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CRISPR Cas for bringing desired traits in crops. 1 2 Abstract 3 In the edge of present day agribusiness, confronting the projected surge in global population 4 concomitantly with escalating environmental perturbations, the sustainable production of 5 high quality food has emerged as a substantial challenge. In order to overcome this major 6 undertaking, adaptation of innovative, resilient and science driven agricultural strategies has 7 been an essential step in plant biotechnology. Advanced biotechnological approaches like 8 genome editing with site directed mutagenesis permit to step forward towards new cultivar 9 1 with desirable traits in economically important crops Latterly, Zinc-finger nucleases (ZFNs), 10 transcription activator-like effector nucleases (TALENs), and clustered regularly interspersed 11 short palindromic repeats (CRISPR)/CRISPR associated protein 9 system has revolutionised 12 the plant breeding system with exceptional achievements. On that account, the present review 13 re-evaluates and discusses the information on genome editing technologies specially CRISPR 14 /Cas 9 approach in detailtogether with complete mechanism of CRISPR /Cas, problem and 15 future aspects of the approach aiming to develop desirable agronomic genetic traits. 16 17 Introduction 18 19 The achievement of plant breeding mainly depends on development of a wide hereditary 20 variation. Plant breeders from decades are using several techniques cross breeding, mutation 21 breeding and transgenic breeding for enhancing quantitative and qualitative traits of the crop. 22 . Cross breeding involves improving trait by crossing genetic material from one variety to 23 another and further choosing outstanding progeny with the preferred trait, followed by 24 backcrossing with the recipient line for subsequent generations to eliminate the linked traits 25 (Mangrauthia et al., 2024). But it takes several years to incorporate desirable alleles and to 26 enhance variability in the plant by the use of cross breeding and moreover, the genetic 27 variability of the crops has been greatly reduced by the large fixed parts of the genome 28 (Swarup et al., 2021). Mutation breeding involves the enhancement of traits by creating 29 random mutations in the plant material by the use of chemical mutagens or physical 30 irradiations (Yali et al., 2022), However,the screening of such a large number of mutants is 31 time taking, laborious and challengingtogether with the burdens for

increased crop production 32

(Sarsu et al., 2023). Transgenic breeding involves transferring of exogenous genes into crop 33 to obtain the enhanced trait and can be useful, but the long and cost regulatory estimation 34 processes and the public concerns in the commercialization of the produced genetically 35 modified crops acts as a barrier for the use of the technology (Marone et al., 2023). Thus, the 36 novelties in the technology with development of new, fast and efficient techniques to create 37 advanced crops with better characteristics are a need of the hour, and are essential to enhance 38 the agricultural yield and accelerate sustainable agricultural development (Patel et al., 2023). 39 Zinc finger nucleases 40 The research in the genetics has been revolutionized by the use of genome editing approaches 41 using high throughput sequencing and computational analysis. Several gene 42 editingtechniques leading to site directed mutagenesis has been developed during last few 43 years. Zinc finger nucleases (ZFNs) were the first engineered endonucleases which are site 44 directed and are created by fusion of the DNA-binding domain of the artificial array of the 45 zinc fingers and the non-specific cleavage domain of FokI, which cleave the target DNA by 46 protein-DNA binding (Limbalkaret al., 2025). ZFNs firstly came from the study on FokI 47 restriction enzymes, when it was figured out that the recognition and 1 the cleavage domain of 48 FokI are physically separable and, if the non-specific nuclease domain from FokI are put 49 away from its natural recognition domain, other recognition domains can be incorporated. 50 Using this strategy set of zing fingers were chosen, as zinc fingers naturally occur in 51 eukaryotic sequence specific DNA binding transcription factors (TF) (Zess and Begemann 52 2021). Each module of these TF is the finger that recognizes principle three base pair of 53 DNA, and successive finger recognize successive triplet in DNA target. So FokI cleavage 54 domain cuts double stranded DNA by assembling different combination of zinc finger on 55 them (Abdelrahmanet al., 2021) (Figure 1).. Despite of its uses, potential of ZFNs technology 56 has not been fulfilled as its nucleases requires protein engineering for recognizing target 57 DNA sequence (Liu et al.,

2024). Therefore, due to construction complexity, high but 58 variable off-target rate, high cost and skill, difficulty in customization, its application in the 59 field of genome editing gets restricted (Castro et al., 2021). 60

61 Figure 1: Comparative mechanism of Zinc finger nucleases and TALENS. ZFN-Zinc 62 finger nuclease 63 64 Transcription activator-like effector nucleases (TALENs) 65 TALENs system arose from studies of bacterial plant pathogen of the genus Xanthomonas, 66 these bacteria produce proteins that they secrete into plant host cell. In the host cells these 67 protein binds upstream of the host gene and regulates its transcription in a way that promotes 68 the bacterial infection (Teperet al., 2023). TALENs are chimeric enzymes formed by binding 69 of transcription activator like (TALE) protein fused with catalytic domain of the FokI 70 endonuclease to cleave the target DNA. The specificity in recognizing the target DNA is due 71 to the central repeat region of 33 to 35 amino acid tandem repeats, which differ at two amino 72 acids which determines at which nucleotide it will bind. By combining 12 to 31 of these 73 repeats, TALENs can be customized to bind to specific DNA template.Two sets of TALEN 74 system must dimerize to cleave the target DNA resulting into double stranded break. The 75 cells repair the DNA by NHEJ mechanism leading to frame shift mutation (Bhardwaj and 76 Nain 2021) (Figure 1). Although the efficiency of the TALENs is high as compared to the 77 ZFNs as it involve no re-engineering of proteins (Mishra et al., 2023), but production of 78

