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Phylogenetic analysis of rice cultivars for *CYP93G1* and functional evaluation for apigenin and biofilm formation

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Abstract

Improving biological nitrogen fixation (BNF) in rice is a long-standing vision for the researchers as it decreases the reliance on surplus usage of nitrogenous fertilizers. Enhancing plant signaling molecules, such as flavonoids, facilitates improved interactions between plants and microbes, thereby promoting increased biofilm formation. Apigenin, a key flavonoid in root exudates, induces the expression of the *gumD* gene in microbes, which is essential for exopolysaccharide synthesis (EPS), promoting microbial colonization of roots, biofilm formation, and potentially enhancing BNF. A phylogenetic analysis of 280 rice lines from the 3K RG panel, targeting the candidate gene *CYP93G1*, which is involved in apigenin synthesis, enabled the selection of nine genotypes for evaluating apigenin levels and biofilm formation. The results revealed that, despite having lower levels of apigenin, the promising genotypes Gokulganja, Local Bhat and Nona Bokra exhibited significant biofilm formation compared to the other genotypes, suggesting their potential as effective cultivars for genome editing to enhance BNF. This is the first study to assess these traits concerning a specific gene in a 3K panel. Our findings demonstrated that targeted manipulation of tricin flavone biosynthetic pathway genes in these selected genotypes could significantly boost BNF, promoting ecological rice cultivation practices and advancing environmental sustainability.

Keywords

Phylogenetic analysis; colonization; environment; naringenin

Introduction

The United Nations has declared that the population of the world will reach 11 billion by the end of the 21st century. In consequence, the global production of food crops needs to be increased by another 40% (1). With rice being the mainstay food for almost one-half of the world's population, it is necessary to boost the productivity of rice (2). Among the various elements that are essential for plant growth, nitrogen (N) holds an indispensable place. Though nitrogen is abundantly available in the atmosphere, plants cannot utilize it directly. Thus fertilizer application becomes inevitable for farmers, to get maximum output from the crop (3). Among the total fertilizer used in rice, nitrogen itself accounts for about 37%, which signifies its impact on rice production (4). Despite having an immense impact on increasing the production of rice, nitrogen fertilizer has its detrimental impacts, which

include reduction in biodiversity, contamination of natural resources, and/or greenhouse gas release. The other effects include an increase in the nitrous oxide (N_2O) concentration in the atmosphere and disruption in the balance of aquatic ecosystems (3). A critical way to counter the negative effects of N fertilizer and reduce its usage is harnessing the benefits of Biological Nitrogen Fixation (BNF). It is a naturally occurring phenomenon that involves the conversion of atmospheric nitrogen (N_2) into ammonium (NH_4^+), a much simpler soluble form with the help of microorganisms. In general, a mutualistic relationship is established between the host plant and microbes with the release of certain flavonoids and isoflavonoids into the rhizosphere. This acts as a signal for the microbe to colonize the host and form an association with them (5). A similar principle was exploited for harnessing the benefits of BNF in the rice using *Gluconacetobacter diazotrophicus*, a diazotrophic bacterium. Through screening of 740 different compounds, apigenin, and luteolin flavonoid were found to be the positive regulators for biofilm formation in *G. diazotrophicus* (6).

Biofilm formation is a natural phenomenon, where bacterial communities are attached to the matrix for their augmented survival in the changing environment to meet their requirements. In a biofilm, the bacterial cells are entrenched in a matrix, which is generally made up of extracellular polymeric substances (EPS). The main components of EPS are proteins, exopolysaccharides and extracellular DNA, even though the composition varies between bacteria (7). In addition to its role as an energy reservoir, biofilms are crucial for the bacteria to combat stress, and also to interact with other living organisms (8). Examining the biofilm formation of plant growth-promoting bacteria (PGPB) is vital due to the numerous benefits they offer. They prevent pathogens from colonizing plants, secrete antimicrobials, provide nutrients and produce substances such as auxin, cytokinin and gibberellins that promote plant growth. Additionally, evidence shows that plants with PGPB exhibiting biofilm-forming potential can better withstand salinity, drought and other abiotic stresses (9). In biofilm formation, exopolysaccharide production aids the microbes in their surface colonization and the *gumD* gene is responsible for initiating the process of its production (10). Even though both apigenin and luteolin have the potential to increase biofilm formation, genome editing to increase the flavonoid content is only effective in the case of apigenin, as there are large gene families in rice encoding for O-methyltransferases, which facilitates the conversion of luteolin.

