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Applications of Gene Editing for Improving Climate Change Resilience in Agriculture

By

Nicholas G. Karavolias

A dissertation submitted in partial satisfaction of the
requirements for the degree of
Doctor of Philosophy
in
Plant Biology
in the
Graduate Division
of the
University of California, Berkeley

Committee in Charge:
Professor Brian J. Staskawicz, Chair
Professor Sarah Hake
Professor Benjamin Blackman

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Applications of Gene Editing towards Improving Climate Change Resilience in Agriculture

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By
Nicholas G. Karavolias

Abstract

Application of Gene Editing for Climate Change in Agriculture

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Nicholas G. Karavolias
Doctor of Philosophy in Plant Biology
University of California, Berkeley
Professor Brian J. Staskawicz, Chair

Climate change imposes a severe threat to agricultural systems, food security, and human nutrition. Meanwhile, efforts in crop and livestock gene editing have been undertaken to improve performance across a range of traits. Gene editing applications for climate change specifically have converged on four major traits: nutritional quality improvement, yield enhancement, disease tolerance, and abiotic stress tolerance, with the fewest current applications directed towards abiotic stress tolerance. While only few applications of gene editing have been translated to agricultural production thus far, numerous studies in research settings have demonstrated the potential for potent gene editing based solutions to address climate change in the near future.

Gene editing of rice (*Oryza sativa*) specifically holds promise for generating climate resilient foodscapes. Rice is of paramount importance for global nutrition, supplying at least 20% of global calories. However, water scarcity and increased drought severity are anticipated to reduce rice yields globally. Rice stomatal developmental genetics were explored as a mechanism to improve drought resilience while maintaining yield under climate stress. CRISPR/Cas9-mediated knockouts of EPFL10 and STOMAGEN yielded lines with c. 80% and 25% of wild-type stomatal density, respectively. *epfl10* lines with moderate reductions in stomatal densities are able to conserve water to similar extents as *stomagen* lines, but do not suffer from the concomitant reductions in stomatal conductance, carbon assimilation, or thermoregulation observed in *stomagen* knockouts. Moderate reductions in stomatal densities achieved by editing EPFL10 may be a climate-adaptive approach in rice that can safeguard yield. Editing the paralog of STOMAGEN in other species may provide a means to tune stomatal density in agriculturally important crops beyond rice.

Negative pleiotropic effects of gene editing may be mitigated by editing a single copy of a duplicated gene underlying a trait of interest. However, this approach is limited by a narrow set of duplicated genes whose null phenotype is not deleterious to overall plant fitness. Promoter editing is emerging as an increasingly relevant tool to generate subtle trait variation while mitigating against harmful pleiotropy. We applied a multiplexed, guide design approach informed by bioinformatic analyses to generate genotypic variation in the promoter region of *OsSTOMAGEN*. Engineered genotypic variation corresponded to continuous variation stomatal density and size. This near-isogenic panel of stomatal variants was leveraged in physiological assays

to establish discrete relationships of stomatal density with a range of gas exchange parameters. Developmental plasticity in response to vegetative drought was inhibited in some promoter alleles and in *stomagen*. Derived stomatal variants can be matched with similarly broad environmental conditions to optimize. Collectively our data suggest a role of promoter editing as a tool for establishing trait variation including phenotypic gain-of-function that can be leveraged for establishing relationships of anatomy and physiology and for crop improvement along diverse environmental clines.

In securing food systems against the severe implications of climate change, gene editing approaches towards the adaptation of rice to abiotic stress has shown promise. An additional approach makes use of gene editing for improving crop quality in crops with existing tolerance. To this end, we sought to improve the safety of the drought-stress-tolerant cassava crop, for human consumption using CRISPR/Cas9. Cassava accumulates cyanogenic glucosides which are human-toxic-metabolites that must be removed to avoid severe human health consequences. Cyanogenic glucosides may play an important physiological role in cassava plants, so eliminating their synthesis entirely may also limit overall productivity. Our work sought to engineer tissue specific accumulation of cyanogenic glucosides by editing, *MeCGTR1*, a putative systemic transporter using CRISPR/Cas9. *cgtr1* lines exhibited depletions of cyanogenic glucosides in upper leaves while maintaining wild-type levels in tuberous roots. Together with a phloem girdling assay, our data indicated a root-to-shoot mode of cyanogenic transport which stands in contrast to previously documented modes of detected movement. Our work provides the first in-vivo validation of a cyanogenic glucoside transporter in cassava providing evidence for the de novo biosynthesis of cyanogenic glucosides in roots.

Dedication

This dissertation is dedicated to my loving parents, George and Antonia, and all other immigrants, brave enough to dream.

And to their kids, bold enough to realize those dreams while discovering their own.

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Chapter One

A systematic review of the applications of gene editing for climate change in agriculture

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1.1 Introduction

Climate change poses a severe threat to the future of the environment as it pertains to agriculture, biodiversity, human society, and nearly every facet of our world. The primary cause of climate change is the anthropogenic addition of greenhouse gases to the atmosphere. Due to these human emissions, the average temperature of the planet has risen by nearly 1°C since 1850 [1,2]. Even if warming were to be halted at 1.5°C, which would require drastic and immediate global action, long-term effects of past emissions would linger for centuries or millennia[2]. The magnitude of the effects depends on the amount of emissions; in general, more frequent heatwaves, droughts, floods, and persistent sea level rise and global temperature increases are expected[2]. Indeed, many of these effects are already being observed [1–3].
In both natural ecosystems and agricultural settings, plants and animals are being forced to contend with novel conditions that change more quickly than their pace of adaptation. Rising temperatures and shifting precipitation regimes will drastically alter the biological landscape, resulting in species migration, invasion, and extinction[1,4]. One meta-review of more than 130 studies has estimated that one in six species may go extinct due to the changing climate[4]. Simultaneously, global food supplies are declining as droughts and floods impact agricultural output. Under a range of warming scenarios, agricultural output is expected to decline globally. Productivity of major commodity crops will be affected, especially in lower latitudes where the effects of climate change on yield will be more severe[3].

In response to these challenges, the use of gene editing, also referred to as genome editing or genome engineering, has emerged as a method to either aid in the adaptation of organisms to climate change or help mitigate the effects of climate change on agriculture.
Gene editing is a method to generate DNA modifications at precise genomic locations. These modifications can result in knockout or knockdown of one or multiple genes without the permanent insertion of any foreign DNA. Alternatively, genes from within the organism’s gene pool or from other organisms can be inserted into precise locations within the genome to knock-in a new trait. Transcription

activator-like effector nucleases (TALENs), Zinc Finger Nucleases (ZFNs), and CRISPR/Cas systems have all been utilized to achieve precise gene edits[5,6]. The precision and efficiency of generating edits has been tremendously improved by the introduction of CRISPR/Cas systems, although there is certainly still a role for other gene editing technologies. The application of gene editing techniques has generated great potential for developing crops and livestock that can better manage the impositions of climate change.

We seek here to illuminate the ways in which gene editing may help combat the deleterious effects of climate change by highlighting current efforts to apply these techniques in crops and livestock. We will summarize the efforts undertaken thus far and describe the limitations and opportunities that exist with gene editing technologies. Tables 1-4, provided at the end of the review, summarizes the breadth of applications of gene editing in crops and livestock.

1.1.1 Climate change will inhibit agricultural productivity

The effects of climate change have already started to emerge and will undoubtedly worsen. Currently, crops in lower latitude regions have begun to experience yield declines, while higher-latitude regions have experienced an increase in yield[3,7]. However, global declines in yield and crop suitability are projected over the course of the century as a direct result of climate change. According to the Intergovernmental Panel on Climate Change (IPCC), extreme weather events will disrupt and decrease global food supply and drive higher food prices[3]. In dry areas of the planet especially, climate change and desertification are likely to reduce agricultural productivity. Areas closer to the equator will be most vulnerable to declines in crop yield as temperature increases, with the continents of Asia and Africa having the largest populations vulnerable to increased desertification. Indeed, desertification has already started to reduce agricultural productivity and biodiversity, compounded by unsustainable land management and increased population pressure. While it is unclear to what extent aridity will increase on a global scale, it is likely that the area at risk of salinization will increase. Climate change will also contribute to current land degradation with increased droughts, floods, rising seas, and more intense tropical storms[3].

1.1.2 Effects of climate change on crops

The major contributing greenhouse gas to climate change is carbon dioxide (CO_2)[3], which generally has a positive effect on plant growth. As CO_2 concentration increases, so too does the rate of photosynthesis and carbon assimilation (an effect known as CO_2 fertilization)[8]. Simultaneously, however, the nutritional quality of food decreases in response to heightened CO_2[3]. Furthermore, the increased growth associated with higher CO_2 may be offset by other environmental factors; there has been an observed decline in this CO_2 fertilization effect in the past 30 years, likely due to shifting nutrient concentrations and lower availability of water[8]. Given the aforementioned increase in extreme temperature and precipitation events, combined

with the shifting prevalence and range of diseases across the globe[9,10], the overall effect of climate change on crops will be detrimental[3,7]. Already, global yields of maize, wheat, and soybeans have slightly decreased from 1981-2010 relative to the pre-industrial climate[7].

1.1.3 Effects of climate change on livestock

Livestock will similarly be negatively affected by climate change. Increasing temperature and shifting precipitation directly impact livestock themselves, the crops grown for their feed, and diseases that infect them [3,11]. Increasing temperatures will have perhaps the most profound effects on livestock: heat stress impacts feed intake and can reduce weight gain, decreases reproductive efficiency, has multiple negative health effects, and increases mortality in many livestock species[11].