novel TALE arrays can be long and relatively costly, due to large size of TALEN, along with 79 a pair of proteins to identify the antiparallel DNA strand and create DSB, restricts its use as 80 the gene editing technology (Bhagtaney and Sundarrajan 2023). To overcome the 81 shortcomings of these techniques, a new technique known as CRISPR came into existence. 82 83 Figure2: CRISPR- Cas system- CRISPR locus organisation and complex of different 84 Cas proteins like 9, 12, 13 and 14 forming the guide RNA . NUC- Nuclease lobe, HNH- 85 Ribonuclease domain and RuVC-Ribonuclease, REC1 and

REC 2-Recognition lobe86 HEPN- Higher eukaryotes and prokaryotes nucleotide binding domain. 87 88 4 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) 89 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR90 associated (Cas) proteins are extensively found in bacterial and archaeabacterial genomes as 91 adaptive immune systems (Ganger et al., 2023).The first CRISPR/ Cas system known to 92 cleave the target DNA was the type II system which belonged to Streptococcus pyogenes 93 (Varble and Marraffini 2022). 94

The CRISPR system mainly consists of two components Cas 9 endonuclease and guide RNA. 95 The guide RNA guides the Cas 9 endonuclease to create double stranded breaks in the 96 targeted DNA (Aksoyet al., 2022). This is the crucial step as after the cleavage of DNA the 97 DNA repair mechanism comes in activity and start repairing the DNA either by NHEJ 98 orHDR resulting into either deletion or insertion mutations (Nambiaret al., 2022)(Figure 2). 99 In recent years, CRISPR/Cas9 genome editing has emerged as a promising alternative due to 100 its precise editing capabilities (Cheng et al., 2025). This innovative tool enables targeted 101 modification of DNA sequences. The ground-breaking technology is poised to revolutionize 102 biotechnological research by facilitating the manipulation of target genes associated with 103 plant metabolism, immunity, and stress resistance, thus facilitating the development of 104 improved crops (Kumar et al., 2023).This technology has been observed to be better than the 105 traditional breeding technologies(Kumar et al., 2020).the system precisely edits the genome 106 of the plants with great efficiency, thus making it a suitable technology for use (Chib et al., 107 2020).However the technology still possesses certain limitations like off targeting as the 108 CRISPR/Cas system is still under modifications to bring maximum application advantages to 109 the researchers (Ali et al., 2023). 110 Mechanism of action in CRISPR 111 CRISPR is originally found in bacteria and archae as an adaptive immune system to protect 112 from the invading virus(Garcia-Robledoet al., 2020).CRISPR systems have been classified 113 into two; Class 1 and Class 2, further categorized into three types

each. Class 1 (Types I, III, 114 and IV) utilize multiple Cas proteins for RNA guided cleavage, while Class 2 (Types II, V, 115 and VI) rely on only one endonuclease protein for the DNA cleavage (Hillary and Ceasar 116 2023). 1 The most commonly used system is type IICRISPR/Cas 9 system adapted from 117 Streptococcus pyognesisand repurposed for genome editing by modifying the DNA 118 sequences by either deleting or introducing new gene sequences (Liang and Corn 2022).The 119 CRISPR array is mainly composed of two components; first component includes two type of 120 RNAs i.e CRISPR RNA or crRNA, Tracer RNA or tracrRNA and the other component is 121 RNA dependent endonuclease i.e Cas (CRISPR associated protein) (Mohamadi et al., 2020). 122 The CRISPR immunity works in three phases, phase 1 is adaptation, phase 2 involves 123 expression and processing and phase 3 proceeds with recognition and cleavage of the target 124 (Mosterd et al., 2021).In phase 1 when the bacteria is infected by the phage the Cas, such as 125 Cas1 and Cas2 recognize the Protospacer (a 20 -50 bp short sequence similar to the spacer 126 sequence in CRISPR array) from the invading phage or virus and store it as a new spacer on 127

CRISPR locus as a result an memory for the invader is formed for future recognition 128 (McGinn and Marraffini 2021) (Figure 3).. 129 Phase 2 transcription of CRISPR array occurs resulting into the formation of the long 130 precursor RNA (pre-crRNA). The pre-crRNA is processed by RNase III and Cas proteins into 131 crRNA, the crRNA further combines with the trans-activating CRISPR RNA (tracrRNA) to 132 form a duplex form complex. Finally the Cas 9 protein binds to this duplex resulting into 133 formation of the single guide RNA (sgRNA) (Munawar and Ahmad 2021). 134 Phase 3 involves the target recognition and cleavage with the help of Cas9. It contains a 135 sequence known as PAM (Protospacer Adjacent Motif) which is adjacent to the target 136 sequences in the foreign DNA in most of the CRISPR -Cas systems (Ding et al., 2021). It 137 also contains a guide RNA, which is almost 20nt and leads Cas 9 to the target DNA adjacent 138 to the PAM and has the sequence complementary to the part of the sequence of the invading 139 DNA

