

Rice breeding in the new era: comparison of useful agronomic traits

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1 **Rice breeding in the new era: comparison of useful agronomic traits**

2 **Abstract**

3 Understanding agronomic traits at a genetic level enables the leveraging of this knowledge
4 to produce crops that are more productive and resilient, have better quality and are adjusted
5 for consumer preferences. In the last decade, rice has become a model to validate the function
6 of specific genes, resulting in valuable but scattered information. Here, we aimed to identify
7 particular genes in rice related to traits that can be targeted by different mutation techniques
8 in the breeding of crops. We selected gain of function, malfunction, and specific mutations
9 associated with phenotypes of agronomic interest. The review includes specific trait-related
10 genes involved in domestication, stress, herbicide tolerance, pathogen resistance, grain
11 number/quality/weight, plant structure, nitrogen use, and others. The information presented
12 can be used for rice, other cereals, and orphan crops to achieve a superior and sustainable
13 production in challenging farming conditions.

14 **Keywords.** Mutagenesis, domestication, CRISPR, gamma radiation, chemical mutagen,
15 biotic stress, abiotic stress

16

17 **Introduction**

18 Induced mutagenesis is a valuable tool to support functional genomics studies and the
19 development of new genotypes. Rice serves as an outstanding model because of its impact
20 on the worldwide food supply chain and the availability of genomic and agronomic resources
21 to utilize. Rice was the first crop sequenced in 2004 (Matsumoto *et al.*, 2005),
22 biotechnological techniques are available, and the genomic information is available to search
23 for specific target mutations, such as from the Rice Genome Annotation Project and Oryza
24 Genome which can contribute to the precise engineering of the crop (Kajiya-Kanegae *et al.*,
25 2021; Kawahara *et al.*, 2013; Tanaka *et al.*, 2020). Biological, chemical, and physical agents
26 can induce mutagenesis. Typical methods are radiation (first used on vegetables in 1928),
27 ethyl methanesulfonate (EMS) (which produces 2-10 mutations per Mb), and new breeding
28 techniques to introduce specific mutations via genetic engineering (Romero & Gatica-Arias,
29 2019; Serrat *et al.*, 2014; Soriano, 1961; Viana *et al.*, 2019). In this review, we present rice
30 traits that have emerged or have been validated in the last decade (2010-2021) and were
31 derived from technological advances in genomics (Benavente & Giménez, 2021). This paper
32 is focused on characteristics that could be targeted by mutagenesis of rice lines and related
33 crops to produce predictable changes in gains or losses of function. We present traits that
34 could result from the use of different techniques; remarkably, genome editing represents an
35 exciting opportunity to transfer the information about gene-trait relationships to other crops
36 to improve their traits. Consequently, they represent a challenge from a regulatory point of
37 view for countries that have established a different regulation for genome-edited plants in
38 contrast to other mutagenesis techniques.

39 **Methods**

40 The methodology applied a search based on PubMed articles and keywords: rice, traits, stress
41 tolerance, resistance, breeding; selection of papers with agronomic traits linked to specific
42 genes described within 2010-2021. Finally, verification of each gene, trait, and mutation was
43 performed using specialized web servers such as Gramene, EnsemblPlants, Rice Diversity,
44 FunRiceGenes, Rice Genome Annotation Project, Oryza Base, and Rice Information
45 GateWay. The search resulted in a selection of 117 papers out of 500.

Webserver	Link
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Gramene	https://ensembl.gramene.org/genome_browser/index.html
EnsemblPlants	http://plants.ensembl.org/index.html
Rice Diversity	http://www.ricediversity.org/data/index.cfm
FunRiceGenes	http://funricegenes.ncpgr.cn/
Rice Genome Annotation Project, Michigan State University	http://rice.plantbiology.msu.edu
Oryza Base	https://shigen.nig.ac.jp/rice/oryzabase/
Rice Information GateWay	http://rice.hzau.edu.cn/

46

47 **Importance for breeding rice**

48 Rice, such as many other tropical crops, is susceptible to a large set of biotic (fungi, bacteria,
49 nematodes, insects, and viruses) and abiotic (salinity, drought, heat, and cold) stresses that
50 cause yield and economic losses (Fig. 1). In general, biotic stress cause losses worldwide up
51 to 35% of the total food production (Bainsla & Meena, 2016). As an example, losses in rice
52 due to insects can account for over 40%. Moreover, losses caused by the fungal pathogens
53 *Magnaporthe grisea*, *Thanatephorus cucumeris*, and *C. miyabeanus* have been estimated
54 worldwide at 35%, 24%, and 16%, respectively (Oerke & Dehne, 2004). On the other hand,
55 abiotic stress represents the primary cause of crop losses worldwide, and yield losses can be
56 as high as 50% of crop production (Ashraf *et al.*, 2008).

57 In this regard, the generation of rice-resistant varieties to biotic and abiotic conditions
58 represents one of the major challenges that breeders face. For decades, breeding strategies
59 include selection, hybridization, mutation induction using chemical and physical agents, and
60 somaclonal variation. More recently, the availability of genome editing technologies,
61 genome sequences, efficient tissue culture, and transformation methodologies could
62 remarkably facilitate the breeding of rice (Fig. 2).

63 **Rice breeding systems**

64 Several methods are available for breeding rice with natural or induced mutagenesis; among
65 them, we can mention mutation breeding, tissue culture, and new breeding techniques
66 (CRISPR mutagenesis, base editing, and prime editing) (Fig. 2).

67 Mutation breeding

68 The mutation breeding principle is to generate heritable changes in the DNA by external
69 agents. The changes result by exposing plant cells to physical (UV, X-ray, gamma radiation)

70 or chemical (sodium azide and ethyl methanesulfonate) agents (Mba *et al.*, 2010). Induced
71 mutagenesis offers a promising alternative for developing rice varieties resistant to biotic and
72 abiotic stresses since it could accelerate the spontaneous mutation process and increase the
73 pool of allelic variants available for genetic improvement (Gressel & Levy, 2006; Oladosu
74 *et al.*, 2016; Viana *et al.*, 2019).

75 Tissue culture

76 Totipotency, a distinguishable characteristic of plant cells, in principle allows each cell to
77 regenerate an entire plant. This process involves the culture of plant tissue fragments or
78 individual cells on special growth media enabling the cells to grow, divide, and differentiate
79 into organs (Fehér, 2019). Among the techniques available, somaclonal variation are
80 spontaneous changes in the DNA leading to genetic and phenotypic variations among
81 clonally propagated plants. The somaclonal variants obtained could be detected using in vitro
82 selection by applying selective pressure in culture conditions (Bairu *et al.*, 2011; Larkin &
83 Scowcroft, 1981).