1.1.5 Climate change impacts biodiversity and food systems

Beyond agriculture, the effects of climate change on biodiversity are no less severe. A recent meta-review of 97 studies found that even with only moderate increases of global temperature, biodiversity will suffer significant declines[1]. The pressures of climate change on biodiversity, in combination with increased agricultural demand, have also served to exacerbate the oftentimes antagonistic relationship between agricultural and natural landscapes. The impact of individual climate change effects and their intersections are complex. Broadly, the direct and indirect consequences of climate change will be deleterious to plant and animal performance in cultivated systems (Figure 1).

Mitigating the deleterious impacts of climate change on biodiversity is paramount. However, most applications of gene editing have converged on agricultural commodities: there are very few instances of gene editing for climate change in non-commodity organisms. This review therefore focuses on how gene editing solutions can address the broad effects of climate change on agriculture while maintaining the importance of applying these transformative technologies to the totality of biodiversity threatened by climate change.

1.2 Applications of Gene Editing in Agriculture

Here we present an extensive exploration of gene editing-based solutions in response to the daunting limitations to agricultural productivity imposed by climate change. We note that these examples are mostly from public institutions and represent proof-of-concept experiments rather than commercialized technologies.

1.2.1 Increasing Abiotic Stress Tolerance

Abiotic stresses, including but not limited to drought, salinity, and flooding, pose some of the most severe threats to agricultural productivity in the face of climate change. Abiotic stress is anticipated to become more severe in agricultural systems

as a result of climate change. Current research efforts demonstrate that gene editing is an effective tool in broadening resistance of crop tolerance as described in the following examples (Table 1).

1.2.2 Salinity tolerance in rice

Rice, a staple food for more than half of the world's population, is of primary importance for global food security[12]. Two major abiotic stresses that affect rice are drought and salinity, necessitating research explorations into the potential of leveraging gene editing for developing tolerant varieties. One such exploration was the use of CRISPR/Cas9 to knockout OsRR22, a gene associated with salt susceptibility in rice[13]. Rice plant performance in high salinity environments (0.75% NaCl) was improved with no concomitant decreases in grain yield, plant biomass, or grain quality. Edited lines were on average 19% shorter in saline solution whereas wild type plants were 32% shorter. Edited plants also had much less severe biomass reductions due to salt exposure compared to unedited plants, and showed no significant differences to unedited plants in the absence of saline. Saline studies were conducted in the greenhouse and overall agronomic performance was evaluated in the field. Researchers found that edited plants had much less severe biomass reductions due to salt exposure compared to wild type plants[13].

1.2.3 Drought tolerance in rice

Rice has also been engineered to improve drought and high temperature tolerance by targeting stomatal development. Stomata, which are anatomical features on the surface of all crop plant tissues, serve as the major sites of water loss. In this study researchers targeted a positive regulator of stomatal density in rice[14]; while they did not explicitly test the effects of this editing on water use efficiency, other research has shown that stomatal reductions in rice have clear, positive implications for water use efficiency[15]. Rice lines with reductions in stomatal densities had better yield in severe drought and were able to maintain lower temperatures despite no differences in yield. Stomatal density reductions achieved by a cisgenic approach in this case mirrored stomatal density reductions achieved by a knockout-based, gene-editing approach. Thus, reducing stomatal densities by gene-editing or cisgenic approaches could enable plants to resist water deficits and could also increase heat tolerance.

1.2.4 Enabling northern production of rice

An additional editing effort in rice produced early maturing rice that is more amenable to production in northern latitudes [16]. Northern latitudes experience longer day lengths and cooler temperatures. Applications of CRISPR/Cas9 to the flowering related genes Hd2, Hd4, and Hd5 generated rice plants that flowered significantly earlier than their wild-type counterparts, making them more fit for northern production. These varieties may be well suited for use in future conditions where temperatures and other climatic conditions near equatorial regions render farmlands less fertile. Early flowering plants may also be a useful adaptation against water

deficit: by shortening the life span of crops through early flowering, less cumulative water may be required.

1.2.5 Semi-dwarf banana varieties

In banana, gene editing using CRISPR/Cas9 to generate knockouts of genes for the biosynthesis of gibberellins has facilitated the development of a semi-dwarfed variety. This variety may be more resistant to lodging as a result of intense winds, typhoons, and storms, anticipated to increase in severity as a result of climate change[17]. Semi-dwarfed varieties have historically been an important trait in crop improvement, as was the case with rice and wheat that enabled the Green Revolution[18].

1.2.6 Promoter editing for drought tolerance in maize

Beyond generating knockouts, gene editing tools can also facilitate knock-ins. Researchers have used CRISPR/Cas9 to insert a promoter at a specific maize locus to increase drought tolerance[19]. Specifically, an alternate maize promoter was inserted before ARGOS8, a gene associated with drought tolerance. This precise insertion enabled greater grain yield during flowering water stress, while maintaining yields in normal growth conditions. This approach represents an intragenic technique facilitated by gene-editing in which a native maize genetic sequence was introduced at a new locus to increase plant adaptation to an abiotic stressor.

1.2.7 Enhancing thermotolerance of cattle

In animals, gene editing has also been applied to mitigate abiotic stress imposed by climate change. Acceligen, a subsidiary of Recombinetics Inc., has undertaken an initiative to improve the thermotolerance of cattle, with support from the Foundation for Food and Agriculture Research (FFAR) and Semex. Researchers are focused on replicating the SLICK phenotype originally identified in Senepol cattle through gene editing. Variations of this phenotype in cattle contribute to thermotolerance [20,21]. Conventionally bred cattle possessing SLICK genetics are more thermotolerant, as exhibited by lower vaginal temperatures, lower rectal temperatures, lower respiration rates, and more sweating, thus leading to increased milk production during summer months[20]. Using gene editing approaches, Acceligen seeks to replicate SLICK genetics, to improve thermotolerance of important cattle breeds[21].

1.3 Managing Disease

A majority of diseases affecting plants and animals are anticipated to become more widespread with climate change [9,10,22–25]. Fortunately, many current gene editing efforts have shown promise in conferring disease resistance. This work will become even more necessary in coming years as climate change increases disease severity and incidence. Increased range of vectors, rising temperatures fostering reproduction of pathogens, and host organisms becoming more susceptible to

pathogens are some of many climate change driven causes of worsening disease. Gene editing can provide a solution to managing these current and emerging global threats to agricultural productivity precipitated by climate change (Table 2).

1.3.1.1 Plant gene editing for disease resistance

In plants, genes have been identified that increase disease resistance when knocked out. Altering genetic elements involved in susceptibility has thus far been the primary form of disease mitigation through gene editing. While there are few such genes available for increasing disease resistance, researchers have already successfully leveraged many of these loci for heightened resistance. The following examples demonstrate the effectiveness of knocking-out susceptibility loci for enhanced resistance .

1.3.1.2 Increased rice resistance to a range of disease

Climate change may have varying effects on diseases across geographies and temporal scales, as was indicated by modeling two prevalent rice diseases in Tanzania: leaf blast and bacterial leaf blight[26]. It should be noted that these diseases are two of the most devastating rice diseases globally. The model indicated that bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (PthXo2) will likely become more severe due to climate change in Tanzania, whereas rice infection by leaf blast caused by *Magnaporthe oryzae* will decrease due to changing climate. Modeling two diseases in the same geography revealed that climate change will not always result in increased disease severity, thereby necessitating careful considerations of diseases that will indeed become worse due to climate change. Gene editing for a range of rice diseases has proven remarkably effective. CRISPR/Cas9 was used to produce knockouts of OsSWEET13. SWEET family genes encode sucrose transporters that can be exploited by pathogens[27]. Mutating this gene increased disease resistance dramatically[28]. A similar approach to address bacterial leaf blight used CRISPR/Cas9 to target the promoter region of multiple OsSWEET genes in two studies[29,30]. Lines that had been edited were broadly resistant to many Xanthomonas pathogens. The knockout of an alternative gene, Os8N3, similarly resulted in plants that were also resistant to this pathogen. Os8N3 was selected as a target based on naturally occurring resistance alleles in this gene despite the mechanisms of resistance not being well-defined[31]. In the case of leaf blight, gene editing effectively reduced rice susceptibility to a pathogen that is anticipated to become more damaging in some regions of the world due to climate change.

CRISPR/Cas9 mediated knockout of OsERF922, an ethylene response gene previously implicated in blast resistance, had significantly reduced lesion sizes in response to being infected with leaf blast with no concomitant alterations to agronomic traits[32].

Editing a eukaryotic elongation factor, eIF4G, in rice, using CRISPR/Cas9 yielded plants entirely resistant to rice tungro virus. Edited plants infected with virus had no

detectable viral proteins and exhibited higher yields relative to wild type[33]. Additional efforts to engineer broad-scale resistance to multiple rice pathogens simultaneously are described below.

1.3.1.3 Engineering broad-scale resistance in rice, barley, and tomato

Some crops have been edited to establish resistance to numerous diseases simultaneously. Engineering broad-scale resistance to disease in staple crops could provide a single approach to addressing many simultaneously worsening diseases[30]. An example of this approach is the editing of bsr-k1, a rice gene identified to be important in disease resistance. Bsr-k1 binds to defense-related genes and promotes their turnover[34]. Editing this gene using CRISPR/Cas9 yielded rice plants that were simultaneously resistant to leaf blast and bacterial leaf blight by stabilizing important defense genes. Field trials of edited lines yielded 50% greater when challenged with rice leaf blast in the field. Other agronomic properties of the edited rice plants were not affected[34].