which is to be edited (Gupta et al., 2021). In many cases, a chimeric sgRNA, formed by 140 the association of the crRNA and the tracr RNA into a single transcript is used, which is 141 accurately functional in causing Cas mediated sequence-specific DNA cleavage. This 142 simplified two-component system CRISPR Cas 9, 2 can be used to target any DNA of interest 143 merely by changing the 20nt guide RNA sequence (Liao and Beisel 2021). 144 The Cas9 enzyme consists of two well defined lobes: the alpha-helical recognition lobe 145 (REC) and the nuclease lobe (NUC). The NUC has the HNH nuclease domain (motif with 146 three conserved amino acids; Histidine (H) Asparagine (N) and Histidine (H), the RuvC 147 nuclease domain and the CTD (C- terminal domain) (Palanivelu, 2021). The Cas 9 enzyme 148 snips the target DNA 3bp upstream of the PAM with these two nuclease domains. The HNH 149 like-nuclease domain cuts the target strand (the strand complementary to the guide RNA) of 150 the DNA and the RuvC like-nuclease domain which cuts the non-target strand (strand 151 opposite to the complementary strand of the guide RNA) (Chuang et al., 2021). The RuvC 152 domain of the enzyme has three motifs: Motif I, Motif II, and Motif III. The HNH domain of 153 the nuclease enzyme interrupts motif I and III, and the larger lobe made of alpha-helices 154 (REC) interrupts motif I and II (Nasrallah et al., 2022). The two domains of the enzyme 155 (REC and NUC) 1 are connected to each other by two linkers, one formed by the arginine-rich 156 bridge and the other formed by the disordered linker. The CTD domain of the enzyme 157 contains a PAM- interacting site, which is essential for PAM recognition (Jiang and Doudna 158 2017).Studies show that mutating any of the two domains of the Cas 9 converts it to nickase 159 while mutating any of the two domains stops its endonuclease activity but its RNA - guided 160 DNA binding ability remains as it is (Deb et al., 2022). The Cas 9 enzyme is present in an 161

inactive state and undergoes extensive rearrangements on binding with the sgRNA, thus 162 making the enzyme compatible for the recognition of the target DNA(Montecillo et al., 163 2020). 164 165 Figure 3: Mechanism of CRISPR-Cas9 activity highlighting three

phases- Adaptation – 166 Integration of protospacer in CRISPR array, Expression- Generation of Guide RNA (complex 167 of Cas9-with crRNA and tracRNA, Interference phase- Degradation of target DNA 168 169 Binding of the Cas 9 with the sgRNA indicates preparedness of the enzyme to search for the 170 complementary sites in the target DNA (David et al., 2022). The two prerequisite conditions 171 for the recognition of the target sequence are: The complementary base pairing between the 172 20nt spacer and the complementary protospacer is the target DNA and the presence of PAM 173 sequence adjacent to the target site (Karvelis et al., 2017). The most commonly studied and 174 used CRISPR Cas 9 system for genome editing belongs to Streptococcus pyogeneswhich has 175 a 5'-NGG-3' PAM sequence in the target DNA. The PAM sequence is of immense 176 importance in the type II CRISPR Cas 9 system as it helps in distinguishing between the self 177 and non-self-sequences. A single mutation in PAM can inhibit the cleavage of the target 178 DNA (Gupta et al., 2021). DNA melting starts after the Cas 9 has found a suitable PAM in 179 the target site,and is followed by the formation of RNA-DNA hybrid, and the formation of 180

the R-loop by the non-target DNA strand. The R-loop is formed when the guide RNA of the 181 crRNA array, combines with the ds target DNA to form a RNA-DNA heteroduplex(Pacesa et 182 al., 2022).The guide RNA pairs with the target DNA strand (complementary to the guide 183 RNA), thus replacing the opposite non-target DNA strand (non-complementary to the guide 184 RNA) (Gorski et al., 2017). The interaction of the R-loop with the target DNA and the 185 complementary guide RNA, leads to the double stranded breaks, 3bp upstream of PAM by 186 the HNH and the RuvC nuclease domains of the Cas 9 (Allemailem et al., 2024). 187 After the site specific double stranded break (DSB), the cell undergoes repair mechanism. 188 Non homologous end joining (NHEJ) and homologous directed repair (HDR) in DNA repair 189 mechanism are the two most commonly used methods to induce sequence specific changes 190 during genome engineering (Yang et al., 2020) (Figure 4). 191 192 Figure 4: Categorization of site specific

nuclease approach(SSN), depicting double 193 stranded break repair (DSB) by non-homologous end joining (NHEJ) or homologous 194 repair (HR) method in ZFNs / TALENs and CRISPR, SSN 1 result in small 195 deletion/insertion via NHEJ, SSN 2 for point mutation via HR and SSN 3 for complete 196 gene edit using HR. 197 198

