Background:
Environmental risk scenarios linked to NGT rice with modified flavone biosynthesis

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Abstract

Rice is globally one of the most important staple foods and also an important model plant for applications of new genomic techniques (NGTs). In the recent study of Yan et al., researchers generated an NGT rice variant, which is unlikely to be achieved by conventional breeding methods, including non-targeted mutagenesis. They knocked two genes involved in flavone biosynthesis to produce an NGT rice with increased apigenin content. The elevated content of this flavone was shown to increase the biofilm formation of nitrogen-fixing bacteria in soil and, therefore, to increase the nitrogen availability for the rice plant. However, the altered composition of associated microbiota could also have negative consequences, i.e. to affect the plants' susceptibility to biotic or abiotic stressors, yield and greenhouse gas emissions. The ability of the NGT rice to hybridize with wild and weedy rice could also lead to the uncontrolled spread of the newly introduced genotypes into the environment, with unpredictable consequences, e.g. weedy rice could potentially become an increasingly serious threat to rice farming.

Introduction

Rice is a major food source especially in Asia, Africa and South America. In recent decades, breeding approaches have successfully increased rice productivity. However, weedy rice, climate change, pathogens and other factors still regularly threaten rice harvests.

NGT applications are currently being used in many plant species to develop new varieties, including rice. Its relatively small-sized diploid genome and the availability of genomic data (Sasaki, 2005) has made rice a model plant for functional and structural genomics studies (see e.g. Mishra et al., 2018; Zafar et al., 2020; Zegeye et al., 2022). At present, there are more than two hundred published studies on NGT rice variants (EU-SAGE), some of which have already been tested in field trials. The first EU field trial with NGT rice is currently being conducted in Italy (B/IT/24/01, 2024).

This factsheet presents a real case of an NGT rice variant for which an international patent application has been filed (WO 2022/015762). It seems to be questionable, if this rice could be obtained from conventional breeding, including non-targeted mutagenesis. We include an overview of related hazards and environmental risk scenarios for this NGT rice to demonstrate the need for in-depth risk assessment.

Biology and ecology of rice

Rice belongs to the grass family of Poaceae. Two domesticated rice species are cultivated as a cereal: *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice). There are many related subspecies and varieties that have been described. The cultivation of African rice is

currently in decline and has been partially replaced by higher-yielding Asian rice subspecies, e.g. *Oryza sativa japonica* and *Oryza sativa indica*.

Apart from domesticated rice, more than 20 species of wild rice have been described. Amongst them, *O. rufipogon* and *O. barthii* are thought to be the wild progenitors of *O. sativa* and *O. glaberrima*, respectively. Continuous hybridization and de-domestication between cultivated varieties and wild progenitors have resulted in various weedy rice types (Roma-Burgos et al., 2021). Weedy rice produces fewer grains but shares characteristics and appearance with cultivated rice species – it is therefore very difficult to identify and combat. In addition, severe seed shattering and deep seed dormancy in wild rice means that it remains viable for years in seed banks (Grimm et al., 2020). Populations of weedy rice are found worldwide and substantially diminish rice yields.

Rice is a principally self-pollinating crop that produces large quantities of pollen grains. However, cross-pollination can occur either by wind or insect pollination, e.g. honey bees can carry a large amount of viable rice pollen over long distances (Pu et al., 2014), thus also contributing to gene flow between cultivated, wild and weedy rice.

The interaction of rice plants with associated microbiota is also very important both agriculturally and ecologically, as the plants and their associated microbiota act as a functional entity, also known as a 'holobiont' (Sánchez-Cañizares et al., 2017). Rice plants form symbiotic associations, e.g. with mycorrhizal fungi or nitrogen-fixing bacteria, thus providing benefits by improving nutrition and fitness (Campo et al., 2020; Masuda et al., 2023). However, we still have only limited knowledge about these complex interactions.

Transgenic rice

Projects to genetically engineer rice using established genomic techniques started decades ago and resulted in different transgenic rice events. However, very few events have been granted market authorisation, with traits comprising mostly the production of insecticidal Bt toxins or herbicide tolerance. Bt rice was authorised in China in 2007, but there appears to be no commercial cultivation. Only very few herbicide (glufosinate) tolerant events have been approved for the US market. In the Philippines, rice with enhanced provitamin A content (Golden Rice) was introduced after decades of discussion.