In barley, CRISPR/Cas9-mediated editing of MORC1, a defense related gene previously identified in Arabidopsis thaliana, simultaneously increased resistance to *Blumeria graminis* f. sp. *hordei* the causative agent of barley powdery mildew and *Fusarium graminearum*. Edited barley plants contained less fungal DNA and exhibited fewer lesions[35].

Likewise, editing of a single locus in tomato conferred broad-spectrum resistance. Mutations to SlDMR6-1. Loss of function mutants in Arabidopsis thaliana maintain higher levels of salicylic acid. CRISPR/Cas9 edited tomato lines were more resistant to P. syringae, P. capsici and Xanthomonas spp., indicated by markedly less severe disease symptoms and lowered pathogen presence[36].

1.3.1.4 Managing cassava brown streak virus

In cassava, gene editing was used to address brown streak virus which can cause yield reductions of 70% in severe cases. Similar to host eukaryotic translation initiation factors in rice (eiF), eIF4E isoforms encoded by the cassava genome are required by Potyviridae viruses for infection. Simultaneous targeting of ncbp1 and ncbp2, two such eiF4E genes, by CRISPR/Cas9 enhanced plant resistance: root disease severity and viral titre were lowered significantly in edited cassava lines.

1.3.1.5 Engineering cucumber viral resistance

Likewise in cucumber, CRISPR/Cas9 was used to generate deletions in the eIF4e gene to inhibit viral infections. Lines with homozygous mutations were resistant to cucumber vein yellowing virus, zucchini yellow mosaic virus, and papaya ringspot virus-W as demonstrated by reduced symptoms and viral accumulation[37].

1.3.1.6 Wheat powdery mildew mitigation

Wheat powdery mildew is also anticipated to become increasingly severe on winter wheat in China with the changing climate[24]. In anticipation of this increased disease pressure, researchers in China have undertaken a gene editing effort to address wheat susceptibility to this disease. Employing TALENs and CRISPR/Cas9, researchers successfully edited the mildew resistance locus (MLO) in the wheat genome[38]. As a result, the percentage of viable powdery mildew-causing pathogens was effectively 0% in edited lines and nearly 20% in wild type. Edited plants exhibited marked improvements in powdery mildew resistance relative to wild type. An additional effort targeting EDR1, as an alternate mechanism for achieving powdery mildew resistance was undertaken using CRISPR/Cas9: edited wheat plants like the MLO edited lines were resistant to powdery mildew indicated by reductions in fungal structures and microcolonies[39].

1.3.1.7 Powdery mildew mitigation in tomato and grape

Gene editing for disease resistance in non-grain crops has also been a successful endeavor. For example, in tomato, the MLO locus was also edited using CRISPR/Cas9, leading to the development of elite tomato varieties that are resistant to the powdery mildew disease as indicated by heightened hydrogen peroxide accumulation after infection with powdery mildew[40]. Targeting of MLO homologs in grapevine similarly increased powdery mildew resistance; in grapevine there was about a two-fold reduction in powdery mildew sporulation in an edited line[41]. It is noteworthy that homologous loci in alternate crop species gave rise to powdery mildew resistance when edited, providing an example of transferable applications of gene editing among distantly related species.

1.3.1.8 Enhancing resistance of tomato to alternate diseases

Also in tomato, gene editing approaches have improved resistance to bacterial speck and tomato yellow leaf curl virus[42,43]. Editing of JAZ2 in tomato using CRISPR/Cas9 reduced infection by Pseudomonas syringae pv. tomato, the causal agent of bacterial speck, by reducing pathogen mediated stomatal opening. Edited plants maintained significantly reduced levels of *Pseudomonas syringae* pv. tomato relative to wild type. CRISPR/Cas9 mediated resistance in tomato has also been effectively applied to manage yellow leaf curl virus. Tomato plants were engineered to contain guides targeting multiple sequences in the TYCV genome. Viral accumulation was markedly decreased in engineered tomatoes and this resistance was heritable over many generations.

1.3.1.9 Securing banana resistance to BSV

In bananas, banana streak virus (BSV) presents a major barrier to breeding and distribution of banana cultivars (*Musa spp.)* in various parts of the world[44]. This virus integrates into the B subgenome of *Musa* species and remains latent until plants encounter stress such as drought. Many important agronomic species of banana such as plantains are affected by this virus, and breeding programs are restricted in their ability to use *Musa balbisiana* as source material due to the presence of the

latent virus. Knockouts of the endogenous virus produced lines in which 75% of edited plants remained asymptomatic after being exposed to water stress. This is the first study to show the efficacy of targeting an integrated plant virus in a plant genome and provides a promising mechanism to address a significant barrier in banana production and breeding[44].

1.3.1.10 Transient resistance in cacao

In cacao, knockout-based improvement of resistance to *Phytopthora tropicalis* was achieved in a transient assay. Cacao plants transiently expressing CRISPR/Cas9 targeting the TcNPR3 gene, a suppressor of disease response, exhibited smaller lesions after infection with *Phytopthora tropicalis*. This work represents the first application of gene editing in cacao, and paves the way for future stably heritable edited lines of cacao[45].

1.3.1.11 Grapefruit resistance to citrus canker

Citrus canker is a devastating disease in most citrus fruits especially as most commercial varieties remain susceptible to infection. Application of CRISPR/Cas9 to grapefruit has successfully increased resistance of edited fruit to infection. The causative agent of citrus canker, *Xanthomonas citri subsp. citri* (Xcc), is able to increase expression of CsLOB1, thus generating cankers[46]. CRISPR/Cas9 mediated editing of CsLOB1 promoter binding sites[46] and the CsLOB1 coding region[47] were both effective in generating resistant grapefruit lines indicated by tremendously reduced symptoms.

1.3.1.12 Increased virus and abiotic stress resistance in potato

CRISPR/Cas9 mediated editing of a gene encoding coilin in potato plants has facilitated increased resistance to potato virus Y. Coilin is a major structural component of the subnuclear Cajal bodies previously implicated in virus interactions *in planta*. Modification of the potato coilin gene C-terminal domain significantly increased resistance of potato to Potato virus Y and also increased salt and drought tolerance[48].

1.3.1.13 Curing cotton cancer

Cotton verticillium or “cotton cancer” is a severe disease of cotton caused by *Verticillium dahliaeng* [49]with little resistant germplasm available in natural populations. 14-3-3 proteins c and d had been previously identified as negative regulators of disease response. Knockouts of 14-3-3 c and 14-3-3 d simultaneously yielded cotton plants that were more resistant to cotton verticillium indicated by fewer disease symptoms and lowered pathogen presence.

1.3.2 Animal gene editing for disease resistance

Diseases affecting animal hosts are projected to be affected by climate change as well. The intersection of multiple climate and human variables makes it difficult to determine how climate change will affect animal pathogens[10,50]. It is likely that currently temperate locations will become more favorable to tropical vector-borne

diseases, exposing new host populations with no previous immunity and potentially creating new disease transmission modes and patterns[51]. Overall, there is high confidence that diseases and disease vectors will worsen due to climate change[52]. Gene editing has been used to target several animal diseases; several such studies are described below to demonstrate the state of the field. These examples are meant to demonstrate a substantial basis for the capacity of gene editing to ameliorate diseases that may potentially worsen due to climate change. The vast majority of editing for animal disease resistance centers on the creation of disease-resistant livestock, with the exception of CRISPR-based gene drives in mosquitoes and other vector and reservoir species [53–57].

1.3.2.1 Viral resistance in chicken

In chickens, avian leukosis viral subgroup J is a disease that can infect meat and laying chickens, resulting in relatively high mortality rates. Researchers used CRISPR/Cas9 and homologous recombination to create an amino acid deletion in the extracellular portion of the gene *chNHE1* (chicken Na^+/H^+ exchanger type 1), which encodes the virus receptor in chickens that allows the virus to infect cells. The deletion was completed in chicken primordial germ cells, which through transplantation and subsequent breeding resulted in chickens resistant to infection by the virus[58].

1.3.2.2 Tuberculosis and mastitis resistance in cattle

Tuberculosis resistance in cattle has been addressed in two studies. In the first study, researchers focused on the mouse gene *SP110* (SP110 Nuclear Body Protein), which controls *Mycobacterium tuberculosis (MTB)* infections and induces apoptosis in infected cells[59]. The authors used TALENickases to insert the gene into a specific location in the bovine genome via homologous recombination; the knock-in of this resistance gene improved tuberculosis resistance[59]. A second study using CRISPR/Cas9 was able to knock-in the *NRAMP1* gene (natural resistance-associated macrophage protein-1, a gene associated with innate immunity) from bovine via homologous recombination. The resulting cattle likewise exhibited increased tuberculosis resistance[60].

Similarly, gene editing has been utilized to prevent mastitis, the most significant disease of dairy cows. In two studies, homologous recombination in cattle facilitated by ZFNickases enabled the insertion of two genes that confer resistance to infection from *S. aureus*, a causative agent of mastitis: the gene encoding lysostaphin from *Staphylococcus simulans*[61] and the human lysozyme (hLYZ) gene[62] into an intron of the β-casein locus of dairy cattle. Because casein is a protein found in dairy milk, the genes inserted into this locus would mimic expression of β-casein and the exogenous proteins would be present in milk produced from the edited cows[61]. Both of these studies yielded dairy cows with milk that was able to prevent *S. aureus* infection of the lactating cow.