All cells face double stranded breaks during cell cycle that are repaired preponderantly by the 199 NHEJ. The DNA ends are identified, selected, polymerised, and ligated in a versatile manner 200 by theproteins involved in the NHEJ mechanism. Ku70/Ku80 proteins bind to the blunt ends 201 of cleaved double stranded DNA and ligation of the DNA occurs by ligase IV (Meyenberg et 202 al., 2021). This adaptability licenses NHEJ to operate on 8 a wide range of DNA end 203 arrangements, ending with mutation in the repaired DNA (Chang et al., 2017). This repair 204 mechanism occurs in the absence of any homologous DNA template, which makes the 205 process little error prone also resulting into random mutations sometimes (Chatterjee and 206 Walker, 2017). 207 HDR is another mechanism for the repair of the targeted DNA.As compared to NHEJ, HDR 208 happens at lower and significantly more variable frequency. It uses externally added target 209 DNA template or a homologous sequence for the repair mechanism. The externally added 210 target DNA can either be single stranded or double stranded oligonucleotides. This 211 mechanism creates more precise and specific genetic alteration (Jin et al., 2025). 212 Other systems of CRISPR 213 Although type II CRISPR/Cas system is most commonly used for genome editing, but there 214 exist other systems also particularly in class 2 of CRISPR system such as Cas12(belonging 215 from type V) and Cas13 (belongs from type VI). The system varies in structural organization, 216 target specificity, cleavage mechanism, PAM requirement and repair pathway (Chaudhary et 217 al., 2024). 218 Cas12- The system belongs from type V CRISPR system exhibiting cleavage system 219 different to Cas9 system. Cas12 system (Figure 2).cleave both of the DNA strand by a single 220 RuvC- like catalytic domain unlike Cas9 system where two nuclease domains are 221 responsible for cleavage of the DNA strand (Lei et al., 2017). It recognizes T-rich PAM 222 located at 5´

end of target sequence, unlike G-rich PAM of Cas9. The system generates 223 staggered cuts in the DNA and follows HDR system for DNA repair (Teng et al., 2019). 224 Cas13 225 Cas13- Cas13 has been added to the CRISPR list very recently, it belongs toclass 2 type VI 226 of CRISPR system. The system works only on RNA and is catalysed by its HEPN (Higher 227 eukaryotes and prokaryotes nucleotide-binding domains. The Cas13 system involves the use 228 of only crRNA for cleavage of RNA (Ashraf et al., 2022). Since the system works on the 229 RNA only so no DNA strand cleavage occurs and hence no DSB mechanism takes place in 230 Cas13 system and the system does not rely on host repair mechanism or pathway. But since 231 the HEPN domain of the Cas13 system is present on the outer surface, there are high chances 232

of cleavage of RNA other than target RNA resulting in programmed cell deaths (Yang and 233 Patel, 2024) (Figure 2).. 234 Cas14- very recently Doudna's group explored a new protein named as Cas14, a 400-700 235 amino acids small protein coding for small protein with molecular weight of 40-70 kd. The 236 small size of this Cas14 protein make it efficient for targeting ssDNAusing only T-rich 237 sequences without a requirement of PAM sequence, but cannot target dsRNA or ssRNA 238 (Savage, 2019). For cleavage of ssDNA , similar to Cas9, Cas14 also require the tracrRNA 239 and crRNA complex. In term of efficiency Cas14 system 6 has proven to be more efficient and 240 specific than Cas9, Cas12 and Cas13. Due to its small size it 1 can be used to target any type of 241 tissue and improves SNP (single nucleotide polymorphism) (Hillary and Ceasar, 2023) 242 (Figure 2).. 243 244 Application of CRISPR/Cas 9 in plants 245 Concerning the surging need of food resources with increasing population, the food and 246 agricultural researchers are showing significant attention to explore CRISPR/Cas technology, 247 which was first used by Feng et al., 2013 for modifying genome of world's largest staple food 248 crop, rice. Several studies have proven 1 the efficiency of this technology in plant genome 249 editing compared to other technologies existing previously. The CRISPR/Cas9 genome 250 editing has been used to edit plant genome more precisely

and efficiently (Chib et al., 2020). 251 The main applications are as below:- 252 □ Crop trait improvement via knockout: Eradicating the undesirable gene is the most 253 promising approach for advancement in the field of genetic. The most widely used 254 application of CRISPR/Cas9 comprises knocking out the unwanted genes. There have 255 been several traits which are being enhanced and amended by the use of gene 256 knockout. Better yield, quality, biotic- and abiotic-stress resistance, hybrid-breeding 257 techniques and several other aspects of crop productivity have been enhanced using 258 this approach (Rao and Wang, 2021). For eg. Knocking out of OsERF922 gene was 259 done in rice to achieve bacterial blight disease resistance a major cause of loss of 260 almost 50% rice yield (Wang et al., 2016) 261 □ Knock-In and Replacement by CRISPR/ Cas:Several traits in the crop plants are 262 altered by single-nucleotide substitutions, gene expression changes, or the addition of 263 new genes with favorable traits. Introduction of new alleles without linkage drag or 264 generating allelic variants that do not exist naturally has been made simple by knock265