84 New breeding techniques

85 *CRISPR/Cas9*

86 The clustered regularly interspaced short palindromic repeats (CRISPR)-associated
87 endonuclease Cas9 (CRISPR/Cas9) system from *Streptococcus pyogenes* targets a specific
88 genomic sequence using an engineered 20 base pair (bp) RNA guide sequence that binds to
89 matching DNA and the Cas9 protein, upon recognition of an additional 3' localized PAM
90 sequence 5'-NGG-3', generates a double-strand break at a desired location in the genome.
91 This genome editing method allows the insertion, deletion, or modification of DNA with high
92 specificity and efficiency (Romero & Gatica-Arias, 2019).

93 *CRISPR/Cpf1 system*

94 The nuclease Cas12a requires a small crRNA for inducing double strand breaks with
95 efficiencies similar to those of CRISPR/Cas9. Moreover, this nuclease uses a 18-23 nt spacer
96 for its maximum efficiency and specificity and identifies a T-rich PAM located 5' upstream
97 of the guide and generates staggered ends with 5' overhangs (Schindele *et al.*, 2018).

98 *Base editing*

99 This system allows the conversion of nucleotides without inducing double-stranded DNA
100 breaks or using donor templates. It is based on Cas9 nickase fusions to a nucleotide
101 deaminase domain and has been used for changing a C-G base pairs into T-A (cytidine
102 deaminase base editor), or A-T into G-C (adenosine deaminase base editor) (Marx, 2018).

103 *Prime editing*

104 This system uses a catalytically impaired Cas9 endonuclease fused to a reverse transcriptase
105 enzyme, and a prime editing guide RNA (pegRNA). This complex is capable of identifying
106 a target site and replace the target DNA nucleotides without double-stranded DNA breaks or
107 using donor templates (Anzalone *et al.*, 2019; Lin *et al.*, 2020).

108 **Agronomic traits of interest**

109 **1. Domestication genes**

110 The *Oryza* genus is composed of species with a variety of genome structures, including six
111 diploids (n = 12; named AA, BB, CC, ee, ff, gg) and five polyploids (n = 24, named BBCC,
112 CCDD, HHJJ, HHKK, and KKLL) (E. Chen *et al.*, 2019; Kim *et al.*, 2015; Nadir *et al.*, 2017;
113 Wing *et al.*, 2018). Only two diploid (2n=24) species of rice have been domesticated and
114 used for cultivation: *Oryza sativa* and African *O. glaberrima*. Rice domestication favored the
115 selection of specific loss of function alleles. Wild relatives typically have functional versions
116 of these genes such as *sh4*, *waxy*, *BH4*, *qSH1*, *AN1*, *brown pericarp*, *PROG1*, and *OsG1*. The
117 *sh4* gene is related to reduced seed shattering (*Os04g0670900*). The *waxy* gene controls the
118 amylose content (*Os06g0133000*). *BH4* is related to the hull color of the seeds
119 (*Os04g0460200*). The gene *qSH1* is involved in seed shattering (*Os01g0848400*). The *AN1*
120 gene is related to seeds, morphology, and grain shape (*Os04g0350700*). *RC Brown pericarp*
121 is involved in the seed coat (*Os07g0211500*). *PROG1* is related to an erect plant structure
122 (*Os07g0153600*). *OsLG1* is related to a closed-panicle structure (*Os04g0656500*) (Li *et al.*,
123 2017). The importance of such genes and their domesticated alleles is critical in
124 understanding how *de novo* domestication can be achieved from wild *Oryza* varieties and
125 how such genes can be further used for breeding of rice and other crops.

126 This concept was demonstrated in polyploid *O. alta* (CCDD) by (H. Yu *et al.*, 2021),
127 targeting *SD1*, *GS3*, *IPAI*, *Ghd7*, *Gn1a*, *Wx*, *Bh4*, *TAC1*, *An-1* homologs, as well as African

128 landraces of *Oryza glaberrima* by disrupting the *HTD1* (*O. sativa* Os04g0550600), *GS3* (*O.*
129 *sativa* Os03g0407400), *GW2* (*O. sativa* Os01g0197700) and *GNIA* (*O. sativa*
130 Os02g0244100) genes (Lacchini *et al.*, 2020). For plant breeding, the use of non-
131 domesticated, more genetically diverse rice species that better adapt to stress conditions, such
132 as African landraces *O. glaberrima*, *O. barthii*, *O. meridionalis* (AA), Australian landraces
133 *O. longistaminata* (AA), *O. australiensis* (EE), and Asian landraces *O. rufipogon* (AA) or
134 *Porteresia coarctata* (*O. coarctata*) (KKLL), harbors a valuable potential of developing more
135 sustainable rice crops (Mammadov *et al.*, 2018; Reddy *et al.*, 2017).

136 **2. Stress tolerance**

137 Rice susceptibility to salt is evidenced by a yield decrease due to delays in heading and
138 panicle sterility especially in salt-sensitive varieties like MI48. In contrast, salt tolerance in
139 varieties like IR29, Fatmawati, Pokkali, Cheriviroppu, FL478, IR651, CSR27, FL30, Fontan,
140 SR86, IR9884-54-3 results from ion exclusion, osmotic and tissue tolerance with multiple
141 genes involved in the process, which confers agronomic stability of this trait (Qin *et al.*, 2020;
142 Reddy *et al.*, 2017; Takagi *et al.*, 2015; A. Zhang *et al.*, 2019). The orchestrated stress system
143 can be targeted for achieving salt tolerance by mutating genes encoding key transcription
144 factors, specifically *OsRR22* (Os06g0183100), *STL1* (Os04g0110600), and the zinc finger
145 transcription factor encoded by *DST* (Os03g0786400) (Cui *et al.*, 2015; X. Liu *et al.*, 2020;
146 Santosh Kumar *et al.*, 2020; Yuan *et al.*, 2020; A. Zhang *et al.*, 2019). Other transcription
147 factors are critical in stress adaptation, which results in stress sensitivity when inactivated.
148 This is the case for *MSL37* (Os11g0163500) which encodes a positive salt stress transcription
149 factor response by regulating ion transporters, *P5CS* (Os05g0455500), which causes
150 accumulation of the osmoprotectant proline, the transcription factor *SNAC2*
151 (Os01g0884300), which is key in root adaptation, and *OsNAP* (Os03g0327800), which
152 triggers a stress response mediated by ABA (X. Chen *et al.*, 2014; Lee *et al.*, 2017; Nuñez-
153 Muñoz *et al.*, 2021; Sripinyowanich *et al.*, 2013; Takasaki *et al.*, 2010). For details, see
154 Figure 3 and Table 1.

155 Osmoprotection by accumulating molecules such as trehalose or proline is a possible
156 pathway leading to salt tolerance, as proven currently in plants like rice and *Arabidopsis*
157 (Garg *et al.*, 2002; H. W. Li *et al.*, 2011; Nuñez-Muñoz *et al.*, 2021; Paul *et al.*, 2018). Other

158 individual genes can confer osmoprotection, such as the Na⁺ transporter *SKCI*
159 (*Os01g0307500*) with a V395L that provides salt tolerance (Jayabalan *et al.*, 2019).
160 Knocking out an independent gene, *OsEPFL9* (*Os01g0824500*), results in increased water
161 use efficiency under stress because of the reduced stomatal count (Yin *et al.*, 2017, 2019).