M. haemolytica is a causative agent of pneumonia in cattle. ZFNs were used to make a precise edit in *CD18*, a gene encoding a surface protein on cattle leukocytes. Leukocytes extracted from the edited cow exhibited very low levels of cell toxicity in the presence of *M. haemolytica* leukotoxin relative to wild type cattle. Thus, a gene edit markedly improved the tolerance of gene edited cattle to *M. haemolytica*, a pervasive agent of disease[63].

1.3.2.3 Disease resistance in pigs
Progress has also been made in developing disease resistance in pigs using gene editing. In a 2014 study, researchers were able to knock out two genes, *CD163* and *CD1D*[64]. The former is required for infection by porcine reproductive and respiratory syndrome virus (PRRS virus), and the latter is involved in innate immunity. In a follow-up to this study, the researchers assessed the *Cd163* knockout pigs for resistance to PRRS, finding that they displayed no symptoms when infected. In comparison, wild-type offspring developed severe symptoms necessitating their euthanization[65]. Similar results were also obtained by a later study, also using CRISPR/Cas9 editing to knock out *CD163* to produce pigs fully resistant to PRRSV[66,67].

Further work in pigs was able to demonstrate the utility of CRISPR/Cas9 to knock-in resistance to classical swine fever virus (CSFV) at the *Rosa26* locus[68]. This locus is a preferred site for transgene insertion due to its ubiquitous and strong expression, coupled with a lack of gene-silencing effects[68,69]. The edited pigs were resistant to CSFV, whereas all wild type pigs exhibited 100% mortality. Researchers were also able to knock-in the *C. elegans fat-1* gene into the *Rosa26* locus in pigs[68]. As *fat-1* is implicated in both disease resistance and nutritive quality of meat, this study served as a proof-of-concept to demonstrate the possibility of simultaneously improving the nutritional value of pork and increasing general disease resistance in pigs. Finally, a recent study was able to use CRISPR/Cas9 editing to knock out the *ANPEP* (aminopeptidase N) gene, conferring resistance to infections caused by coronaviruses[70].

1.3.2.4 Viral resistance in aquatic species
Use of gene editing to combat disease in aquatic species is more limited; the first use of a CRISPR system to test enhanced disease resistance was in 2018[71]. Here, researchers were able to use CRISPR/Cas9 in grass carp cell lines to knock out *gcJAM-A* (grass carp Junctional Adhesion Molecule-A), a gene involved in grass carp reovirus (GCRV) infection. When challenged with GCRV, the edited cells were shown to suppress viral replication[71].

In crops and livestock, genes have been identified that increase disease resistance when knocked out. Altering genetic elements involved in susceptibility has thus far been the primary form of disease mitigation through gene editing for crops. Whereas, in livestock, gene editing has facilitated the knockout and knock-in of genes to improve disease resistance. Successful gene editing studies in crops and

animals provide a reasonable foundation for further use of this technology to address a range of diseases, some of which may be exacerbated by climate change.

1.4 Increasing Yields

Global climate change will continue to broadly reduce crop and livestock yields[72]. Some landscapes will experience yield improvements, but largely climate change stands to lower productivity[52]. Coupled with population increases, these coinciding phenomena will necessitate the expansion of agricultural lands into currently non-cultivated geographical areas. This expansion imposes a severe threat to biodiversity and the associated ecological services of non-agricultural lands. Land sparing through the augmentation of yield can mitigate the deleterious effects of agricultural expansion, with gene editing as a potential tool toward this solution (Table 3).

1.4.1 Crop yield improvement

1.4.1.1 Improving rice yields

In crop plants, a variety of gene edits have been produced to increase yields. In rice, for example, targeting different combinations of genes associated with yield restrictions has produced lines with 11-68% increased yields[73–75]. DEP1 and Gn1a are yield-associated genes that have been previously characterized for their involvement in yield attributes. One novel allele of Gn1A and three novel alleles of DEP1 generated by CRISPR/Cas9 produced significantly higher yields relative to wild type alleles.Gn1A novel mutants yielded >24% which was slightly better than the 21% yield advantage conferred by natural mutant Gn1A. Novel mutants in DEP1 yielded up to 51% greater than WT which is 11% greater than the naturally occurring mutants[75].

In another case simultaneous and individual knockouts of three yielded related genes, GS3, GW2, and Gn1a, identified to negatively regulate grain size, width, and number, respectively, increased yield in rice in three different cultivars. Simultaneous KOs generated greatest yield increases, with gains of up to 68% in one cultivar. Multiple knockouts can thus be additive in yield improvements[74].

CRISPR-Cas9 induced mutations in class I PYL genes were also able to increase yield. Abscisic acid (ABA) is a phythormone associated with abiotic stress . PYL/RCARs are genes that encode receptors for ABA, a phytohormone essential in drought responses and in seed dormancy. Simultaneous mutations of class I PYL genes resulted in rice plants that were higher yielding in paddy conditions. The stomata in PYL mutants were much less responsive to the addition of ABA, maintaining larger apertures despite the presence of the drought signal. Thus, the mutant plants lost water more readily. The resultant plants had larger panicles, greater panicle branches, more tillers, and overall higher yield when tested in field

conditions. This approach could be appropriate for rice grown in paddy conditions but would be deleterious in conditions where water is limited. Triple knockout of PYLs 1,4,6 afforded a 30% increase in yield in well-watered conditions[73].

1.4.1.2 Knockout-based wheat yield improvement

In wheat, simultaneous knockouts of GW2, LPX-1 and MLO were meant to enhance yield and disease resistance. Genes selected were previously shown in separate studies to increase yield and wheat pathogen resistance. Homozygous, simultaneous mutations in these three loci yielded wheat plants that had significantly elevated grain weights and size, while disease resistance was not evaluated[78].

1.4.1.3 Improving yields of waxy maize

In maize, CRISPR/Cas9 was used to generate high amylopectin varieties from elite cultivars by knocking out the waxy gene. Gene edited varieties yielded 5.5 bushels more per acre relative to high amylopectin varieties generated by conventional breeding and were produced in less time, highlighting the throughput and utility of gene editing relative to conventional breeding in certain specific applications[76].

1.4.1.4 Engineering tomato architecture and domestication for yield

Engineering of tomato architecture using CRISPR/Cas9 has enabled improvements such as drastically increased fruit size and altered plant morphology better suited for urban environments[77,78]. Promoter editing of SlCLV3, a mobile peptide important in floral stem cell regulation; S, an inflorescence architecture gene; and SP, an overall architecture gene were able to generate novel variation and enhancement in fruit size, floral architecture and overall architecture in tomato. This study also provided major breakthroughs in the use of gene editing cis-regulatory elements for crop improvements.

Efforts have been made to increase yield as well as other agronomic properties of tomatoes using CRISPR/Cas9 to domesticate a wild tomato variety. By identifying and editing six genes associated with key domestication traits, researchers were able to domesticate a wild tomato relative, increasing fruit size threefold and fruit number tenfold. and yield while also improving nutrition, abiotic stress tolerance, and disease tolerance. This study also provides the basis for future gene editing mediated wild relative domestications[79].

1.4.2 Livestock yield improvement

1.4.2.1 Enhancing livestock yield through MSTN knockouts

In animal gene editing, many efforts have converged on targeting the *MSTN* gene in species such as pig, cattle, sheep, goat, rabbit, and several aquatic animals including carp, catfish, and red sea bream[80–104]. The *MSTN* gene (also known as *GDF8*) encodes the gene for myostatin, a growth differentiation factor that inhibits

muscle growth. In natural cattle populations MSTN mutations underlie the double-muscled phenotype[105]. Successful *MSTN* knockout animals exhibit significantly higher muscle mass than those with the functional *MSTN* gene. Assessment of *MSTN* knockouts tend to vary, with some studies comparing birth weight, body weight-to-muscle mass, muscle fiber number, muscle weight, and muscle size between edited and unedited animals. Combined with several bottlenecks in efficiency of editing, comparing the outcomes of each study to another can be challenging. ZFN, TALENs, and CRISPR/Cas9 have all been utilized to achieve *MSTN*-edited animals.

Studies that assessed the phenotypes of edited MSTN pigs reported increased birth weight[102], body weight-to-muscle mass ratio equaling 170% that of unedited pigs[84], and upwards of 100% increased muscle mass[101]. Other studies reported an obvious double-muscled phenotype[85,90], or significantly larger muscles[104], but not all studies reported an assessment of the edited pig phenotype[94]. Comparatively fewer studies on MSTN knockouts have been conducted in cattle, although two performed in 2014 resulted in edited animals with an obvious double-muscled phenotype[92,100]. Faster growth[99] and increased body weight of up to 60%[83] were found in sheep, with similar results in goats[86,91,98]. Studies performed as early as 2014 reported successful editing in sheep[101], but phenotypic assessment in all cases is still lacking[100] or limited to microscopy of muscle tissue[81]. One study edited both goats and rabbits; while both exhibited increased weight ratios of biceps and quadriceps upwards of 50%, the rabbits tended to have very large tongues and low viability[82]. Other studies in goats successfully targeted another gene, *FGF5*, in addition to *MSTN*[103], or inserted an additional gene, *fat-1,* into the *MSTN* locus[95].

MSTN has also been targeted in several aquatic species, with the first heritable MSTN knockout in an aquaculture species being performed by ZFN in 2011[80]. TALENs and CRISPR/Cas9 were later used to edit carp, a tetraploid species, although severe bone defects were present in addition to enhanced muscle mass[88]. Successful editing followed in several aquaculture species such as catfish, which resulted in a 29.7% increase in catfish fry body weight[89], red sea bream, which resulted in a 16% increase in muscle mass[106], and blunt snout bream[93], which resulted in a 7% increase in body weight[97]. Outside of fish there has also been one successful *MSTN* knockout in pacific oyster, a major aquaculture bivalve[87].