By knocking out the gene, *CYP75B3* and *CYP75B4*, through genome editing, the apigenin content of the Kitaake rice variety was increased, thereby biofilm formation by *G. diazotrophicus* was enhanced (6). A similar strategy can be incorporated in other rice varieties to enhance the BNF in them. In this study, we have analyzed various rice varieties for their scope in enhancement of BNF, based on the results obtained from phylogenetic analysis of *CYP93G1*, which aids the conversion of naringenin to apigenin (11) by evaluating apigenin concentration and biofilm formation for the selected genotypes. In addition to these selected genotypes, the study includes four local cultivars: ASD 16, a widely cultivated and high-yielding variety in South India (12); CO 51, known for its adaptability across seasons in Tamil Nadu (13); Kavuni, a traditional variety recognized for its anti-diabetic properties (14); and the recently released CO 58. These varieties were selected for their diverse attributes.

Materials and Methods

Phylogenetic analysis

A total of 280 genotypes from the 3K - RG rice panel were chosen for the phylogenetic analysis. The genotypes were analyzed for the gene *CYP93G1* (LOC_Os04g01140) using the SNP seek database (https://snp-seek.irri.org/). The results of the phylogenetic analysis were obtained in NEWICK file format and were visualized using iTOL v6 (https://itol.embl.de/).

Biofilm assay

The biofilm assay was carried out with some modifications to the formerly mentioned method (6). In the modified media of ATCC (1.5% mannitol, 1.5% sucrose, 0.5% yeast extract and 0.3% peptone; pH of 6.0), *G. diazotrophicus* strain was grown overnight at 180 rpm; 28 °C. Then the secondary culture was initiated in fresh ATCC media at a ratio of 1:50, and incubated in the same condition for six hours. The culture was then pelleted at 6000 rpm for one minute, which was completely dissolved by adding the root exudates of the rice plant. For the collection of root exudates, the plants were grown hydroponically (15) for four weeks in a controlled greenhouse condition. The following protocol collected exudates from the roots with some modifications (16). The roots of the plants kept in tubes containing 15 ml of water, surrounded by cold water were shaken for two hours at 60 rpm (18 °C) and the exudates were filtered using a 0.22 µm membrane filter. About 198 µl of the dissolved solution and 2 µl of apigenin (10 mM) were added to the wells of a sterile 96-well plate and shaken at 150 rpm; 28°C for three days. The solutions were discarded after incubation and 200 µl of crystal violet solution (0.2% w/v crystal violet and 2% v/v ethanol in water) was added to each well. Again, the plate was incubated at 150 rpm at 28°C, for 30 minutes. The solutions were discarded as described earlier, followed by a three-sterile water wash and air drying. 200 µl of 95% ethanol was added to dissolve crystal violet into ethanol and the plate was again shaken at 150 rpm at 28°C for 15 minutes. Then, the absorbance was measured at 540 nm in a Biotek microplate reader (Fig. 1).

Quantification of apigenin

Sample preparation

Apigenin extraction from rice roots was performed based on (17) with slight modification. The roots of the plants were harvested and snap-frozen in liquid nitrogen. The freeze-dried samples were powdered using a pestle and mortar in the presence of liquid nitrogen. Then, 200 mg of powdered sample was taken in a 15 ml tube and 1.2 ml of acidic methanol (1% HCl in methanol) was added to it. It was left undisturbed for eight hours and the extract was separated

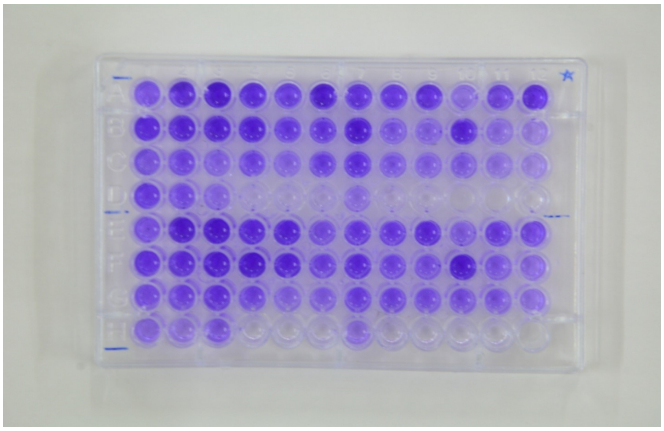

Fig. 1. Biofilm formation analysis: Rice root exudates were combined with *Gluconacetobacter diazotrophicus* on a 96-well plate and incubated for three days. The measurement was taken using a BioTek microplate reader at 540 nm. Each genotype was tested in triplicate, alongside control, to ensure reliable and accurate results.