So far, only one market application has been filed for import into the EU, for rice resistant to the herbicide glufosinate (LL62). The application was withdrawn in 2007.

The relatively small number of transgenic rice authorisations worldwide may be associated with several incidents of contamination, which affected the markets globally. Interestingly, many cases appear to involve accidental releases of unapproved GMOs (Clapp, 2008). For example, in 2006, glufosinate tolerant rice, LL601, was detected in parts of the US harvest, and later found to have originated from a few small-scale field trials conducted by Aventis (now Bayer). By the autumn of 2006, LL601 had been found in shipments of imported rice in countries throughout the world. The costs incurred globally as a result of LL601 rice contamination were estimated to range from 741 million to 1.285 billion US dollars (Blue, 2007).

In the EU, from 2002 to 2023, rice was found to be responsible for more than half of all cases of food imports containing unlabelled GMOs (Eissa et al., 2024), many of which were imports from China. Bt rice from China was first detected in 2005, even though there is no commercial cultivation of transgenic rice in China until today. The rice events appear to have originated from field trials – from where they permanently introgressed Chinese rice seed systems.

NGT rice with increased apigenin content (Yan et al., 2022)

NGTs have been increasingly applied in rice in recent years, and rice plants have become a model organism for many genome editing studies. As a result, there are now a large number of NGT rice variants, one of which is analyzed in more detail below.

The cultivation of crops like rice is heavily dependent on the application of nitrogen fertilizers, as most plants are not able to directly use molecular nitrogen in the atmosphere (N_2) (Pittol et al., 2016). They rely on other sources, such as nitrogen fertilizer, decomposed biomass or certain, diazotrophic bacteria, which convert atmospheric nitrogen into ammonium nitrogen (NH_4^+), and thus make it available for plants.

The study published by Yan et al. pursued an indirect approach to increasing nitrogen availability in rice plants, i.e. the plants were genetically modified with NGTs to attract more nitrogen-fixing bacteria.

Interactions between plant roots and bacteria in the soil are, i. a. supported by the formation of biofilms (Flemming and Wingender, 2010; Meneses et al., 2011). Therefore, Yan et al. screened for plant compounds that stimulated biofilm formation of nitrogen-fixing bacteria promoting the colonization around the plant roots and subsequent biological nitrogen fixation. They identified, amongst others, the flavone apigenin which induces the biofilm formation of nitrogen-fixing bacteria.
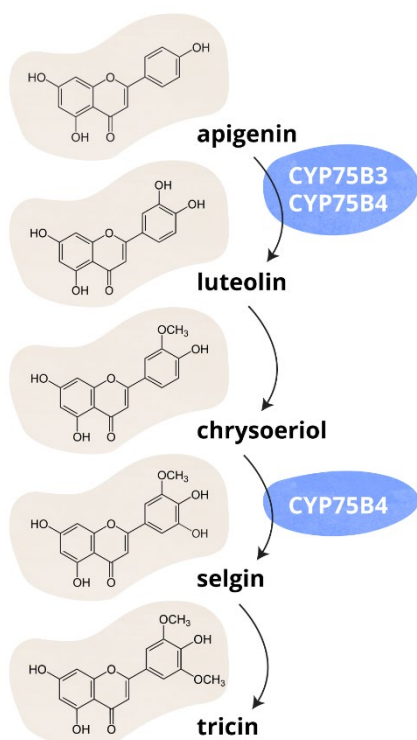

Figure 1: The role of the enzymes the flavonoid hydroxylases CYP75B3 and CYP75B4 in the tricin biosynthesis pathway. The hydroxylases CYP75B3 and CYP75B4 mediate the conversion of apigenin into luteolin. Chrysoeriol is an intermediate which is further 5'-hydroxylated by CYP75B4 to selgin, the precursor of tricin.

In order to increase the amount of apigenin in rice plants (*O. sativa japonica cv* Kitaake), the researchers used CRISPR/Cas9 to switch off enzymes that degrade apigenin or convert it into other substances. The authors simultaneously targeted two genes encoding flavonoid hydroxylases (CYP75B3 and CYP75B4) that convert apigenin to luteolin and chrysoeriol to selgin (CYP75B4) (Figure 1). The resulting NGT rice extruded more apigenin from the roots, promoting the biofilm formation of nitrogen-fixing bacteria around the roots. In addition, the NGT rice plants assimilate more nitrogen than the control rice. Under limited soil nitrogen conditions, the NGT rice had more panicles and their yield increased by 20-30 %.