While *MSTN* editing appears quite promising for improving animal yields, the drawbacks of this gene target must also be considered. For example, increased birth weight of edited animals can result in birthing challenges, and viability has been an issue in several studies[84,103]. Fine-tuning *MSTN* mutations beyond complete knockouts could serve to optimize the use of this gene as a land sparing tool. Additional targets for increasing biomass beyond MSTN should also be considered. For instance, of *MSTN* mutations, other studies in pigs have targeted knockouts for increased muscle mass[107–109].

1.4.2.2 Enhancing livestock yields through alteration of sex ratios

Livestock gene editing has also been employed to alter sex ratios of offspring. In many production schemes, only a single sex is required (i.e., female chickens in laying operations). Increasing ratios of the preferred sex in offspring stands to lower inputs and space typically allocated to rearing animals which are unfit for the desired production. An effort in chicken editing used CRISPR/Cas9 to insert a fluorescent protein into the male sex chromosomes thus facilitating high-throughput sex determination during embryogenesis[110]. A system to produce exclusively female offspring in mice by targeting exclusively male genes has been developed with potential for transferal to alternate mammalian species[111]. Ongoing works seeks to improve sex-determining and sex-biasing technologies to facilitate land and resource sparing in livestock operations[112], and avenues for increasing litter size in general are being explored: one study succeeded in mutating the *GDF9* gene to increase litter size in goats[113].

Land sparing is not the only method by which the ecological consequences of agriculture can be buffered; one alternative is land sharing[114,115]. Most of the efforts undertaken by researchers to date are intended to be compatible with a land sparing approach, however. Gene editing for increased yields of plants and animals has been considerably effective in research studies and could act to prevent the sprawl of agricultural production.

1.5 Enhancing nutrition

At the end of 2019, 690 million people worldwide were suffering from undernourishment, or the insufficient consumption of calories [116]. This pandemic stands to worsen as climate change exacerbates yield deficits and pest pressure. Much more pervasive however is malnutrition, which encompasses undernourishment as well as micronutrient deficiencies and overconsumption of calories. As of 2014, approximately 2 billion individuals suffered from micronutrient deficiencies[116].

Climate change is currently contributing to malnourishment in several ways and is predicted to worsen. More extreme climatic conditions will disrupt food chains and increase food prices, with tropical and subtropical regions experiencing the worst effects of crop yield decline[52]. Prolonged droughts, which are projected to increase, reduce root acquisition of water-soluble nutrients such as nitrate, sulfate, calcium, magnesium, and silicon. Additionally as erratic rainfall episodes worsen, more nitrate is expected to leach from soils[117].

The increased level of CO_2 will also be detrimental to nutritional quality of many crops[52,118]. Wheat grown at projected mid-to-late 21st century CO_2 levels has been found to have reduced protein, zinc, and iron[52], and similar nutrient decreases have been observed in rice, legumes, and several vegetables[118]. A modelling study predicted that climate change has placed many fruits and vegetable crops at a high risk of going extinct[119]. In addition, lack of reduced intake of fruits and vegetables caused by limited access could double the number of deaths caused by malnutrition

by 2050[119]. Projections indicate that at the current rate of CO_2 emissions, an additional 1.9% of the global population will become deficient in zinc, 1.3% will be protein deficient, and 57% of children and childbearing-aged women will live in geographies at high-risk of iron deficiencies, by 2050[120].

Gene editing may play a role in ameliorating the current and future states of the malnourishment pandemic beyond providing increased yields. Recent work applied to plants and livestock has indicated that gene editing may effectively increase desirable nutritional metabolites, reduce anti-nutrients, and alter macronutrients in ways that can be advantageous for human health (Table 4).

1.5.1 Increasing beneficial metabolites

The color pigments of crop plants such as anthocyanins, lycopene, carotenoids are known for possessing high antioxidant vitamins, necessary for improving nutrition and fighting disease. Anthocyanins help in reducing inflammation and preventing oxidative damage to cells while beta-carotene (a precursor to vitamin A) is essential for vision and other immune functions [121]. In rice, CRISPR/Cas9 editing facilitated the insertion of carotenoid biosynthesis genes in a precise genomic location to increase carotenoid accumulation[121] that could further advance earlier efforts to engineer “Golden Rice”. These fortified lines stand to benefit the poorest women and children in developing countries with rice-dominant diets, such as Bangladesh. Likewise, proanthocyanins, and anthocyanins augmentation has been pursued in rice[122] and other species. In tomato, CRISPR/Cas9 editing has been used to target a series of genes, resulting in yellow, pink and purple colored tomatoes respectively[123–125]. The insertion of a strong promoter via CRISPR/Cas9 upstream of ANT1, which codes for a Myb transcription factor, resulted in anthocyanin accumulation manifested by an intense purple color in tomatoes[123]. The Phytoene synthase gene (Psy1) and other carotenoid biosynthesis genes have also been targets of genome editing that significantly increase lycopene content in tomatoes[126]. In addition, domestication of a wild tomato using gene editing produced tomatoes with 500% increased accumulation of lycopene[79]. Dramatic enhancements of beta-carotene in banana fruit were facilitated by a CRISPR/Cas9 mediated knockout[127]. This color variation holds a great potential for improving consumers’ appeal for variety, while broadening intake of healthy pigments.

Gene editing approaches have been most commonly applied to commodity crops. However, great potential of these tools lies in improving nutrition of non-staple, regionally relevant fruits and vegetables. For example, the tools of gene editing are being applied to further domesticate and improve new “super foods” from tomato’s lesser-known relatives in the *Physalis* genus such as goldenberry and groundcherry[78]. Many of these species are rich in minerals, macro- and micronutrients and bioactive compounds such as antioxidants, vitamins A, B, and C, and have been long-used as folk remedies for diseases still relevant today[128]. Early results suggest that CRISPR/Cas9-mediated deletions of previously identified agronomic genes may improve the cultivability of nutritional berries such as the ground cherry[78], rendering it more economically viable for commercial production.

The Fatty Acid Desaturase 2 (FAD2) gene determines the levels of monounsaturated fats in most oil producing crops; gene editing tools have been used to induce mutations in this gene, leading to a significant change in their level. In the emerging oil seed crop, *Camelina sativa*, targeted CRISPR/Cas9 editing, increased total seed oleic oil composition by more than 50% in some cases while reducing linoleic and linolenic acid levels[129]. Similarly, oleic acid content has been improved in *Brassica napus*[136]. Shifts in seed oil composition are thought to be advantageous nutritionally and to extend shelf-life of oil extracts[134,135]. Applications of TALEN-mediated editing to soybean facilitated the development of a high oleic and low linolenic acid soybeans (HOLL). Oil extracted from these modified soybeans could bypass the need for hydrogenation, a process which generates unhealthy trans-fats[137]. HOLL soybeans are being developed for commercialization by Calyxt Inc. with hopes of release by 2022.
Similar efforts are underway using TALEN-mediated mutagenesis of FAD2 gene to increase oleic acid content of peanut[130].

1.5.2 Modifying macronutrients

In addition to augmenting levels of beneficial metabolites, gene editing has made improvements to macronutrients possible. In rice, knockouts of the SBEIIb gene associated with amylopectin biosynthesis decreased levels of amylopectin in favor of amylose in the grain endosperm[131]. Conversely, other food crops such as cassava have been targeted for reduced starchy content through editing two genes involved in amylose biosynthesis[132]. In strawberry, gene editing has been used to develop a continuum of altered sugar content[133]. As demonstrated, gene editing can be used to fundamentally alter the composition of macronutrients for nutritional improvements.

1.5.3 Lowering anti-nutritional compounds

Gene editing has also been applied to numerous plant species to limit the accumulation of anti-nutritional components such as phytic acid in maize, which is disruptive to the nutrition of monogastric animals [134,135]. To mitigate the deleterious effects of phytic acid on iron, zinc and calcium absorption, CRISPR/Cas9 and ZFNs were used in separate studies to mutagenize genes in the phytic acid pathway, indicating the potential utility of gene editing for anti-nutritional mitigation[135]. In sorghum, an important food crop in drought-prone areas, a major class of storage proteins called kafirins leads to a poor protein digestibility. By targeting genes that synthesize kafirins, researchers have successfully reduced kafirin levels and improved protein quality and digestibility[136]. In a similar effort, Tang *et al.* engineered a rice variety to prevent the accumulation of cadmium in rice grains using CRISPR/Cas9[137].

In chickens, gene editing has driven improvements of nutritive properties by reducing fat. Using CRISPR/Cas9 to target the GOS2 gene known to influence lipid catabolism yielded chickens with dramatically reduced abdominal fat deposition, with no observed side-effects[138]. As previously mentioned, the knock-in of fat-1 in pigs enhanced the nutritional value of pork by altering accumulation of beneficial fatty

acids[68]. Gene editing has also been used to reduce the allergenicity of globally important commodities such as milk and wheat[139–141].

In animals and crops, gene editing has been leveraged to improve the nutritional aspects of these foodstuffs. This technology has enabled micronutrient improvements, anti-nutritional component reduction, macronutrient modifications, and removal of allergens. In anticipation of the severe impacts of climate change on nutrition, gene editing could serve as a potent adaptive mechanism across a suite of commodities and nutritional traits.