162 Other stress tolerance pathways can be modified by specific alleles, too. Low cadmium
163 accumulation occurs after mutating the metal transporter genes *OsNramp5* (*Os07g0257200*)
164 and *OsNramp1* (*Os07g0258400*). Plants are able to resist heat stress only when the gene
165 *OsNTL3* (*Os01g0261200*) is functioning correctly, whereas cold tolerance can result from
166 mutation of the *OsMYB30* (*Os02g0624300*) gene. Finally, more cuticle wax is deposited
167 when the gene *DHS* (*Os02g0682300*) is mutated (Chang *et al.*, 2020; X. H. Liu *et al.*, 2020;
168 Sasaki *et al.*, 2012; L. Tang *et al.*, 2017; Z. Wang *et al.*, 2018; Zeng *et al.*, 2020).

169 **3. Herbicide resistance monogenic traits**

170 Rice is usually cultivated under two agronomical systems: paddy transplanted rice (PTR) and
171 dry seeded rice (DSR). The first is the conventional method, which requires water flooding
172 and represents a sustainability issue because of water scarcity, methane production, and the
173 consumption of nonrenewable energy (W. Wang *et al.*, 2017). DSR, on the other hand,
174 represents opportunities for efficient water and nitrogen use, and a reduction of both
175 greenhouse gas emissions and labor demand, especially in countries such as China, where
176 90% of rice is currently produced under PTR (Shekhawat *et al.*, 2020). However, weed
177 management is a challenge in DRS, specifically during the first 41 days after sowing (DAS).
178 This includes complication by weedy rice (*O. sativa f. spontanea*), which is a variety of rice
179 that is morphologically similar to cultivated rice, but grows as a weed. It produces far fewer
180 grains than cultivated rice, and can result in rice yield losses of up to 50% (Nadir *et al.*, 2017).
181 Weedy rice usually has increased seed longevity, seed shattering and stress tolerance which
182 makes it difficult to control (Durand-Morat *et al.*, 2018). The use of chemical control
183 represents a tool to manage weeds including weedy rice, but poses additional challenges.

184 The Herbicide Resistance Action Committee (HRAC) and the Weed Science Society of
185 America (WSSA) classify herbicides into 34 groups and one unknown group based on their
186 "mode of action" (MoA) at the biochemical level (Dayan *et al.*, 2019; Forouzesh *et al.*, 2015;
187 Gaines *et al.*, 2020). The discovery of a new mode of action has been rare in the last 30 years.

188 A good example is leptospermone, and its analogous inhibitors that act as
189 hydroxyphenylpyruvate inhibitors of dioxygenase (HPPD) (Dayan & Duke, 2020). Different
190 modes of herbicide use, such as rotations, delay the emergence of herbicide-resistant weeds.
191 However, weeds are evolving to resist multiple MoA types of herbicides. For example,
192 *Chloris radiata* is found in Colombian rice fields with dual resistance to glyphosate (mode
193 of action 9) and the acetolactate synthase (ALS) inhibitor imazomox (mode of action 2)
194 (Hoyos *et al.*, 2021). Weedy rice infestation in the USA resulted in 5.7 million tons of harvest
195 lost and \$457 million in environmental costs between 2002-2014 (Bzour *et al.*, 2018). To
196 control weedy rice, herbicide tolerance was introduced into cultivated rice 20 years ago based
197 on an *acetohydroxy acid synthase AHAS/ALS (Os02g0510200)* gene mutation, providing
198 tolerance to the mode of action 2 (F. Li *et al.*, 2019). Currently, rice herbicide tolerant
199 varieties are used in the USA (700,000 Ha), Brazil (600,000 Ha), Uruguay (70,000 Ha),
200 Argentina (32,000 Ha), Malaysia (95,000 Ha), and Italy (60,000 Ha), as well as in many
201 Central America countries, such as Costa Rica, Honduras, Panamá, and the Dominican
202 Republic (Singh *et al.*, 2017). The incorrect use of this variety allowed introgression and
203 outcrossing of the resistance into weedy rice, which means that weed herbicide control
204 requires stricter farming practices, such as herbicide rotation (Dauer *et al.*, 2018).
205 Alternatives such as aryloxyphenoxy propionate-resistant rice (mode of action 1), which is the
206 result of mutations in the *ACCcase2 (Os5g0295300)* gene, already exist and will allow for
207 herbicide rotation (Camacho *et al.*, 2019; de Andrade *et al.*, 2018).

208 According to the literature, at least five target genes have the potential to develop herbicide-
209 resistant rice varieties with a different mode of action. Two of those have already been
210 described above: *ACCcase2* on aryloxyphenoxy propionates (*MoA-1*) and *AHAS/ALS* on ALS
211 (*MoA-2*). For *ACCcase2*, mutations such as I1781L, S1866F, I1879V, A1884P, W2027C,
212 W2125S, D2176G, and C2186R/P1927F/G2201A/W2125C in exon 32 provide herbicide
213 tolerance at a different level. *AHAS/ALS* alleles cause ALS (*MoA-2*) resistance when carrying
214 the following mutations: A96V/A122T/P171H/P171S/P197S/C287T and
215 W548L/W574L/S627I/S653I/S653N/G654E. *OsTubA2 (Os11g0247300)* provides tolerance
216 to dinitroanilines (*MoA-3*) with a mutation in the fourth exon, M268T. *HPPD*
217 (*Os02g0280700*), provides tolerance to triketones with a natural insertion
218 (GGAACCAAAAGAATTAGAGACGATATCA) in the fourth exon. Finally, the double

219 mutation known as “TIPS” (T102I+P106S) in the *OsEPSPS* (*Os02g0510200*) gene provides
220 tolerance to *MoA-9* (glyphosate). For details, see Figure 4 and Table 2.

221 Weeds that are tolerant to the inhibition of photosynthesis at PSII by herbicides can also
222 provide insights to generate herbicide tolerant crops. The S264G mutation in *psbA* increases
223 tolerance more than 50-fold to triazine in herbicide-tolerant radish (*MoA-5*). However, it can
224 also compromise fitness because of less efficient photosynthesis (Lu *et al.*, 2019). Other
225 mutations, such as Val219Ile, Asn266Thr, Phe255Ile, and Ala251Val, can also provide
226 tolerance (Gaines *et al.*, 2020). It is important to note that the *psbA* mutation Val-219-Ile
227 provides tolerance to the amide propanil *MoA-5* on *Cyperus difformis* (Pedroso *et al.*, 2016).
228 Propanil is widely used in rice cultivation because the crop is naturally capable of degrading
229 the molecule by a putative enzyme located in the mitochondria, and an additional pathway
230 could increase its tolerance (J. J. Chen & Matsunaka, 1990; Matsunaka, 1967). The described
231 mutations could also result in herbicide tolerance in rice when targeting the homologous gene
232 AAS46167, encoding protein P0C434, to address an additional *MoA*.