ins and replacements via CRISPR/Cas system. Moreover, multiple traits can be 266 modified via knock-inby introducing multiple genes in a single variety (Rozov et al., 267 2019). Herbicide tolerance in rice by knock-in of mutated acetolactate synthase (ALS) 268 gene to confer resistance to herbicide bispyribac-sodium is one of the example for 269 gene knock-in (Ouyang et al., 2025). 270 □ Use of Base editors for Genome editing: As many agriculturally essential traits can 271 be altered by single-nucleotide polymorphisms in either coding or noncoding regions, 272 base editing is fairly convenient for plant breeding and improved crop development. 273 For single-base substitution, base editing is developing as a substitute and is an 274 effective, influential tool to HDR-mediated precise gene editing in plants (Molla et 275 al., 2021). The most commonly used base editors include the cytidine base editor 276 (CBE) and adenine base editor (ABE) (Bharat et al., 2020). 277 □ Fine-Tuning Gene Regulation via CRISPR/Cas in Plants: Besides generating 278 mutations in coding sequences, modifying gene expression is a beneficial

method for 279 investigating role of a gene and can significantly enable plant breeding (Sun et al., 280 2024). Gene expression can be modified at several levels, including transcription, 281 mRNA processing, and mRNA translation. These processes are under the control of a 282 series of cis-regulatory elements, which can be modified by genome editing. Till date, 283 the alteration for modifying the gene expression mainly included promoters replacing 284 and deleting cis-regulatory elements (Cui et al., 2023). 285 □ Antiviral Plant Breeding Strategies: The viral infections are amongst the chief 286 reasons that lead to damage of valuable crops in natural ecosystems. These infections 287 significantly decrease harvest by displaying different symptoms in plants and 288 therefore bring economic burden (Nazarov et al., 2020). The CRISPR/Cas system 289 delivers a resistance mechanism that cleaves plasmids, DNA viruses , and RNA 290 viruses. Opportunely, genetic engineering by the use of CRISPR/ Cas 9 has been 291 supporting as a potent tool to increase plant resistance against a broad range of viral 292 infections (Tyagi et al., 2021). 293 □ Highly efficient plant mutant libraries via CRISPR/Cas: The most appreciated tool 294 for functional genomics is whole-genome-scale mutant libraries. Traditional mutant 295 libraries contain the data based on random mutations encouraged by agents such as 296 irradiation, T-DNA insertions, ethyl methyl sulfonate (EMS) mutagenesis, and 297 transposons (Santosh, 2020). The use of these methods to achieve stabilized mutations 298

requires many generations; moreover finding the genotypic and phenotypic 299 associations amongst the mutants is a time-consuming and laborious process.The 300 availability of high-quality, high-coverage, uniformly dispersed mutant libraries 301 generated via CRISPR/Cas could simplify the expansion of advanced germplasm 302 approaches as well as crop trait improvement (Tan et al., 2024). 303 304 305 Figure5 : Impact of CRISPR/Cas 9 on genetic engineering in plants 306 307 Table1. Targeted mutagenesis 2 using the CRISPR/Cas system in rice 308 Name Targeted Gene Effect of editing on plant Reference Rice (Oryza sativa) OsPHO1;2 Improved phosphate uptake

Mayura et al., 2025 OsALS Herbicide resistance Ouyang et al., 2025

OsACA9 Disease resistance and regulates leaf senescence Wang et al., 2024 OsCPR5.1 Resistance against yellow mottle virus Arra et al., 2024 OsPUB9 Resistance against bacterial leaf blight Kim et al., 2024 OsCOP1 Improved UV protection Hu et al., 2024 OsCAT2 Improved scavenging of ROS Shen et al., 2024 OsMYB84 Controlled uptake and transport of copper Ding et al., 2024 OsNIP3 Reduced arsenic accumulation Xu et al., 2024 SD1, Wx Increased semi-dwarf glutinous traits Wang et al., 2024 OsNAS2 Increased uptake and translocation of zinc Ludwig et al., 2024 OsRR22 Salinity tolerance Sheng et al., 2023 OsAUX5, OsWRKY78 Regulated amino acid accumulation Shi et al., 2023 Pi21, OsSULTR3;6 Resistance against rice blast Yang et al., 2023 OsCKX Stress tolerance and enhanced growth Zheng et al., 2023 OsWRKY71, Bph15 Resistance from brown plant hopper Li et al., 2023 OsHPP04 Resistance against rice rootknot nematode Huand et al., 2023 OsTPP3 Improved salt resistance Ye el al., 2023 OsLCD Low cadmium accumulation

 Chen et al., 2023 bHLH57 Increased yield even under cold condition Zhang et al., 2023 OsPUB7 Enhanced drought resistance Kim et al., 2023 OsGER4 Enhanced heat resistance Nguyen et al., 2023 NRAMP1, FRO2 Improved Fe uptake Krishna et al., 2023 OsPDR7, OsZIP9 Improved zinc accumulation Lu et al., 2023

OsGS2/GRF4 Improved size and yield Wang et al., 2022 PR10/Bet vl-like protein gene Resistance from Meloidogynegraminicola Li et al., 2022 CRTL, PSY Enhanced vitamin A content Dong et al., 2020 OsDEPI, OsROCs Enhanced heat resistance Malzahn et al., 2019 OsSWEET 11, OsSWEET 13, OsSWEET 14, Os8N3 Resistance against Bacterial Blight Olivaet al., 2019 ISA-1 Reduced starch content and increased sugar content Shufenet al., 2019 eIF4G Resistance against Rice Tungro Disease Macoveiet al., 2018 Waxy Low Amylose content Zanget al., 2018 SBEI and SBEIIb Increased amylose content and the resistant starch content Sun et al., 2017 OsAnn3 Increased relative electrical conductivity and reduced survival ratio after exposure to cold treatment Shenet

al., 2017 OsNramp5 Reduced Cadmium content Tang et al., 2017 Gn1a, DEP1, GS3, and IPA1 Alteration in grain number, panicle architecture, grain size and plant architecture Xuet al., 2016 OsERF922 Resistance against Rice blast Wang et al., 2016 309 Table 2.Targeted mutagenesis 2 using the CRISPR/Cas system in wheat 310 Name Targeted Gene Effect of editing on plant Reference Wheat S Disease resistance Waites et al., 2025