1.6 Limitations and opportunities

Gene editing has already been applied successfully to plants and animals in research settings to address the effects of climate change. However, there are still significant limitations to its efficacy in enabling climate change mitigation and adaptation. One of the most prominent limitations of current applications is the narrow scope of potential solutions without the use of intra-, cis-, and transgenic approaches. In most of the examples described, researchers produce mutations of genes whose knockout contributes positively to a trait. However, it is more common that loss-of-function mutation detracts from the organism's performance. Thus, seeking to produce improved plants solely through loss-of-function mutation significantly limits the range of possible improvements. Intragenics, cisgenics, and transgenics have a proven record in research settings of improving plant performance in a range of environments and conditions[142,143]. These approaches are able to leverage a vast range of genomic sequences for use in plant and animal improvements. Gene editing in conjunction with intra-, cis-, and transgenic approaches is especially beneficial. For example, CRISPR/Cas9 was used to precisely insert a promoter present in the maize genome upstream of a gene that contributes to drought tolerance[19]. This novel recombination of genetic elements native to the maize genome via CRISPR/Cas9 in an intragenic approach improved drought tolerance. However, genetic elements from sexually incompatible organisms can also provide potent mechanisms for improving performance. In cows for instance, a knock-in of a mouse resistance gene using TALENs enhanced tuberculosis resistance[59]. Gene editing in conjunction with ICT approaches is especially beneficial. While significant improvements through loss-of-function gene editing and intragenic approaches are demonstrably effective, greater opportunity exists in leveraging the totality of genetic diversity. Thus, regulatory barriers to use of gene editing in conjunction with ICT approaches must be addressed to maximize the potential of agricultural improvements.

Opportunities to address climate change with gene editing continue to expand as new technologies emerge. For example, alternatives to the traditional Cas9 protein for editing are being developed. Base editors that facilitate precise nucleotide modifications, epigenome modifiers that alter DNA confirmation and associated expression levels, and prime editing for precise insertion of short DNA fragments are promising candidates in this regard[144–146]. Additionally, new techniques are emerging

to improve the rates of homologous recombination, a current major limitation in plants[147].

To maximize the utility of these emerging editing tools, bottlenecks in delivery must be surmounted. For livestock, the current largest barrier to editing tends to be the production of homozygous, non-mosaic, gene edited animals. Two methods currently exist to generate edited embryos prior to their transfer to a surrogate mother: one uses somatic cell nuclear transfer (SCNT) from an edited cell line to produce an edited embryo, while the other uses direct editing of a zygote. In the former case, sequencing can quickly confirm homozygous edits prior to embryo implantation, but is burdened by the inefficiency of SCNT. In the latter case, breeders can forgo SCNT but are unable to verify homozygous edits until the animal is born[148].

Similarly in plant regeneration, tissue culture is limiting in its species range and efficacy. To enable a broad spectrum of solutions in this space, tissue culture resources for a broader set of species must be developed. Tools for increasing the transformability of recalcitrant varieties and species are currently underway and show great promise for gene editing-based climate change solutions[149,150]. These emerging technologies could foster further improvements in climate change performance.

Explorations of limitations beyond technical applications are not within the scope of this review, but are germane, nonetheless. Consumer acceptance, policy frameworks, and economic feasibility will all factor into the ultimate success of the applications discussed.

1.7 Conclusion

Gene editing is an emerging and increasingly prominent approach in plants and animals applied in response to current, and anticipation of future, climate change. Solutions provided by gene editing have the potential to stand alone or be adopted concomitantly with other climate-smart solutions. The range of gene editing applications for climate change is summarized in Tables 1-4. Many of these applications have converged on traits such as disease resistance, nutritional improvements, abiotic stress tolerance and yield increases, but these are by no means representative of the breadth of opportunities that exist. Notably, there is a dearth of applications dedicated to abiotic stress tolerance. A meager 7% of total solutions have been generated for this pressing collection of stresses. The emergence and application of CRISPR/Cas9 based editing in crops and livestock has facilitated a recent increase in the application of gene editing for climate change. Despite its success in a research context, gene editing for climate change has largely not yet transitioned to real application. Adoption of these technological innovations has been stifled by regulatory barriers, social barriers, and prohibitive policies, among other externalities beyond the technical limitations described. A majority of the advances in gene editing applications for agriculture have occurred

recently, which also explains, in part, the relatively low throughput to agricultural production. The ongoing efforts of public and private institutions alike, are rapidly expanding on current technological innovations. While it should be noted that gene editing is not the sole promising agricultural improvement mechanism, the potency of this technology in providing solutions for climate change in agriculture cannot be overlooked. Gene editing, as demonstrated by the numerous studies summarized herein, stands to provide marked enhancements in agriculture to address climate change.

1.8 Glossary

Cisgenic: an organism engineered to contain genetic material derived from a sexually compatible species.

Climate adaptation: the process of adjusting to an anticipated climate and its associated conditions, including but not limited to altered temperature and precipitation.

Climate mitigation: the process of limiting the magnitude of climate change, usually by reduction or removal of greenhouse gases.

Homologous recombination: exchange of genetic material between a host organism and a desired template strand of DNA that encodes for the genetic material to be inserted flanked by regions that are complementary to adjacent sites in the host genome.

Intragenic: an organism engineered to contain only native genetic elements in a novel configuration.

Knock-in: a gene-editing approach in which genetic elements of interest are precisely inserted into a pre-defined locus.

Knockdown: a targeted mutation that reduces the expression of a gene.

Knockout: a targeted mutation that yields a non-functional gene product, synonymous with loss of function.

Promoter: the nucleotide sequence preceding a gene that specifies that gene's expression profile.

Transformation: process in which DNA is inserted into the genome of an organism.

Transgenic: an organism engineered to contain genetic material from a sexually incompatible organism.

Wild type: the typical form of a gene or organism in an unedited state.

Table 1: Summary of Gene-Editing Applications for Abiotic Stress

Species	Trait Category	Trait Targeted	Gene(s) Edited*	Method	Year published	Ref.
Banana	Abiotic stress	Semi-dwarfed	Ma04g15900 Ma06g27710 Ma08g32850 Ma11g10500 Ma11g17210	CRISPR/Cas9	2019	17
Maize	Abiotic Stress	Drought tolerance	ARGOS8	CRISPR/Cas9	2016	19
Rice	Abiotic Stress	Drought Tolerance	EPFL9	CRISPR/Cas9, CRISPR/Cpf1	2017	14
Rice	Abiotic stress	Early flowering	Hd2, Hd4, Hd5	CRISPR/Cas9	2017	
Rice	Abiotic Stress	Salt tolerance	OsRR22	CRISPR/Cas9	2019	13
Cattle	Abiotic Stress	Thermotolerance	SLICK	CRISPR/Cas9	2018	21

Table 2: Summary of Gene-Editing Applications for Disease Tolerance

Species	Trait Category	Trait Targeted	Gene(s) Edited*	Method	Year published	Ref.
Banana	Disease	Banana Streak Virus	eBSV	CRISPR /Cas9	2019	[44]
Barley	Disease	Broad Spectrum	MORC1	CRISPR /Cas9	2018	[35]
Cacao	Disease	Resistance to phytopthora	TcNPR3	CRISPR /Cas9	2018	[45]
Cassava	Disease	Cassava brown streak disease	ncbp1/2	CRISPR /Cas9	2019	[151]
Cotton	Disease	*Verticillium dahliae*	Gh14-3-3d	CRISPR /Cas9	2018	[49]
Cucumber	Disease	Broad spectrum viral resistance	eIF4e	CRISPR /Cas9	2016	[37]
Grape	Disease	Powdery Mildew	VvMLO3	CRISPR /Cas9	2020	[41]
Grapefruit	Disease	Citrus Canker	CsLOB1 promoter; CsLOB	CRISPR /Cas9	2016, 2017	[46,47]
Potato	Disease	Potato Virus Y	Coilin C-terminal	CRISPR /Cas9	2019	[48]
Rice	Disease	Bacterial blight resistance	OsSWEET14 promoter; OsSWEET11 promoter. OsSWEET13	CRISPR /Cas9	2013, 2015	[28,29]
Rice	Disease	Rice leaf blast resistance	OsERF922	CRISPR /Cas9	2016	[32]
Rice	Disease	Broad spectrum	bsr-k1	CRISPR /Cas9	2018	[34]

Rice	Disease	Rice tungro spherical virus	eIF4G	CRISPR /Cas9	2018	33
Rice	Disease	Bacterial blight	SWEET11, 13,14 promoters	TALENs	2019	29
Rice	Disease	Bacterial blight	Os8N3	CRISPR /Cas9	2019	31
Tomato	Disease	Broad spectrum	SlDMR6	CRISPR /Cas9	2016	36
Tomato	Disease	Powdery Mildew	SlMLO1	CRISPR /Cas9	2017	40
Tomato	Disease	Tomato yellow leaf curl virus	Coat and replicase protein of TYCV	CRISPR /Cas9	2018	42
Tomato	Disease	Bacterial Speck	SlJAZ2	CRISPR /Cas9	2018	43
Wheat	Disease	Powdery Mildew	TaMLOs	TALENS , CRISPR /Cas9	2014	38
Wheat	Disease	Powdery Mildew	TaEDR1	CRISPR /Cas9	2017	39
Cattle	Disease	Mastitis	Lysostaphin , hLYZ	ZFN	2013, 2014	62
Cattle	Disease	Tuberculosi s	SP110	TALE nickase	2015	59
Cattle	Disease	Leukotoxin resistance	CD18	ZFN	2016	63
Cattle	Disease	Tuberculosi s	NRAMP1	CRISPR /Cas9	2017	60
Chicken	Disease	Avian leukosis	chNHE1	CRISPR /Cas9	2020	58
Grass Carp	Disease	Grass carp reovirus	gcJAM-A	CRISPR /Cas9	2018	71
Pig	Disease	Porcine Respiratory Syndrome Virus, innate	CD163, CD1D	CRISPR /Cas9	2014	64

		immunity				
Pig	Disease	Porcine Respiratory Syndrome Virus	CD163	CRISPR /Cas9	2017	[66,67]
Pig	Disease	Classical Swine Fever	shRNA knock-in to *Rosa26* locus	CRISPR /Cas9	2018	[144]
Pig	Disease, nutrition	Classical Swine Fever, *fat 1*	fat-1 knock-in to *Rosa26* locus	CRISPR /Cas9	2018	[68]
Pig	Disease	Transmissible gastroenteritis virus	ANPEP	CRISPR /Cas9	2019	[70]

