotypes GG, CC, and GG. The positive *qTSN4* genotype in Code-*qTSN4* originated from Japonica rice (Fujita et al. 2013, Tasliah et al. 2020)**.** Similarly, the SSR marker RM17483 for *qTSN4* showed amplicon size differences between Inpari 32 and Code-*qTSN4*, further indicating that only Code-*qTSN4* carried the *qTSN4* gene (Ashan et al. 2023).

The results for the SNAP *xa5* marker showed that Inpari 32 and Code-*qTSN4* displayed negative CC genotypes for *xa5* (Figure 4D). This indicates that the SNAP *xa5* marker cannot be used for selection in crosses involving these parents. Inpari 32 was assumed to harbor the *xa5* gene from its male parent IRBB64 (Suryadi et al. 2016, Thamrin et al. 2023). However, no molecular testing has confirmed whether Inpari 32 carries the favorable allele *xa5*. Therefore, any assumptions regarding the presence of *xa5* in Inpari 32 based solely on genetic background require further assessment.

Genotyping results for the SNAP *Xa21* marker in Inpari 32 and Code-*qTSN4* revealed a favorable CC genotype in Inpari 32. Meanwhile, Code-*qTSN4* had a TT genotype, suggesting that it lacked a favorable allele of the *Xa21* gene (Figure 4E). These SNAP marker results aligned with those of the SSR marker pTA248, which showed amplicon size differences between the two parents (Ashan et al. 2023). Individuals carrying the favorable *Xa21* allele may exhibit resistance beyond BLB, as suggested by parallels with the receptor-like *Cre3* gene, which responds to multiple pathogens (Moatamedi et al. 2018, Ercoli et al. 2022, Qalavand et al. 2022).

The applicability of the developed SNAP markers was validated in BC1F1 pyramided lines derived from Inpari 32, which carries the favorable allele C for *Xa21*, and Code-*qTSN4*, which carries the favorable allele A for *qTSN4*. Our results showed a consistent Mendelian 1:1 segregation of *qTSN4* and *Xa21* in the 72 BC1F1 plants (Supplementary Data). Markers without segregation distortion in Mendel's model are required for use as selection tools (Zhang et al. 2010). Among the 72 BC1F1 lines genotyped using the SNAP markers *qTSN4* and *Xa21*, only 18 plants had favorable alleles, with a heterozygous AG genotype for *qTSN4* and a homozygous CC genotype for *Xa21*. In conclusion, we developed and validated SNAP markers for *qTSN4* and *Xa21*, enabling efficient gene pyramiding under optimized multiplex PCR conditions with reliable internal controls.

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DATA AVAILABILITY

The datasets generated and/or analyzed during the current research are available from the corresponding author upon reasonable request.

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