233 Rice is also known to be resistant to Bentazon (*MoA-6*), as it is degraded by cytochrome
234 P450 *CYP81A6* (Pan *et al.*, 2006). Additionally, the P450 gene *CYP72A31* is responsible for
235 conferring tolerance to bispyribac sodium (BS) in *Oryza sativa* cv. *indica*, while its absence
236 in japonica rice varieties results in BS-sensitivity (Saika *et al.*, 2014; J. Zhang *et al.*, 2002).

237 **4. Bacteria, fungi and virus resistance**

238 Rice breeding of pathogen resistance is possible by mutation of specific promoter regions of
239 the *Sweet 14,11,13* genes (*Os11g0508600*, *Os08g0535200*, *Os12g0476200*), respectively,
240 since they are required for infection by bacterial *Xanthomonas oryzae* pv. *oryzae* pathogens
241 causing bacterial leaf blight (BLB) (Jiang *et al.*, 2013; Oliva *et al.*, 2019; Varshney *et al.*,
242 2019). The pathogen emerges by breaking the resistance of varieties planted in approximately
243 80% of the total crop cultivation area carrying the resistance gene *Xa4* on chromosome 11
244 introduced in the 60s (Quibod *et al.*, 2020). Some *Xanthomonas oryzae* pathovars can also
245 infect wild grasses and could become an emergent pathogen that is difficult to control (Lang
246 *et al.*, 2019).

247 A gene to target for fungal resistance is the transcription factor *IPA1* (*Os08g0509600*); higher
248 expression levels of IPA1 result in increased yield and immunity when tested against the
249 fungal pathogen *Magnaporthe oryzae*. Resistance relies on time- and pathogen-specific
250 phosphorylation and activation of the transcription factor at Ser163. Subsequently,
251 phosphorylated IPA1 activates the WRKY45 promoter and following basal resistant gene
252 expression within 48 hours after infection, while the nonphosphorylated IPA1 protein binds
253 to the DEP1 promoter related to yield (Jing Wang *et al.*, 2018). A different way to achieve
254 *M. oryzae* resistance is by mutation of *OsERF922 ethylene response factor 922*
255 (*Os01g0752500*) (F. Wang *et al.*, 2016). Another important trait is tungro spherical virus
256 resistance which results by mutation of gene eIF4G (*Os07g0555200*) coding a translational
257 factor that is key in the initiation of the virus mRNA (Macovei *et al.*, 2018)

258

259

260 For details, see Table 3.

261 **5. Grain number, quality, weight and plant structure**

262 Rice quality traits are essential to achieve a better yield, consumer preference, and growth
263 efficiency. The genes involved in grain number and size, plant density, structure, panicles,
264 and flowering have complex interactions. However, recent findings and key mutations now
265 provide insight into their regulatory mechanisms and greater predictability in achieving the
266 desired phenotype (for details, see Figure 5 and Table 4).

267 Grain size. The *GS3 Grain Size3* gene (*Os03g0407400*) is responsible for negatively
268 controlling the grain length. Its mutation can result in better or worse weight and size that
269 correlates with the composition of its domains: organ size regulation (OSR), a
270 transmembrane necrosis factor receptor/nerve growth factor receptor (TNFR/NGFR), and a
271 von Willebrand factor type C (VWFC) (Meiru Li *et al.*, 2016; Shen *et al.*, 2017; Takano-Kai
272 *et al.*, 2013; Yang *et al.*, 2019; Zeng *et al.*, 2020). The wild type allele contains all of the
273 domains and results in medium grains (Takano-Kai *et al.*, 2013). Loss of function results in
274 long-grain varieties; for example, Minghui 63 has a stop mutation C165A at the second exon,
275 resulting in a loss of function and a long-grain phenotype (Meiru Li *et al.*, 2016; Zeng *et al.*,

276 2020). In contrast, a mutation or deletion in the fifth exon creates a truncated protein with no
277 VWFC domain and a short seed phenotype (H. Mao *et al.*, 2010; Takano-Kai *et al.*, 2013).
278 Grain size, in general, is controlled by several additional genes: higher expression of *GW6a*
279 (*Os06g0650300*), and knockout of *GW5* (*Os05g0187500*), *GW6* (*Os06g0623700*), and
280 *GW5L* (*Os01g0190500*) results in increased grain size (Ayaad *et al.*, 2021; Ishimaru *et al.*,
281 2013; Song *et al.*, 2015; Tian *et al.*, 2019; L. Zhang *et al.*, 2020).

282 Grain number. Malfunction of the gene *Os01g0197700* (*GN1a*) produces an increment of
283 grain per panicle number and flowering because of a lower degradation of cytokines
284 produced by the corresponding cytokinin oxidation enzyme (Liyu Huang *et al.*, 2018; Meiru
285 Li *et al.*, 2016; Shen *et al.*, 2017). Another gene that correlates with increased production and
286 downregulates cytokine level regulation is *EP3 Erect Panicle 3* (*Os02g0260200*) (Ming Li
287 *et al.*, 2011; Shen *et al.*, 2017).

288 Grain starch. Grain starch quality is an essential trait, which depends on the relative content
289 of amylose and protein. The global starch content relies on the gene *ISA1* (*Os08g0520900*)
290 and the protein content relies on *NAC20-26* (*Os01g0104500*, *Os01g0393100*) (Shufen *et al.*,
291 2019; Juan Wang *et al.*, 2020). The waxy gene *WX1* (*Os06g0133000*) controls the grain
292 amylose content (AC). Mutations in this gene correlate with a phenotype that ranges from
293 opaque (8%), semitranslucent (8-12%), and transparent (12% or more) grains (Lichun Huang
294 *et al.*, 2020; Sano, 1984; Xu *et al.*, 2021; Yunyan *et al.*, 2019; C. Zhang *et al.*, 2019).

295 Flowering. Flowering and photoperiodic insensitivity results from overexpression of
296 *OsMeCP* (*Os12g0620400*) (Qu *et al.*, 2021) or by knocking out several genes. For example,
297 *Se5*, *Hd2* and *Hd1* (Andrés *et al.*, 2009; Gao *et al.*, 2014; Shen *et al.*, 2017; Tanaka *et al.*,
298 2020). Another critical regulator of heading date and grain weight seems to be *HGW*, but its
299 homozygous null mutant is embryonic lethal (J. Li *et al.*, 2012).

300 Structure. Farmers prefer smaller plants with many panicles and fewer tillering traits.
301 Knockout of the *DEP1* (*Os09g0441900*) gene, as well as the loss of function of the *HTD1*
302 (*Os04g0550600*) gene introgressed from landraces produces short, dense, erect panicles
303 (Lacchini *et al.*, 2020; Meiru Li *et al.*, 2016; Zou *et al.*, 2006).