Table 3: Summary of Gene-Editing Applications for Land Sparing

Species	Trait Category	Trait Targeted	Gene(s) Edited*	Method	Year published	Ref
Maize	Land Sparing	Yield	waxy	CRISPR/ Cas9	2020	76
Tomato	Land Sparing	Yield	SlCLV3, -S, -SP.	CRISPR/ Cas9	2017	77
Ground Cherry	Land Sparing. Nutrition	Highly nutritive crop modified for improved agronomic properties	Ppr -AGO7, -SP, -SP5g	CRISPR/ Cas9	2018	78
Tomato	Land Sparing, Nutrition	Yield; Lycopene accumulation	SP, Multiflora, Ovate, Fasciated, Fruit Weight 2.2, Lycopene Beta Cyclase	CRISPR/ Cas9	2018	78
Rice	Land Sparing	Yield	DEP1, Gn1A	CRISPR/ Cas9	2018	75
Rice	Land Sparing	Yield	PYL 1,4,6	CRISPR/ Cas9	2018	73
Rice	Land Sparing	Yield	OsGs3, OsGW2, OsGn1A	CRISPR/ Cas9	2019	74
Wheat	Land Sparing	Yield	GW2, LPX-1, MLO	CRISPR/ Cas9	2018	152

Blunt snout sea bream	Land Sparing	Yield	mstna, mstnb	CRISPR/ Cas9	2020	97
Carp	Land Sparing	Yield	sp7, MSTN	CRISPR/ Cas9	2016	88
Catfish	Land Sparing	Yield	MSTN	CRISPR/ Cas9	2017	89
Cattle	Land Sparing	Yield	MSTN	ZFN, TALENs	2014, 2015	92,100
Chicken	Land Sparing	Yield	G0S2	CRISPR/ Cas9	2019	138
Chicken	Land Sparing	Sex-determination	Fluorescent protein into sex chromosome	CRISPR/ Cas9	2019	110
Goat	Land Sparing	Yield	MSTN, FGF5	CRISPR/ Cas9	2015	103
Goat	Land Sparing	Yield	MSTN	TALENs, CRISPR/ Cas9	2016, 2016, 2018	82,86,95
Goat	Land Sparing	Litter size	GDF9	CRISPR/ Cas9	2018	113
Goat	Land sparing, disease	Yield	Fat-1 into MSTN	CRISPR/ Cas9	2018	95
Oyster	Land Sparing	Yield	MSTN	CRISPR/ Cas9	2019	87
Pig	Land Sparing	Yield	MSTN	TALENs, ZFN, CRISPR/ Cas9	2016, 2018, 2015, 2016, 2016, 2017	90,94,101,104

Pig, Buffalo	Land Sparing	Yield	MSTN	CRISPR/ Cas9	2018	94
Pig	Land Sparing	Yield	IGF2	CRISPR/ Cas9	2018, 2019	107,109
Pig	Land Sparing	Yield	FBXO40	CRISPR/ Cas9	2018	108
Rabbit	Land Sparing	Yield	MSTN	CRISPR/ Cas9	2016	82
Red sea bream	Land Sparing	Yield	MSTN	CRISPR/ Cas9	2018	93
Sheep	Land Sparing	Yield	MSTN	CRISPR/ Cas9, TALENs	2015, 2014, 2015, 2016, 2019	83,96,99,100
Yellow Catfish	Land Sparing	Yield	MSTN	ZFN	2011	80

Table 4: Summary of Gene-Editing Applications for Nutrition

Species	Trait Category	Trait Targeted	Gene(s) Edited*	Method	Year published	Ref.
Banana	Nutrition	Increased beta-carotene	LCYε	CRISPR/Cas9	2020	127
Brassica napus	Nutrition	Increased oleic acid content	FAD2	CRISPR/Cas9	2018	
Camelina sativa	Nutrition	reductions of linoleic acid and linolenic acid	FAD2	CRISPR/Cas9	2017,2017	129,153
Cassava	Nutrition	Reduced starch	PTST1, GBSS	CRISPR/Cas9	2018	132
Maize	Nutrition	Reduced phytate levels	IPK1	ZFN	2009	134
Maize	Nutrition	Reduced phytic acid	ZmPDS, ZmIPK1, ZmIPK, ZmMRP4	TALENs, CRISPR/Cas9	2014	135
Peanut	Nutrition	Increased oleic acid content	FAD2	TALENs	2018	130
Potato	Nutrition	Reduced starch	GBSS	CRISPR/Cas9	2017	
Rice	Nutrition	Increased amylose	SBEI, SBEIIb	CRISPR/Cas9	2017	131
Rice	Nutrition	Prevented cadmium uptake	OsNramp5	CRISPR/Cas9	2017	137

Rice	Nutrition	Increased carotenoids	GR-1 &GR-2 carotenoid biosynthesis cassettes inserted in GSHs	CRISPR/Cas9	2020	154
Sorghum	Nutrition	Reduced kafirins	K1C genes	CRISPR/Cas9	2018	136
Soybean	Nutrition	Altered oil levels	FAD2-1A, FAD2-1B, FAD3A	TALENs	2016	155
Strawberry	Nutrition	Altered sugar content	FvebZIPs1.1	CRISPR/Cas9	2020	133
Tomato	Nutrition	Increased anthocyanin levels	ANT1, PSY1	TALENs; CRISPR/Cas9	2015	123
Wheat	Nutrition	Low gluten wheat for reduced allergenicity	Alpha-gliadin array, Gli-2 locus	CRISPR/Cas9	2018	141
Cattle	Nutrition	Reduction of milk allergen	BLG	ZFN	2011	139
Cattle	Nutrition	Reduction of milk allergen	LacS	TALENs	2018	140
Chicken	Nutrition	Less abdominal fat deposition	G0S2	CRISPR/Cas9	2018	138

*All edits are in coding regions of genes listed unless otherwise indicated

Figure 1

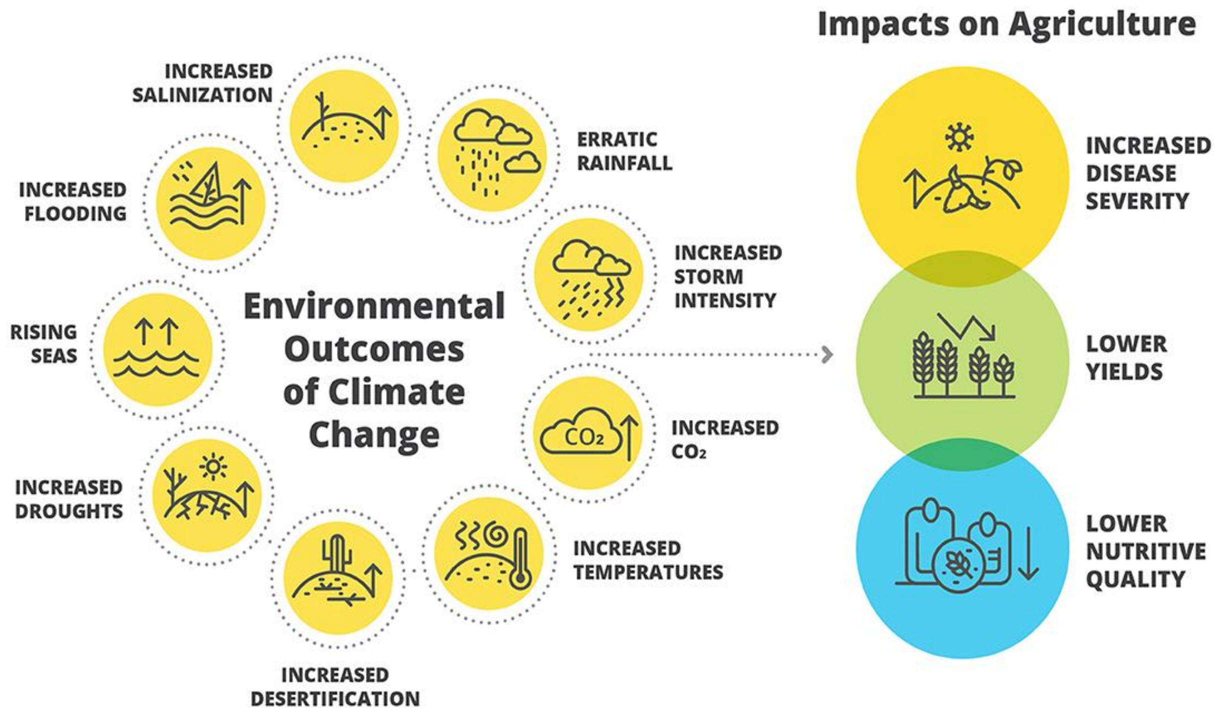

Figure 1| Climate change will negatively impact food systems.