As a side effect of the genetic alteration, the NGT rice was smaller under all tested conditions. In this context it may be interesting that tricin metabolites are incorporated into the cell walls (cell wall lignification) and CYP75B4 disruption strongly affects tricin-lignins and other cell wall components (Lam et al., 2019). The function of tricin in cell wall lignins remain largely unknown (Lam et al., 2021). Furthermore, the analysis of soil samples showed that the composition of the soil bacteria of the NGT rice differs compared to the control plants. The NGT rice recruited a distinct microbiota in the rhizosphere, rhizoplane and endosphere and, in particular, more nitrogen-fixing bacteria.

Difference to conventional breeding

Plant genome organization makes some genomic regions less accessible to conventional breeding methods, including non-targeted mutagenesis. However, NGTs can partially overcome these limitations (Kawall, 2019). In recent years, several research teams have produced rice with genotypes unlikely to be achieved using conventional breeding methods, including non-targeted mutagenesis, (see e.g. (Khanday et al., 2019; Zhang et al., 2020; Wakasa et al., 2024)).

The genotype of the NGT rice with increased apigenin content is not yet known from conventional breeding. There is reason to assume that this genotype would be hardly achievable using conventional breeding methods, as the targeted genes (CYP75B3 and CYP75B4) are probably genetically linked and lie in the genomic region in which recombination is known to occur at a low rate (Figure 2):

The two genes are in close proximity to each other. It is less likely that a recombination event will occur between them and therefore they are referred to as genetically linked. In addition, both genes lie in pericentromeric regions close to the centromere of chromosome 10 with very low-recombination rates (Chen et al., 2002; Sasaki, 2005; Yan et al., 2008; Flowers et al., 2012; Fayos et al., 2019; Peñuela et al., 2023). Suppressed recombination leads to co-segregation of genes in the respective domain (Yan et al., 2008). This means constrains for conventional breeding, because it relies often on the combination of single mutations in one of the respective genes. This is hardly achievable when genes are genetically linked and further lie in regions with low recombination rates that are largely inaccessible to breeding (Chen et al., 2002; Fayos et al., 2019; Song et al., 2021; Shang et al., 2023; Zou et al., 2024). In addition, natural occurrence of large deletions or inversions, which could also lead to the knockout of genes, seem to occur mainly in hotspots outside of (peri-) centromeric regions (Zhou et al., 2023, 2024a, 2024b). Taken all together, it seems unlikely that the knockout of the two flavonoid hydroxylases could be achieved with conventional breeding methods, including non-targeted mutagenesis, at all or in manageable time periods. These findings are also relevant for risk assessment: it should not be overlooked that the rice with the new genotype and its complex interactions with soil bacteria might be introduced into the environment at large scale and may also perform gene flow with wild relatives.
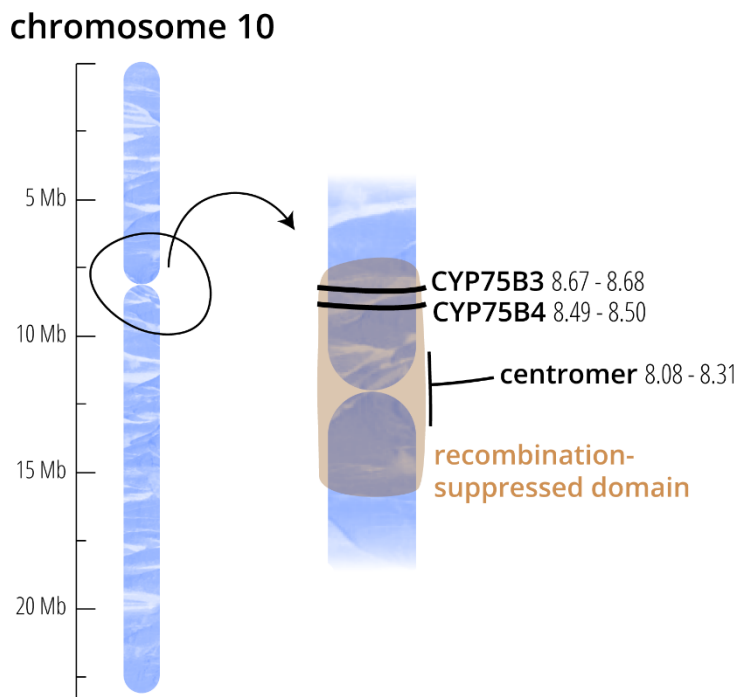
chromosome 10