using a membrane filter of 0.22 µm. To the extract, 2.4 ml of chloroform and 1.2 ml of distilled water were added and vortexed for five minutes followed by centrifugation at 3000 g for five minutes at room temperature. Now the aqueous phase was collected and an equal volume of 2N HCl was added and kept in a water bath at 95°C for an hour. The extract was then vacuum dried in a vacuum concentrator and dissolved in HPLC-grade methanol to half its original volume. Apigenin extract was analyzed by Shimadzu UHPLC as described below.

UHPLC Conditions

The UHPLC analysis was conducted using a Shimadzu UHPLC system, which included a degassing unit (DGU-405), solvent delivery pump (LC-40D xs), autosampler (SIL-40C xs), column oven (CTO-40S) and flow control valve (FCV-20AH2), all coupled to a Shimadzu LCMS-8045 tandem quadrupole mass spectrometer with an electrospray ionization (ESI) source. Shim-pack GISS C18 column (2.1 mm × 150 mm, 3 µm particle size) from Shimadzu, Japan, with a column temperature of 40°C was used for chromatographic separation. Apigenin separation was carried out using a gradient mode with two mobile phases: mobile phase A (0.1% formic acid in water) and mobile phase B (100% acetonitrile), at a constant flow rate of 0.3 ml/min. The samples were injected with a volume of 3 µL with three replications for each sample. The elution gradient program was as follows: mobile phase B was held at 20% for the first minute, raised to 90% from 1 to 8 minutes, maintained at 90% from 8 to 11 minutes, and reduced back to 20% from 11 to 11.25 minutes. The needle was washed with 200 µL of 100% acetonitrile before and after each injection.

ESI-Triple quadrupole mass analyzer

The experiment was conducted using a Shimadzu LCMS-8045 tandem quadrupole mass spectrometer equipped with an ESI ionization source. The analysis was performed in negative mode using Multiple Reaction Monitoring (-ve MRM) after optimizing the precursor and product ions with a standard reference material. The interface, desolvation line, and heat block temperatures were set to 300°C, 280°C and 500°C, respectively. The flow rates for the drying gas, nebulizing gas and heating gas were maintained at 3, 10, and 10 ml/min, respectively. The optimized MRM values for apigenin are provided in Table 1.

Table 1. MRM condition optimized for apigenin analysis.

Name	Precursor ion (m/z)	Product ion (m/z)	Q1 Pre bias (V)	CE (V)	Q1 Pre bias (V)
Apigenin	269.00	117.10	11.00	22.00	21.00
Apigenin	269.00	151.10	39.00	-21.00	14.00

Results and Discussion

Biofilm is a matrix made of extracellular polymeric substances (EPS) where the bacterial colonies are embedded. Biofilm formation by bacteria facilitates their root colonization and boosts the root-microbe interaction (18). Previous findings indicate that flavonoids, apigenin and luteolin, serve as potent inducers of biofilm formation in diazotrophic bacteria (6). Apigenin and luteolin induce the *gumD* expression, which is essential for matrix development, thereby increasing the secretion of exopolysaccharides. With *CYP93G1*, the gene responsible for the conversion of naringenin to apigenin in the tricin biosynthesis pathway (19), phylogenetic analysis was carried out for 280 rice lines. This analysis of 280 rice lines from the 3K-RG panel, focusing on the *CYP93G1* gene, based on SNP data from the SNP-seek database and visualization through the iTOL tool revealed two major clusters of the genotypes (Fig. 2). One of the nodes had 23 genotypes, while the other contained the majority of 257 genotypes. For further studies, three genotypes from the smaller node and six from the larger node were selected for detailed analysis. The inputs for this phylogenetic study were provided as IRIS IDs, for which the corresponding cultivar names are listed. Additionally, four local rice cultivars (ASD 16, CO 51, CO 58 and Kavuni) were selected for further experiments in the assessment of apigenin concentration and biofilm formation and statistically significant differences were analyzed (Table 2). The results of apigenin