304 The transcription factor *IPA1 Ideal Plant Architecture1* (*Os08g0509600*), is related to fungal
305 resistance and yield as mention previously, and its specific mutations between bases 854 and
306 876 can increase the production of the transcription factor protein because they interrupt
307 transcript cleavage due to the micro RNA OsmiR156. For example, C874A in the third exon
308 (leucine to isoleucine) generates a rice plant with a reduced tiller number, increased lodging
309 resistance, and an enhanced grain yield (Meiru Li *et al.*, 2016; Jing Wang *et al.*, 2018).

310 The number of panicles and consequently the yield can be increased by mutating the genes
311 *Pin1A* and *Pin15b* or indirectly blocking their expression. The indirect mechanism results in
312 higher expression of *DEP1* and *LPA1*, which interact to suppress *PIN1a* expression (Fu *et*
313 *al.*, 2019; Liyu Huang *et al.*, 2018; Miao Liu *et al.*, 2021). *LPA1* is also important in the erect
314 phenotype, and its mutation results in lamina inclination (J. M. Liu *et al.*, 2016; X. Mao *et*
315 *al.*, 2018).

316 **6. Other traits**

317 Other rice traits provide value for breeding and for satisfying consumer preferences, such as
318 nitrogen use, fragrance, oleic acid content, and color. Regarding nitrogen provision, there is
319 a better efficiency with a higher expression of the nitrate transporter *OsNPF6.1* and the two
320 transcription factors *OsNAC42* and *OsNLP4* (W. Tang *et al.*, 2019; J. Yu *et al.*, 2021).
321 Mutation of the *FAD2* gene results in an oleic acid increment (Abe *et al.*, 2018; Tiwari *et al.*,
322 2016). Furthermore, a mutation in the *Osor* (*Os02g0651300*) gene results in potential orange-
323 colored rice (Endo *et al.*, 2019), and the fragrance can be increased or decreased by
324 modulating the *BADH2* gene, which prevents the formation of the aromatic compound 2AP
325 (2-acetyl-1-pyrroline) (Shen *et al.*, 2017). For details, check Table 5.

326 **Regulatory approaches**

327 The traits presented in this article can result from the application of conventional or
328 new breeding techniques, such as genome editing. It is important to note that the advance
329 in sequencing technologies allow for a detection of mutations; however, it is unfeasible to
330 identify the specific technique or natural cause that resulted in a mutation like a single
331 nucleotide polymorphism or a few nucleotide variations (H. *et al.*, 2018; ENGL, 2019;
332 Broothaerts *et al.*, 2021). Trying to create a legal system that differentiates between genome

333 editing and other mutagenesis approaches or natural variations represents a challenge, given
334 that detection is not achievable under realistic circumstances (Emons *et al.*, 2018). It is a
335 challenge to regulate a product that cannot be practically distinguished once in the market,
336 but that falls under a norm that requests such a differentiation. Such a legal norm is currently
337 applied in Europe. A supreme court resolution on case C-528/16 enforced that the genetically
338 modified organism (GMO) norm (Directive 2001/18/EC) is applied on genome-edited
339 plants (Purnhagen *et al.*, 2018). A recent study of the European Commission delivered to the
340 Council of the European Union in April 2021 has collected opinions from different
341 stakeholders and concluded that "*similar products with similar risk profiles can be obtained*
342 *with conventional breeding techniques, certain genome editing techniques and cisgenesis. It*
343 *may not be justified to apply different levels of regulatory oversight to similar products with*
344 *similar levels of risk*" (European Commission (EC), 2021). An adjustment of the GMO norms
345 should be endorsed to correspond with such a conclusion.

346 The legal status of a genome editing product depends on norms established at a country level
347 based on a discriminate process to determine whether the final product is a Living Modified
348 Organism (LMO) or not. For countries like Argentina, Australia, Colombia, Brazil, and the
349 United States, a variety is equivalent to conventional in the absence of a foreign
350 DNA (Entine *et al.*, 2021; Gatica-Arias, 2020). For details, see Table 6.

351 The legal frameworks dealing with genome editing plants currently are country-specific.
352 Still, there is some common background in the international definitions of a Living Modified
353 Organism (LMO) given in the Cartagena Protocol on Biosafety as "*any living organism that*
354 *possesses a novel combination of genetic material obtained through the use of modern*
355 *biotechnology*". The keyword in defining such differentiation is "*novel combination of*
356 *genetic material*," which is usually explained in legal terms as the presence of foreign DNA,
357 as described previously.

358 There is an international Central America norm RT 65.06.01:18 approved by Resolution 60-
359 2019 that provides a legally binding definition on Article 4.6 for "*novel combination of*
360 *genetic material*," currently applied in Honduras and Guatemala. The definition states in
361 simple words, that a new combination of genetic material means a stable insertion of DNA
362 that could not be obtained by conventional breeding or available in nature. The procedures

363 and information requested in both countries are aligned with the international definition and
364 are available in decree CD-008-SENASA-2019 for Honduras and 271-MAGA chapter VI for
365 Guatemala. This legal antecedent provides a background for comparative
366 laws within countries with norms still in discussion. The latter is interesting because the
367 Supreme Court of Guatemala endorsed the international standard in Case Resolution 6767-
368 2019. For details, see Table 6.

369

370 **Conclusion**

371 Induced mutations targeting specific genes associated with known phenotypes, as described
372 in this review, will allow for advances in more precise rice breeding to improve varieties that
373 farmers are currently using. It can also result in new varieties and *de novo* domestication
374 from wild relatives and the results can be extrapolated to other crops with homologous traits.
375 Farmers urgently require advanced breeding to respond to the challenges of climate change,
376 consumer demands, water scarcity, nitrogen usage, and sustainable production.

377

378 **List of abbreviations**

379 Ethyl methanesulfonate (EMS)

380 Puddled Transplanted Rice (PTR)

381 Dry Seeded Rice (DSR)

382 Days After Sowing (DAS)

383 The Herbicide Resistance Action Committee (HRAC)

384 Weed Science Society of America (WSSA)

385 Mode of Action (MoA)

386 Bacterial Blight Streak (BLS)

387 Amylose Content (AC)

388 Acetolactate Synthase (ALS)

389 NUE (nitrogen use efficiency).

390 **Declarations**

391 All manuscripts must contain the following sections under the heading 'Declarations':

392 **Ethics approval and consent to participate** 'Not applicable'

393 **Consent for publication** 'Not applicable'

394 **Availability of data and material** 'Not applicable'

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402 edited the paper; A. A-E discussed the results and edited the paper; A.G.-A. wrote, reviewed,
403 discussed the results and edited the paper; M.V.-M. discussed the results and edited the paper;
404 J.B. reviewed, discussed the content and edited the paper . All authors read and approved the
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Figures