(Triticum aestivum) ω- and γ-gliadin Reduced immunotoxicity Yu et al., 2024 TaRR12 Drought resistance Li et al., 2024 TaRPK1 Enhanced yield Rahim et al., 2024 TaHKT1;5 Salt resistance Wang et al., 2024 TaHSFA1 Heat resistance Wang et al., 2023 EIF4E Resistance against yellow mosaic virus Kan et al., 2023 TaPGK Cold resistance Zhang et al., 2023 TaCIPK14 Resistance against stripe rust He et al., 2023 Tamyb10 Reduced pre-harvest sprouting Zhu et al., 2023 PSY Enhanced vitamin and mineral level Narayanan et al., 2023 Ppd-1 Yield enhancement Errum et al., 2023 Sal1 Stress resistance Mohr et al., 2022 TaIPK1 Enhanced uptake of iron and zinc Ibrahim et al., 2022 TaPDI Enhanced protein storage Hu et al., 2022 S Stress resistance Taj et al., 2022 pinb, waxy, ppo and psy Improved grain quality Zhang et al., 2021 TaASN Reduced asparagine accumulation Raffan et al., 2021 TaSBEIIa Regulated starch composition Li et al., 2021 TaNP1 Male sterility Li et al., 2020 TaQsd1 Enhanced seed dormancy Abe et al., 2019 TaMs1 Male sterility Okada et al., 2019 Gli-2 Low gluten wheat Sanchez Leonset al., 2018 TaERF3, TaDREB2 Drought Resistant Kim et al., 2018 Pinb, Waxy and DA1 Yield enhancement Zhang et al., 2018

TaEDR1 Resistance against Powdery Mildew Zhang et al., 2017 311 Table 3.Targeted mutagenesis 2 using the CRISPR/Cas system in other important staple crops 312 Name Targeted Gene Effect of editing on plant Reference Sorghum (Sorghum bicolor) SbLG1 Yield improvement Brant et al., 2021 sbFT Yield improvement Char et al., 2020 SbGA20Ox5 Yield improvement Char et al., 2020 CAD Yield and quality improvement Liu

et al., 2019 K1C Quality improvement Li et al., 2018 Maize (Zea mays) ZmHDT103 Drought resistance Wang et al., 2024 ZmHSPs Enhanced heat resistance Li et al., 2024 Zmpdrp1 Virus resistance Xie et al., 2024 Cry1F Pest resistance Kumari et al., 2024 RZ2MS9 Stress tolerance Figueredo et al., 2023 ZeSWEET1b Enhanced nutrient uptake and accumulation Wu et al., 2023 ZmAGO18b Southern leaf blight resistance Dai et al., 2023 MCMV Reduction in viral infections Lei et al., 2023 ZmG6PDH1 Enhanced cold resistance Li et al., 2023 ZmMYB69 Quality improvement Qiang et al., 2022 Zmbadh2a, Zmbadh2b Improved sugar and acid metabolism Wang et al., 2021 SbLG1 Yield improvement Brant et al., 2021 SbBADH2 Quality improvement Suebpongsang et al., 2020

CRTL, PSY Enhanced vitamin A content Dong et al., 2020 Wx1 Low amylose content Qi et al., 2020 MS8 Male sterility Chen et al., 2018 ARGOS8 Enhanced grain yield in drought condition Shi et al., 2017 ALS1 and ALS2 Resistance against chlorosulfuron herbicides Svitashevet al., 2016 Soybean (Glycine max) GmFAD2 Increased fatty acid Zhou et al., 2023 GmHsp90A2 Heat resistance Jianing et al., 2022 GmSNAP11, αSNAP Soybean cyst nematode resistance Shaibu et al., 2022; Usovsky et al., 2023 Glyma05g29080 White mold resistance Zhang et al., 2022 GmUGT Resistance against insects (leaf chewing) Zhang et al., 2022 GmF3H1, GmF3H2 and GmFNSII-1 Increased isoflavone content and resistance to soya bean mosaic virus [7] Zhang et al., 2020 GmFAD2 Increased oleic acid and decreased linoleic and αlinolenic acid Do et al., 2019 GmFT2a, GmFT5a Late flowering Caiet al., 2018 Barley (Hordeum vulgare) GW2.1 Improved yield Kis et al., 2024 Hina Increased grain hardness Jiang et al., 2022 HGGT, HPT Increased vitamin A biosynthesis Zeng et al., 2020 HvITPK1 Increased phosphate levels Vlčko and Ohnoutková, 2020 HvMORC1 Resistance to Blumeriagraminis and Fusarium graminearum Kumar et al., 2018 HvCKX1 Improved plant productivity and decreased total grain biomass Holubovaet al., 2018