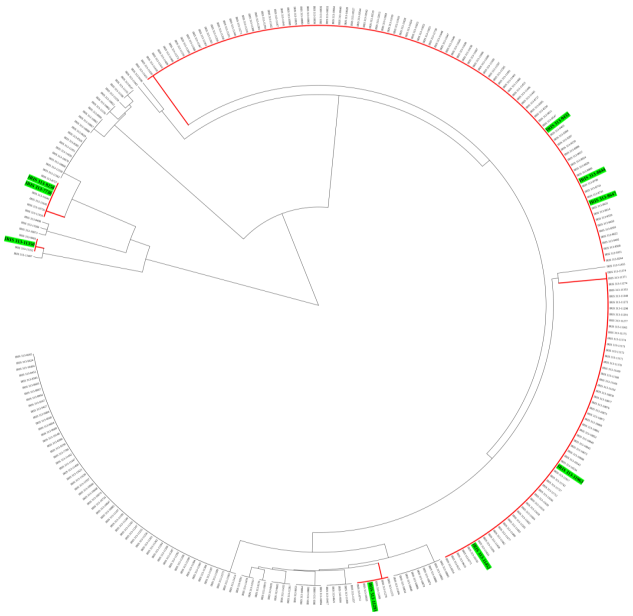

Fig. 2. Phylogenetic analysis of various rice lines for *CYP93G1*: The evolutionary relationship of 280 3K-RG panel rice lines for the gene, *CYP93G1*, was analyzed to select genotypes for further analysis.

Table 2. Genotype name for selected IRIS ID and results obtained from biofilm and apigenin concentration analysis

IRIS ID	Genotypes	Biofilm (abs @ 540nm)	Apigenin conc. (ppb)
IRIS 313-9433	Gokulganja	1.090*	3.069
IRIS 313-7736	Nona Bokra	0.805	Not detected
IRIS 313-8647	Perunel	0.931	9.501
IRIS 313-11350	Bajal	0.651	1.379
IRIS 313-11293	ARC 10975	1.274*	83.069*
IRIS 313-11452	Local Bhat	0.779	Not detected
IRIS 313-8844	ARC 14060	0.619	1.1
IRIS 313-11963	Karangi	0.521	Not detected
IRIS 313-9258	Makro	0.553	Not detected
-	ASD 16	0.497	Not detected
-	CO 58	0.592	30.988*
-	Kavuni	0.314	1.015
-	CO 51	0.647	Not detected

*- Significant at 5% level

estimation of 3K panel genotypes (Fig. 3) were consistent with the phylogenetic analysis. The estimation revealed that 6 of the 13 genotypes were showing less than detectable levels of apigenin, while the other 7 genotypes were showing a considerable amount of apigenin ranging from a maximum of 83.069 ppb in ARC 10975 to a minimum of 1.015 ppb in Kavuni. The genotypes Gokulganja, Perunel, and ARC 14060 which were under the same sub-node, exhibited apigenin concentrations of 3.069, 9.501 and 1.1 ppb respectively. Bajal has an apigenin content of 1.379 ppb and ARC 10975, from a separate sub-node, exhibited the highest concentration among the tested genotypes at 83.069 ppb. The genotypes Nona Bokra and Makro fall under a single sub-node, while Local Bhat and Karangi are grouped under another sub-node. In all these genotypes, apigenin content was not detected or possibly below the detectable threshold, indicating minimal levels across these groups. In the case of local cultivars, apigenin concentrations were not detected for ASD 16 and CO 51. On the other hand, CO 58 recorded the highest concentration of 10.988 ppb with a trace amount in Kavuni (1.015 ppb). Biofilm analysis revealed that all the genotypes revealed significant biofilm formation (Fig. 4), with ARC 10975 showing the maximum biofilm formation with an absorbance value of 1.274 and Kavuni showing the lowest value 0.314 at 540nm. Upon analysis, all the genotypes exhibited considerable absorbance at 540nm among which the genotype with maximum apigenin (ARC 10975) showed a maximum absorbance of 1.274. Rice lines that possess below detectable amounts of apigenin also show biofilm formation. Nona Bokra, with apigenin content below the detectable threshold, shows more biofilm formation than the lines with apigenin. Though the CO 58 genotype's apigenin concentration is next best to ARC 10975, it has less biofilm formation in its root compared to its counterparts. Some of the genotypes that produced higher apigenin exhibited less biofilm-forming ability and this may be attributed to other factors that affect biofilm formation (6, 20-23).