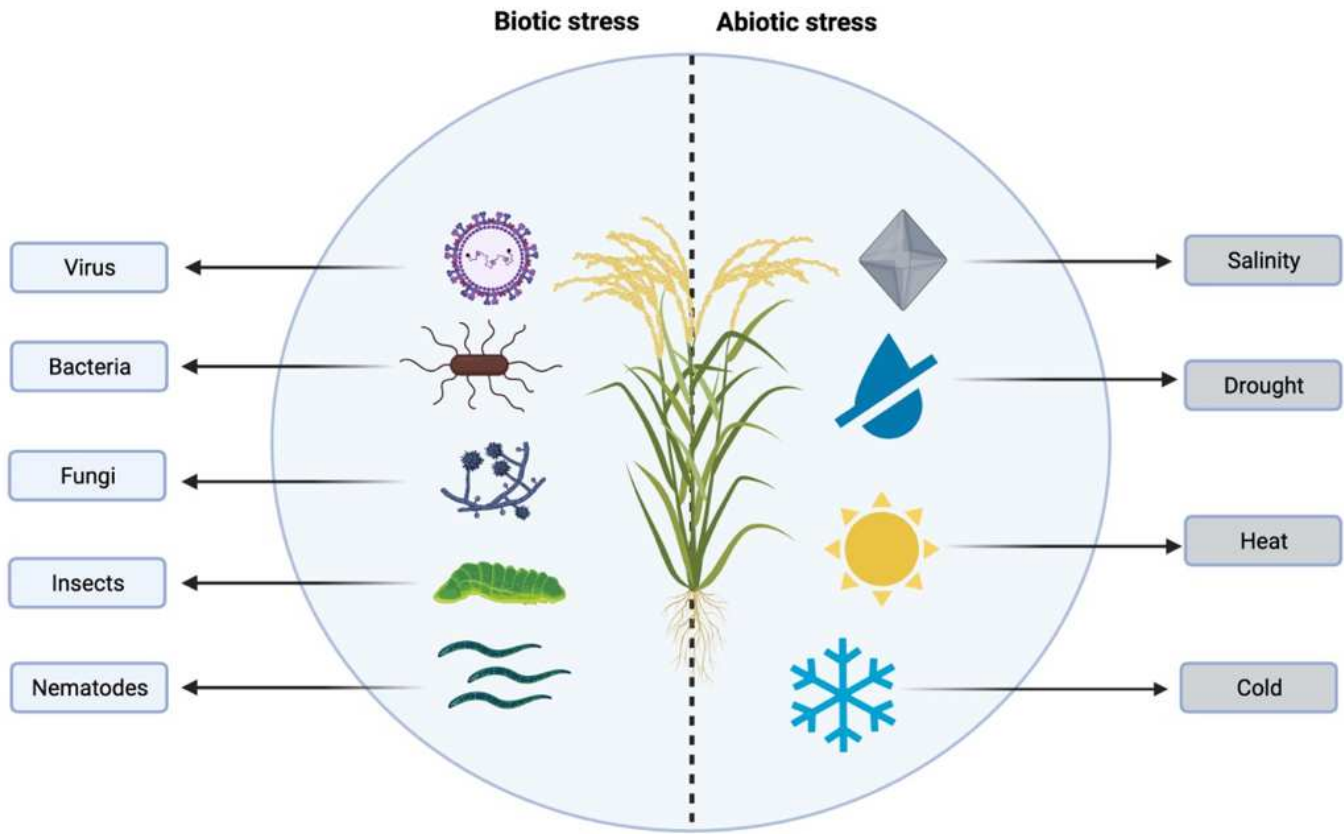


Figure 1

Representation of biotic and abiotic stress factors that affect rice production. Created with BioRender.com

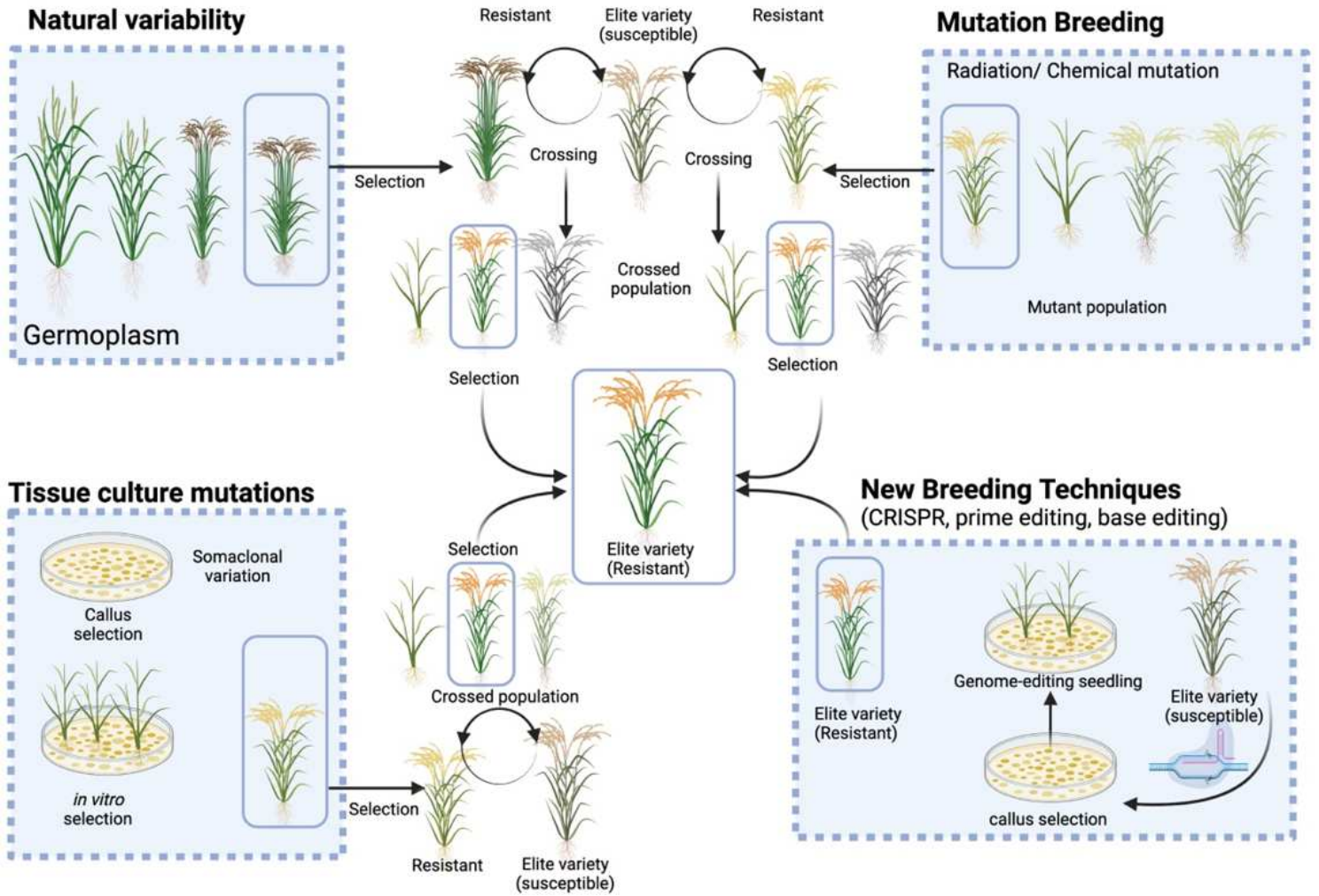


Figure 2

Schematic representation of different systems used for breeding rice: natural variability, mutation breeding, tissue culture mutation, and new breeding techniques. Created with BioRender.com