Fig. 2: CYP75B3 and CYP75B4 lie in close proximity to each other and to the centromere of chromosome 10. Chromosome 10 has a length of 22.9 Mb. The centromere is located at 8.08-8.31 Mb. CYP75B3 (Os10g0320100, position 8.67-8.68 Mb) and CYP75B4 (Os10g0317900, position 8.49-8.50 Mb) lie in close proximity to each other and to the centromere. The size of the crossover-suppressed domain surrounds the centromere.

CYP75B3 8.67 - 8.68
CYP75B4 8.49 - 8.50

centromer 8.08 - 8.31

recombination-suppressed domain

5 Mb
10 Mb
15 Mb
20 Mb

Hazard identification and scenarios relevant to risk assessment

→ *Gene flow and outcrossing*

Rice generally has a high potential for outcrossing/hybridization with wild or weedy rice populations (see biology and ecology of rice). This enables the gene flow of new NGT rice genotypes into the environment (Chen et al., 2004; Lu and Yang, 2009).

The NGT rice genotype developed by Yan et al. has higher ecological fitness due to increased nitrogen assimilation and a higher number of panicles under nitrogen-limiting conditions. In addition, it is smaller, which could also be a fitness advantage under certain environmental conditions (see (Wakasa et al., 2024)). Escapes of this genotype could cause ecological problems (Lu et al., 2003). Hybrids of NGT rice and weedy rice may have an even greater potential to persist and spread in the environment. Consequently, hybrids with enhanced ecological fitness could become much more invasive and difficult to manage (Lu and Snow, 2005). In areas with wild rice populations (e.g. in Asia), outcrossing of NGT rice genotypes may contaminate wild populations, and could possibly lead to displacement of local species (Lu et al., 2003).

In general, outcrossing can result in new combinations of genes in the offspring, and may display next-generation effects associated with unexpected risks resulting from genomic interactions (Bauer-Panskus et al., 2020; Koller et al., 2023). In future, large-scale releases of different NGT rice species into the environment at short intervals may further promote combinatorial effects between NGT plants. The resulting effects may be dependent on the specific gene combinations, the intended traits and exposure to stress conditions in the receiving environment (Koller et al., 2023).

→ *Influence on microbiota*

Switching off the downstream metabolic pathways resulted in an accumulation of the flavone apigenin in the NGT rice discussed here, and thus the enrichment of the nitrogen-fixing bacteria in the root environment of the plant. At the same time, other substances derived from apigenin are either produced less, or not at all, in this rice variant. An earlier study

showed that other flavones, such as tricin, luteolin and chrysoeriol glycosides, were completely absent in a different NGT rice variant when the CYP75B3 and CYP75B4 genes were knocked out (Lam et al., 2019). These other flavones are also known to play essential roles in cross-talk between plants and beneficial microbiota (in the phyllosphere, endosphere and rhizosphere) and defense mechanisms against different pathogens and insects (Peters et al., 1986; Adjei-Afriyie et al., 2000; Kong et al., 2004, 2010; Bing et al., 2007; Bag et al., 2022; Kumar et al., 2024).

Due to the absence of other flavones, the apigenin-rich NGT rice could be more susceptible to infections in the field. In addition, the composition of the microbiota in the root environment is changed. This may be positive for the uptake of nitrogen, but could conceivably be negative for other microbial plant interactions important for soil ecology, plant health and yield.

The interaction of rice plants and their surrounding microbiota is finely-tuned, highly complex and far from fully understood (Pittol et al., 2016; Pantigoso et al., 2022; Santos-Medellín et al., 2022). Therefore, changes in metabolites in genetically modified plants and their impact on the microbiota should be thoroughly investigated during development (Huang et al., 2024; ISPRA, 2024). Moreover, their susceptibility to pathogens or the effects of different environmental conditions are hardly predictable, and will therefore require empirical studies in different environments.