In addition to apigenin, luteolin, which is present in root exudates and synthesized through the tricin biosynthesis pathway, also stimulates the formation of EPS when released into the soil (6). The substantial biofilm formation observed in Nona Bokra and Local Bhat, despite their low levels of apigenin, may be due to higher concentrations of luteolin in these cultivars. Besides flavonoids, root exudates of plants also contain a wide range of compounds like anthocyanins, lignins, carbohydrates, organic acids, amino acids, sterols, fatty acids, glucosinolates, proteins, enzymes and many more

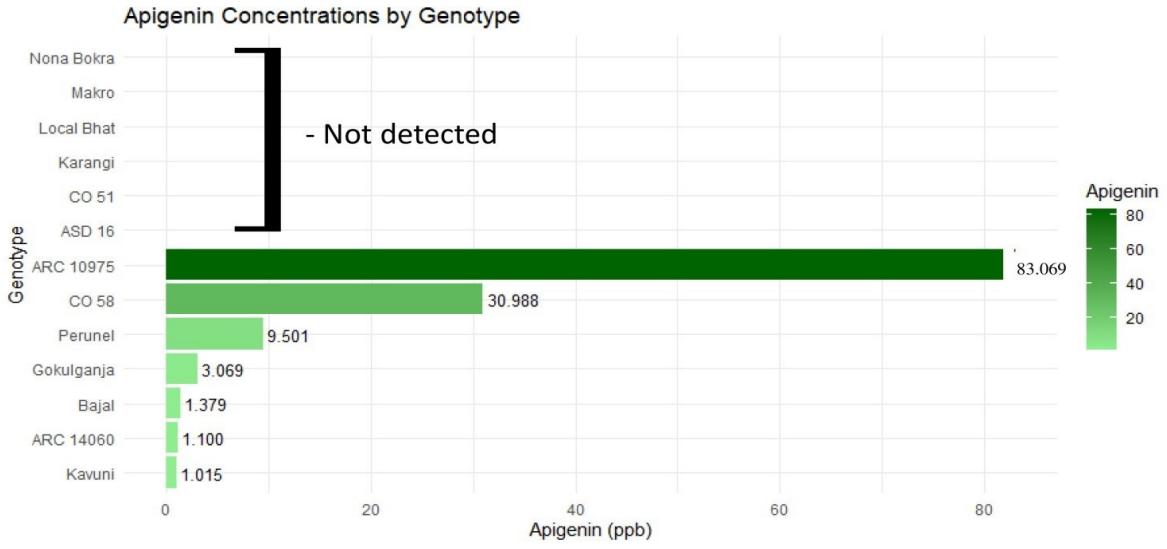

Fig. 3. Color gradient bar graph illustrating Apigenin concentrations (in ppb) across different genotypes: Genotypes with detectable levels of Apigenin are shown in varying shades, with darker color indicating higher concentration. 'ND' (Not Detected) is indicated for genotypes where Apigenin levels were below the detection limit.

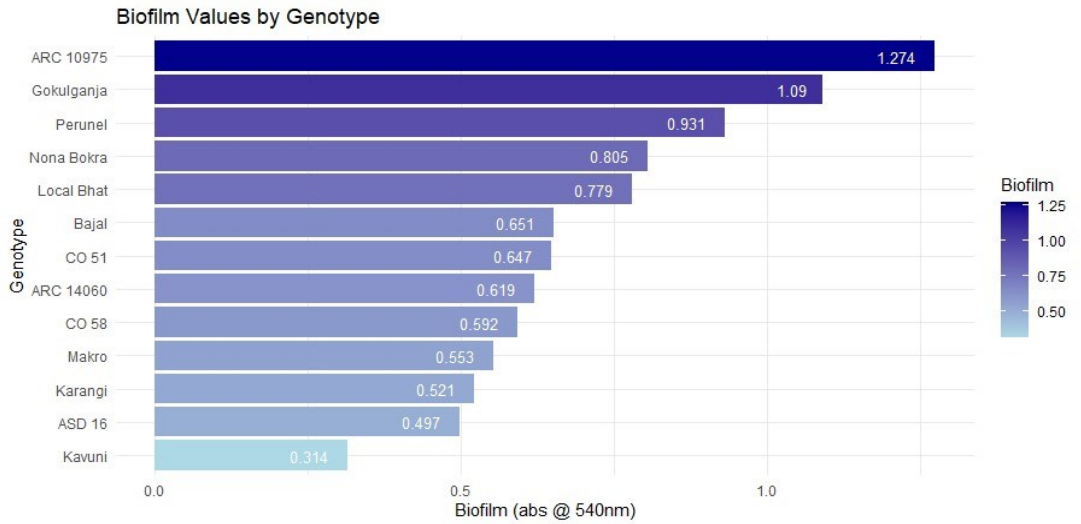

Fig. 4. Color gradient bar graph illustrating biofilm formation: Measured as absorbance at 540 nm across various genotypes with three replications. The gradient ranges from light blue (low biofilm formation) to dark blue (high biofilm formation), indicating differences in biofilm production among the genotypes. The obtained value of each well was normalized with DMSO + water control (absorbance - 0.107)