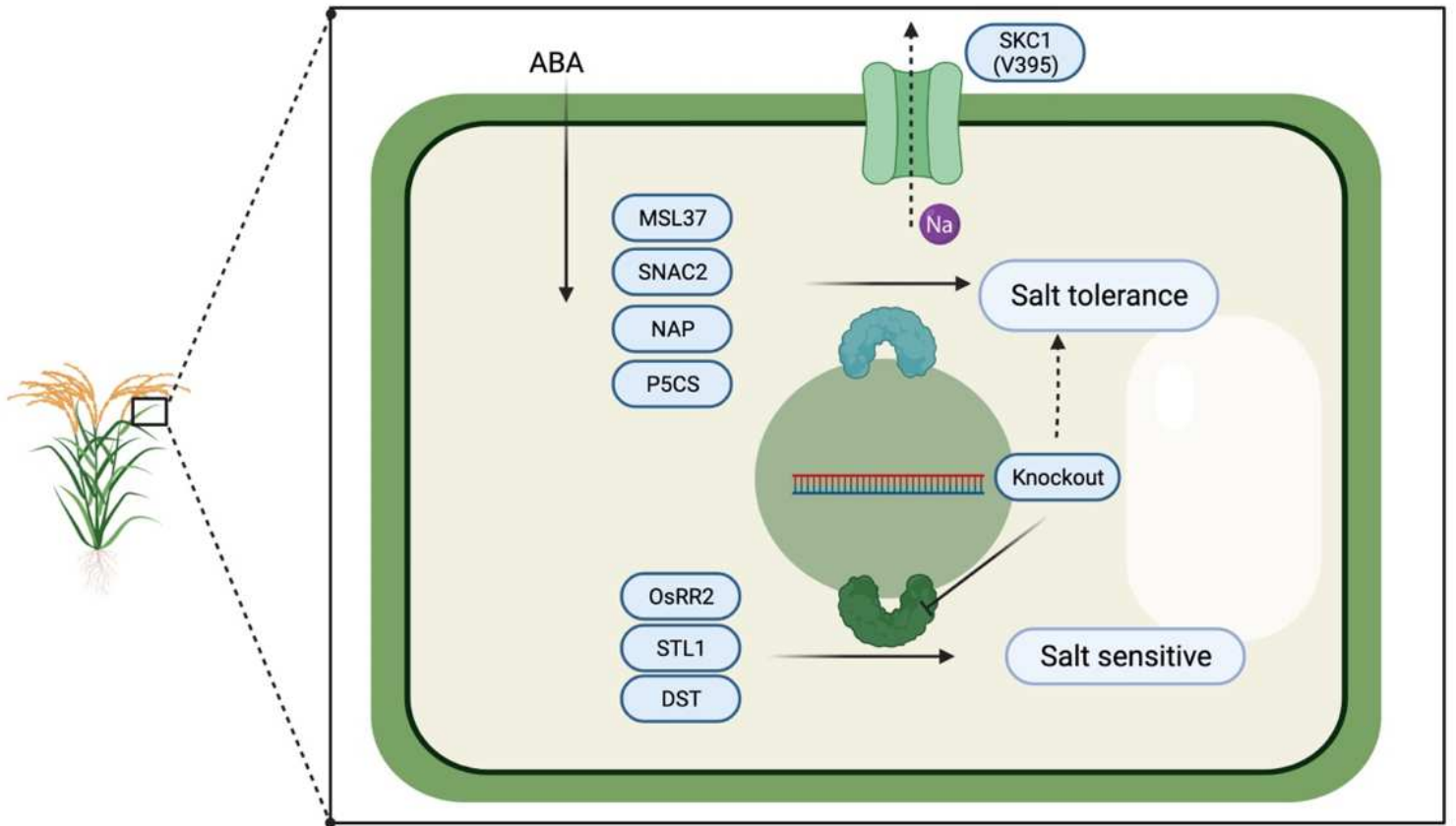


Figure 3

Representation of salt tolerance traits mediated by three different methods: 1) overexpression, 2) knockout of specific genes, and 3) particular sodium channels. Note that the first corresponds to transcription factors that trigger adaptive responses labeled MSL37, NAC2, NAP, and P5CS. The second is a knockout of those that result in salt sensitivity: OsRR2, STL1, DST; and the sodium channel SKC1 in rice. The third is the sodium channel SKC1 containing amino acid V395. Created with BioRender.com.