313 314 Challenges 315 Genome editing technologies like CRISPR/Cas system offer several benefits for the 316 agriculture due to their less-complicated, robust and multiplex targeting. Due to their high 317 efficacy and accuracy, these systems are used to overcome the confines that existed while 318 using the conventional breeding methods for the development of the disease resistant, high 319 yielding and better agricultural crops. In spite of several benefits and enormous application, 320 CRISPR/Cas 9 has quite few shortcomings. The development of the disease resistant crops by 321 the use of CRISPR/Cas 9 system is obstructed by a few important hindrances. 322 These hindrances include: 323 (i) High fitness cost: Some fitness cost may be caused due to the direct targeting of the host 324 susceptible genes as they are linked with the other growth and developing genes in plants. 325 Moreover, the disruption of the any host susceptible genes may affect the formation of 326 several products in the pathway and eventually other products in the plant due to the mutation 327 in the desired target gene. This may cause the insufficiency of several important nutrients and 328 may cause abnormal phenotypic changes. Nevertheless, the fitness cost may modify based 329 on the targeting susceptible gene/s and additionally is governed by numerous elements, i.e. 330 architects of disease susceptibility, defense suppressors, genes or pre-penetration factors 331 involved in the replication machinery. Use of base editing methods, promoter targeting to 332 create immediate alleles, introduction and designing of susceptible gene variants and knock333 in of desired characters is the possible solution to control this phenomenon. Research has 334 been intensively working on natural or synthetic pathogen inducible promoters or regulatory 335 elements from the past two decades. By the use of regulatory elements, pathogen inducible 336 CRISPR/ Cas 9 systems can be shaped, which can quickly knockout a host susceptible gene 337 involved in synthesis of a specific micronutrient or a sugar promoter and eventually evading 338 fitness consequences. Without regarding the barriers of the species, CRISPR/Cas 9 editing 339 can be used for the production of the desired host susceptible gene mutants in most of the 340 plants of interest (Wang et al., 2022). 341 (ii) Off- target mutations: Another

challenge faced by the CRISPR/ Cas9 system, mainly in 342 the construction of transgene-free crops is off-target mutations. Off-targeting can happen due 343

to misguide by the gRNA or may be gRNA independent in nature and refers to the 344 modifications in the DNA at non-specific, unintended and unwanted sites (Hajiahmadi et al., 345 2019). It has become a major barrier in the production of targeted mutations at the desired 346 sites. Several methods are being developed in largely two directions to find a solution to this 347 problem of off-targeting: evolving a technique for distinguishing off-target mutations and 348 developing the CRISPR/Cas system with high precision. Today numerous bioinformatics 349 tools, such as CasOFFinder (http://www.rgenome.net/cas-offinder/) and CCTop 350 (https://crispr.cos.uniheidelberg.de) and several other tools which include SELEX, IDLV 351 capture, Guide-seq, HTGTS, BLESS, Digenome-seq (Wang et al., 2023) and DISCOVER 352 (Zou et al., 2023) have been established as a measure against this matter. As each tool has its 353 individual positive and negative aspects, the researchers have to select their analytical tool 354 depending on their nature of work and their need. 3 On the other hand, many enhancements are 355 being made in the CRISPR/Cas 9 system to reduce the off-target mutations. Firstly, Cas 9 356 proteins including eSpCas9 eSpCas9, HiFiCas9 and HypaCas9 and Sniper Cas9 were created 357 to expand the target specificity of the enzyme. eSpCas9 (Kim et al 2020), HF-Cas9 and 358 HypaCas9 were technologically advanced by structural alterations to enhance specificity, 359 whereas Sniper Cas9 was screened from a library of SpCas9 mutants that displayed enhanced 360 specificity (Moon et al., 2019). The improved Cas proteins exhibited extraordinarily reduced 361 off-target levels whereas retaining on-target activity. Enhanced specificity has also been 362 achieved by gRNA engineering. The synthesis of guide RNA has itself delivered convenient 363 and adaptable opportunities to advance the CRISPR/Cas system. Chemical synthesis, in 364 vitro transcription, or intracellular transcription systems 3 can be used for the synthesis of the 365 gRNAs. Guide RNAs can be engineered in numerous ways, including chemical

alterations, 366 modifications in the spacer length, sequence alterations in the spacer or scaffold, blending 367 with additional DNA or RNA components, and partial replacement with DNA. The 368 engineered guide RNAs are responsible for enhanced genome editing efficiency and target 369 specificity , regulation of biological toxicity, sensitive and specific molecular imaging, 370 multiplexing, and genome editing flexibility (Zhou et al., 2023). Lately, off-target mutations 371 have also been discovered in rice due to cytosine base editors, but no off-target mutations 372 were detected in adenine base editors. This shows that the new tools devised also need 373 developments (Jin et al., 2019). Off-targeting has been removed in many of the important 374 crops. 375