→ *Increasing greenhouse gase emissions*

Rice cultivation represents a significant fraction of agricultural greenhouse gas emissions, in particular nitrous oxide (N_2O) and methane (CH_4). Microorganisms are responsible for a large part of greenhouse gas emissions, as N_2O and CH_4 are products or byproducts in, e.g. nitrification/denitrification and methanogenesis, respectively. The amount of greenhouse gases produced in the soil is strongly influenced by, e.g. climate, plant growth stage, root exudates, crop management practices, microbial population, carbon, nitrogen and oxygen contents (Hassan et al., 2022; Rajendran et al., 2024).

Altered flavone content in the NGT rice strongly influences the recruited microbiota in the rhizosphere, rhizoplane and endosphere (Yan et al., 2022). The changed microbial community could possibly influence greenhouse gas emissions. Since the emissions correlate with microbial composition, a shift in the microbial community can also alter N_2O and CH_4 emissions (see e.g. (Wang et al., 2017; Yin et al., 2023)). Even if the cultivation of the discussed NGT rice could potentially reduce the use of nitrogen fertilizer, changes in the microbial population could negatively influence greenhouse gas emissions if, for example, the population of (de)nitrifying or methanogenic microorganisms grows. In addition, the increased occurrence of nitrogen-fixing bacteria in the rhizocompartments of the NGT rice (Yan et al., 2022) will lead to higher amounts of available ammonium in the soil. Similarly to nitrogen fertilizer, this could also influence N_2O emissions, as ammonium serves as substrate for N_2O producing microorganism. The respective emissions underlie complex interactions further influenced by, e.g. plant growth stage, amount of ammonium, climate and irrigation (oxygen availability) (Hassan et al., 2022; Rajendran et al., 2024) and is very difficult to predict. Therefore, the actual amount of greenhouse gas emissions under the described approach need to be investigated empirically under various conditions.

→ *Possible interaction with genetically engineered microorganisms*

In recent years, genetically engineered (GE) microorganisms have been developed both for release into the environment and as biological agents for bioremediation, biocontrol or biofertilization (Ballester et al., 2023; Miklau et al., 2024). After release into the soil, these GE microorganisms are meant to, for example, produce and excrete bioavailable nitrogen, and

thus promote the growth of crop plants (Das, 2019; Ambrosio and Curatti, 2021; Wen et al., 2021). NGTs and synthetic biology will in future enable the development of an even broader range of GE microorganisms.

Environmental risk assessment and monitoring will be faced with new questions and challenges due to the ability of GM microorganisms to spread, and factors such as dispersal, horizontal gene transfer and rapid evolution (Miklau et al., 2024). Unintended interactions between different GE microorganisms released into the same environment, or with other organisms in the receiving environment, may also occur. Furthermore, it has been found that microorganisms strongly influence the transcriptome of plants (Santos-Medellín et al., 2022). These interactions are highly complex and our knowledge is still limited. The simultaneous release of different GE microorganisms and NGT plants, e.g. NGT rice, will lead to further new and difficult to predict interactions.

Conclusion

Yan et al. were able to generate an NGT rice variant that is unlikely to be achieved from conventional breeding, including non-targeted mutagenesis. The NGT rice produces large amounts of apigenin, but lacks several other flavones. The composition of the associated microbiota is thus also changed.

Symbiotic interactions with microorganisms significantly impact the health and the ecosystem services of plants. Changes in composition and the complex interactions with microbiota may have a broad range of (unintended) effects. Even if the cultivation of this NGT rice variant could potentially reduce the use of nitrogen fertilizer, other unintended effects, e.g. increased greenhouse gases, susceptibility to pathogens or reduced resistance to abiotic stress, may arise and make the beneficial claims of the approach questionable.

Once released, the new NGT genotype can spread into wild and weedy rice, as hybridization often occurs between the rice variants. The intended NGT rice trait also increases its fitness, which may further exacerbate the problems of outcrossing and hybridization. Spontaneous crossings may certainly be relevant for the environment, regional populations of wild rice and rice yields. Damage to biodiversity in and around the fields may possibly be irreversible.

The increasing speed and scale of the introduction of new genotypes, e.g. rice variants and combinations thereof, could overwhelm the natural ecosystems. Furthermore, the simultaneous release of GE microorganisms could lead to unintended and unpredictable interactions.

The above-discussed example of the NGT rice variant shows that a detailed and comprehensive risk assessment is required before any large-scale cultivation of this GMO is allowed. Ultimately, risk assessment should explore unintended direct and indirect, immediate and cumulative long-term effects associated with the environment and other GMOs.

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