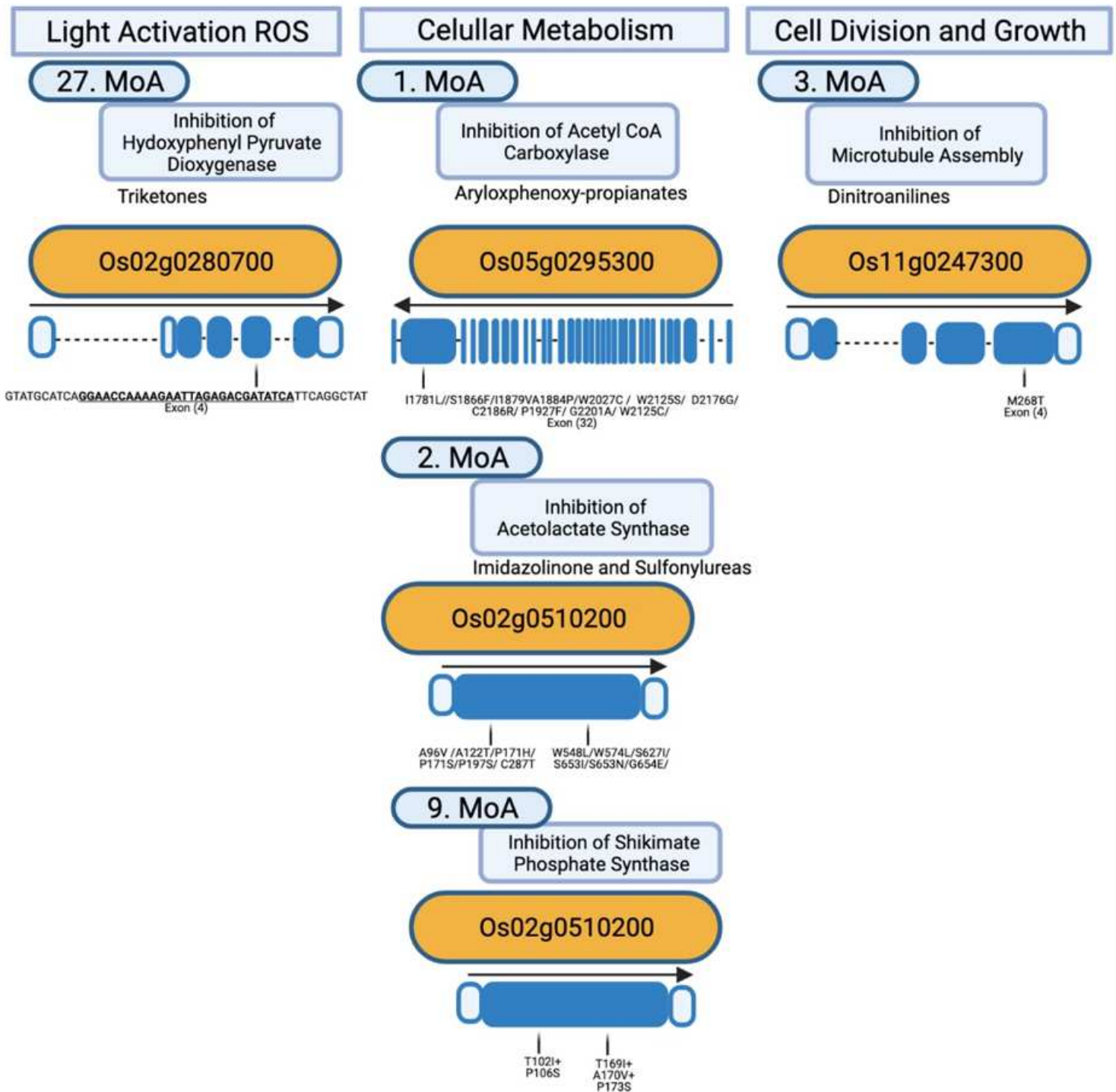


Figure 4

Representation of five rice genes and the corresponding mutation that results in herbicide tolerance. The genes are shown organized by their Mode of Action (MoA). Note the name of the gene in orange circles, the exons in blue filled boxes and the corresponding untranscribed exon regions in the blue empty boxes. Created with BioRender.com.

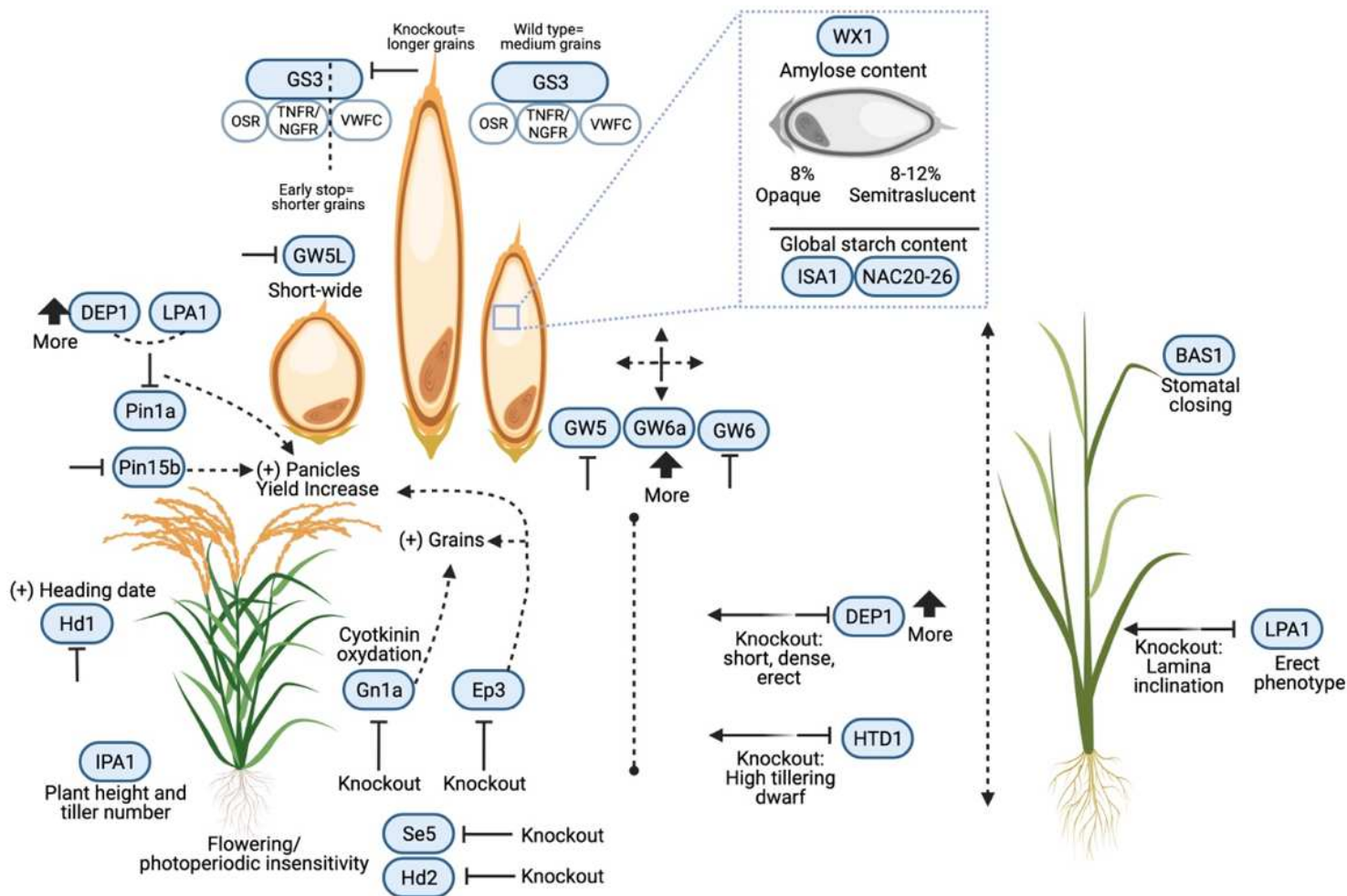


Figure 5

Representation of traits such as grain number, quality, weight and plant structure and gene relationships in rice. Note that heading and flowering are positively influenced by Se5, Hd2, and Hd1 knockout; structure by DEP1, HTD1, IPA1, LPA1, Pin1a, and Pin15b; grain size by Gn1a, and Ep3; grain size by GS3, GW6a, GW5, and GW5L; and grain starch by ISA1, NAC20-26, and WX1. Created with BioRender.com.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [3TablesJB1.pdf](#)