(iii) Commercialization of the crops: The safety and commercialization of the crops 376 generated by the mutations caused by the CRISPR/Cas system are associated with the 377 humans and other living organisms. The crops generated by the editing using the CRISPR/ 378 Cas9 system are transgene-free and thus do not contain any foreign element in their genome. 379 Therefore these crops would not be considered as transgenic and thus their adoption for 380 commercial cultivation becomes easier. But, the adoption of the genome-edited crops is an 381 issue. Concerning about the problem, several countries are debating on this issue due to rules 382 pertaining to GMO crops, while many countries have adopted these genome-edited crops 383 (Turnbull et al., 2021). 384 (iv) Resistance against viruses: DNA/RNA viruses editing: earlier, CRISPR/Cas 9 has been 385 very useful in creating the virus-resistant crops by accurately and efficiently mutating the 386 genetic material of the DNA- and RNA-based viruses. But due to compromise with the virus 387 immunity in several plants, the ability and efficiency of this system has been questioned. 388 Therefore, the expansion of a well-organized, effective and openly and satisfactorily 389 acceptable form of CRISPR such as CRISPR/Cas13a is immediately needed. 390 (v) Plants with unknown genome: The use of CRISPR/Cas 9 system cannot be used on the 391 plants whose genomes are still not known. Moreover, its applicability is also limited for the 392 plants in which the functions of certain proteins are not known. 393

(vi) Delivery systems: Present delivery systems are restricted to explicit plant species, 394 genotypes, and tissues. In addition, more or less all the existing methods need tissue culture, 395 an extensive and laborious process. Refining the current delivery systems and emergence of 396 new systems will be crucial in reducing obstacles to low-cost application of gene editing in 397 plants (Zhang, 2019). To increase the range of delivery systems, both Agrobacterium and 398 plant genes could be manipulated to advance the Agrobacterium-mediated transformation. 399 Plant germline or meristematic cells 3 can be used for establishing genotype-independent, 400 tissue culture–free delivery systems for the delivery of the CRISPR/ Cas 9 in plants (Gordon401 Kamm et al., 2021). The emergence of the sperm cells, the egg cells and the zygote as the 402 realistic target for delivery is a boon for the CRISPR/Cas technology. The limitation of the 403 species specificity can be avoided by the use of pollen mediated transformation and the 404 regeneration using pollination and artificial hybridization. Moreover, the use of shoot apical 405 meristem 3 for the delivery of the CRISPR/ Cas is evident as the stem cells are destined to 406

differentiate into gametes. New delivery systems grounded on nanotechnology and virus 407 particle–like structures too embrace a potential for crop improvement (Abdallah et al., 2025) 408 (viii) Limited PAM sequences: The action of CRISPR/Cas system requires the PAM 409 sequence for the identification and cleavage of the target DNA, and thus the missing of the 410 PAM sequence causes a problem in the action the system. Moreover, there are very limited 411 PAM sites present. 412 (ix) Low HDR efficacy: A challenge of HDR-mediated gene editing is that it needs 413 synchronized introduction of DSBs and delivery of a repair template to one site inside the 414 genome. There are numerous possible ways to increase the frequency of HDR in plant cells, 415 for example, management of DNA repair pathways (Ahmad et al., 2022) 416 Conclusion 417 The CRIPSR/Cas system has permitted cost-effective and efficient gene editing compared 418 with prior technologies, comprising 4 zinc finger nucleases (ZFNs) and transcription

activator419 like effector nucleases (TALENs), making it available to many scientists. The ease, 420 flexibility, and sturdiness of CRISPR/Cas systems make genome editing an influential tool 421 for efficient crop improvement via gene knockout, knock-in, replacement, point mutations, 422 fine-tuning of gene regulation, and other alterations at any gene locus in crops. This system 423 can be further extended to the 2 crops with complex genomes or to the crops with unknown 424 genomes to further extend the technology to a broader prospect, by investigating and 425 developing more efficient delivery systems. 426 Author contributions 427 MKD conceived the review and provided the possible outline. SC, TS, SK, collected the 428 information and wrote the first draft of the manuscript. MKD edited and finished the final 429 draft of the manuscript. 430 Declarations 431 Consent for publication 432 We hereby give our informed consent for the publication of this manuscript and any 433 accompanying materials, including images or data that may directly or indirectly disclose our 434 identity, as part of the publication process. 435

Conflict of interest 436 The authors have no conflicts of interest to declare. All co-authors have seen and agree with 437 the contents of the manuscript. 438 439 References 440 1. Abdelrahman, M., Wei, Z., Rohila, J. S., & Zhao, K. (2021). Multiplex genome-editing 441 technologies for revolutionizing plant biology and crop improvement. Frontiers in Plant 442 Science, 12, 721203. 443 2. Ahmad, Y., Haider, S., Iqbal, J., Abbasi, B. A., Yaseen, T., &Mahmood, T. (2022). The 444 mechanisms of genome editing technologies in crop plants. In Principles and practices of 445 OMICS 2 and genome editing for crop improvement (pp. 295-313). Cham: Springer 446 International Publishing. 447 3. Aksoy, E., Yildirim, K., Kavas, M., Kayihan, C., Yerlikaya, B. A., Çalik, I., ...&Demirel, U. 448 (2022). General guidelines for CRISPR/Cas-based genome editing in plants. Molecular 449 biology reports, 49(12), 12151-12164. 450 4. Ali, A., Zafar, M. M., Farooq, Z., Ahmed, S. R., Ijaz, A., Anwar, Z., ...&Maozhi, R. (2023). 451 Breakthrough in CRISPR/Cas system: Current and future directions and 452 challenges. Biotechnology Journal, 18(8), 2200642.

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