(20). Purified plant polysaccharides, specifically arabinogalactan, pectin and xylan, function as environmental signals that trigger the expression of matrix genes in *Bacillus subtilis*, promoting biofilm formation. However, their subunits and other complex polysaccharides from non-plant sources do not have this effect. In addition to serving as environmental cues, these plant-derived polysaccharides can be broken down and metabolized into UDP-galactose, a sugar nucleotide that is then incorporated into the EPS of the biofilm matrix. Therefore, plant polysaccharides play a dual role in not only initiating the biofilm formation process but also providing necessary building blocks for matrix synthesis in *B. subtilis.* In the presence of plant polysaccharides, other plant growth-promoting strains such as *B. subtilis* GB03 and *Bacillus amyloliquefaciens* FZB42 also produced more durable biofilms (21). Salicylic acid, a significant plant hormone, which is involved in the immune response of plants, influences the root microbiome composition in the case of *Arabidopsis thaliana*. Isogenic mutants of *A. thaliana* with modified immune responses were used to prove that salicylic acid has an impeccable role in the assembly of typical root microbiomes. Drastic changes were seen in the root-associated bacterial populations of plants that are devoid of salicylic acid signaling, indicating that salicylic acid influences the colonization of particular families (22). Other plant compounds like pentoses, aromatic organic acids and gamma-aminobutyric acid influence the microbial population in the rhizosphere and their colonization and these compounds play a pivotal role in determining the dynamics and composition of the rhizosphere-associated microbial community (23). Even though many other determinants are involved in establishing successful colonization and biofilm formation of microbes in roots, (6) genetic modification for increased apigenin proves to be successful for harnessing BNF benefits in rice crops.

Among the 13 genotypes, Nona Bokra shows promising signs for exploiting the benefits of BNF as even with the apigenin concentration below the traceable level, they exhibited biofilm absorbance of 0.805, which is higher than genotypes with apigenin. If we can increase the apigenin content in the Nona Bokra genotype by knocking *CYP75B3* and *CYP75B4* through genome editing, the apigenin content can be increased, thereby BNF can be improved drastically. Another promising genotype in biofilm formation is Gokulganja. Even though the Gokulganja genotype contains only a trace amount of apigenin concentration (3.069 ppb), biofilm formation in them is almost double that of CO 58, where CO 58 has almost 10 times more apigenin compared to Gokulganja. Therefore, Gokulganja can be another suitable candidate for the enhancement of apigenin. Less biofilm formation in the case of CO 58 despite its high apigenin may be due to above mentioned other factors that are present in the root exudates which have a huge impact on microbe-root colonization. Genotype Local Bhat shows similar results to Nona Bokra, as even in reduced apigenin levels, significant biofilm formation is visible. With ASD 16 being the most popularly cultivated and high-yielding *indica* rice variety in southern India (12), there is an imperative need to engineer the variety in such a way that it demands less N fertilizer. Genetic modification of this genotype to boost apigenin production is crucial, as it enhances BNF. This, in turn, would significantly reduce N fertilizer input, promoting more sustainable agriculture.

Conclusion

In this study, we expanded our analysis to assess the potential of 3K RG rice panels for engineering the genome to enhance BNF. The findings were promising with varieties such as Gokulganja, Local Bhat and Nona Bokra showing positive responses that indicate potential for increased BNF through genetic modification. On the other hand, the ASD 16 variety exhibited a need for targeted engineering to achieve similar results. On the successful modification of the rice genome, BNF can be enhanced leading to a significant reduction in fertilizer use, which would have far-reaching environmental benefits.

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Authors' contributions

AB carried out the phylogenetic analysis, biofilm assay, apigenin estimation and drafted the article. RM and RR were the lead for the concept and supervised the study. SA and MS designed the methodology. SM reviewed the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author used ChatGPT in order to improve language and readability, with caution. After using the tool AA reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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