Figure 2:

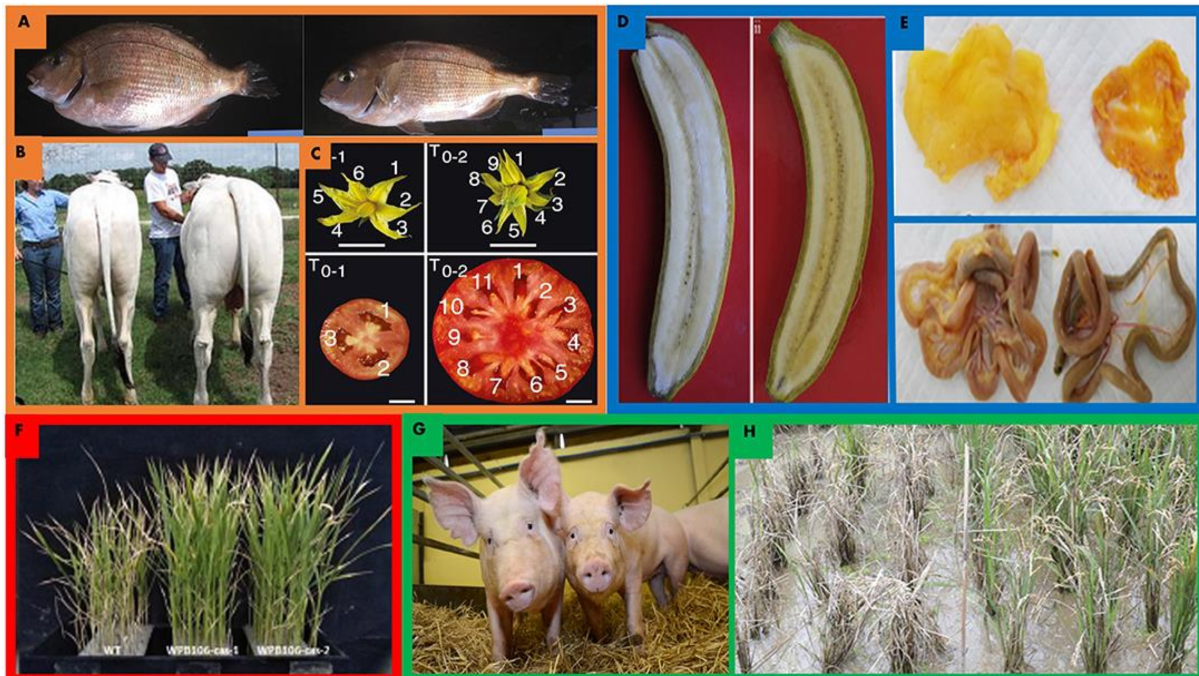

Figure 2| Examples of gene edited crops and livestock.

Gene editing improvements for abiotic stress tolerance are outlined in red. land sparing in orange, nutrition in blue, and disease in green. Improvements to saline tolerance in rice. Knockout of OsRR22 enhances yield in high saline environments. Wild type (left) and edited rice (right) grown in 0.75% saline solution. Wild type plants are 13% shorter than edited plants in saline conditions[13]. B, C.) MSTN gene edits in livestock enhance muscle yields in a variety of organisms B.) CRISPR/Cas9 mediated MSTN edited red sea bream (left) and wild type (right)[107] . Edited red sea bream exhibited 16% skeletal muscle mass increases on average C.) TALEN enabled MSTN edited cow (right) and wild type (left) exhibit increased overall mass and greater muscle mass[101]. D.) CRISPR/Cas9 promoter editing of tomato CLV3, S, SP facilitated novel variation and enhancements to fruit size, floral architecture and overall architecture in tomato. Edited tomato (right) exhibits enlarged fruit size and increased locule number.[81] E.) CRISPR/Cas9 edited LCYε enhanced beta-carotene accumulation in edited banana (right) relative to wild type (Left) by nearly sixfold in some lines[132]. F.) CRISPR/Cas9 mediated editing of G0S2 in chicken (right) accumulates less abdominal and gastrointestinal fat.[148] G.) CD163 locus edited by CRISPR/Cas9 yielded gene edited pigs that are entirely resistant to porcine reproductive and respiratory virus when challenged with the virus [65,66] H.) bsr-k1 edited rice in field (right) greatly outperforms wild type (left) after being challenged with rice blast. Edited lines performed 50% better than wild type in field after inoculation[35].

Figure 3

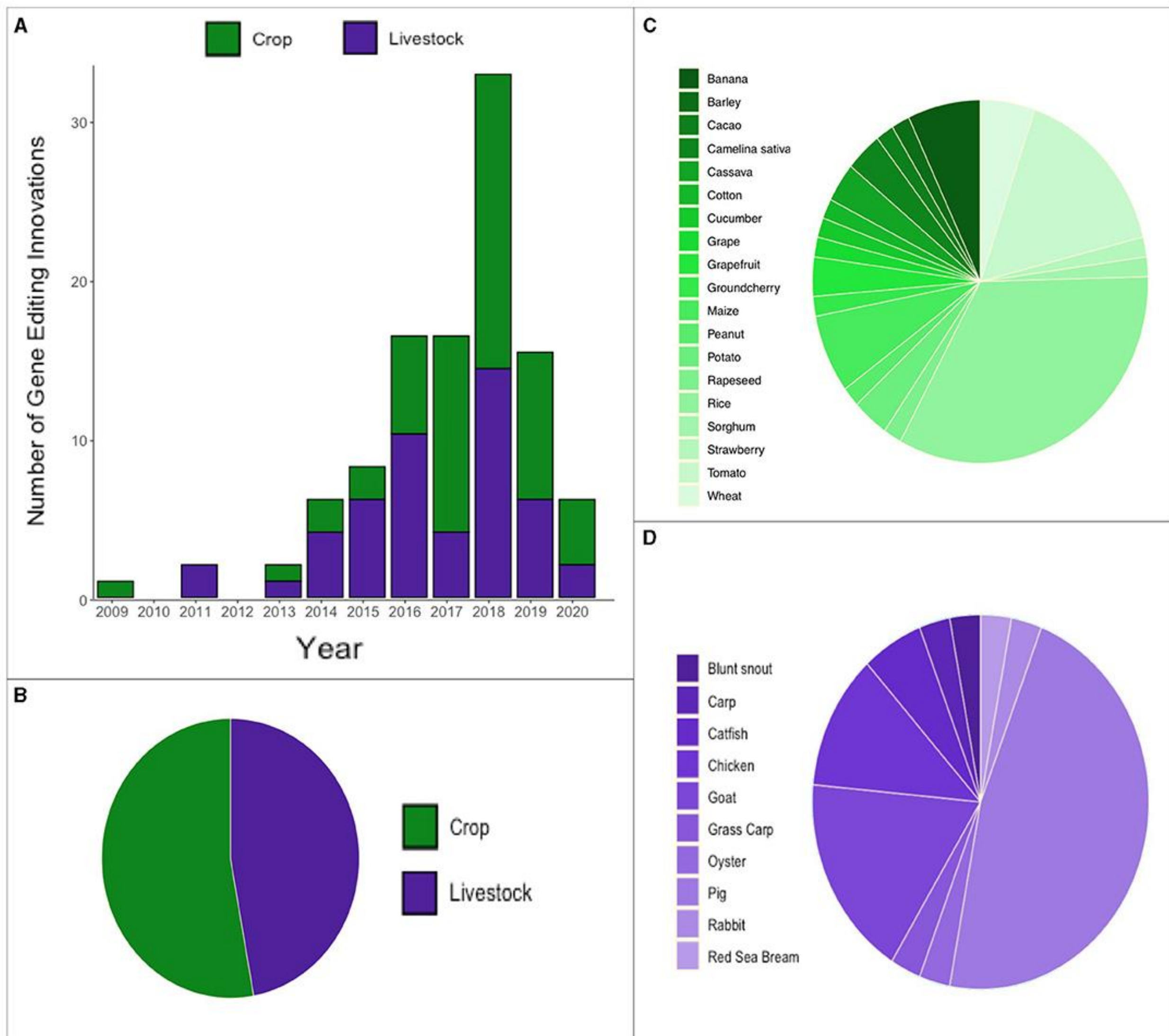

Figure 3 | Overview of gene editing innovation emergence over time and species. Annual count of gene editing innovations since 2009. B. Proportion of gene editing innovations B.) in crops and livestock C.) a diverse array of crops D.)a diverse array of livestock

Chapter Two

Paralog editing tunes rice stomatal density to maintain photosynthesis and improve drought tolerance

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2.1 Introduction

Prolonged periods of drought and increased desertification are anticipated to become more prevalent in the next century[52]. Climate change modeling predicts increases in global temperatures by 2-4 °C by the end of the 21st century. Increased temperatures alone and in combination with limited water will negatively impact crop yields[156–158]. The development of climate change adapted crops is essential to maintain crop yields in the face of rapid global population growth and worsening climates.

Rice (*Oryza sativa)* is one of the most widely consumed crops globally and trails only wheat and maize in area harvested[159]. Originally domesticated in semi-aquatic habitats, rice is especially sensitive to drought relative to other C3 cereal crops[160–162]. Future water limitations may necessitate transitions of fully flooded paddy fields to water-saving production schemes[158]. Rain-fed production, which comprises about 45% of total rice grown, is particularly susceptible to drought as a result of unpredictable precipitation[163–165]. Furthermore, most regions where irrigated rice is produced are currently experiencing or projected to experience water scarcity[164]. Thus, all rice, regardless of production methods, would benefit from improvements that maintain yields with less water.

Stomata are at the nexus of plants and the atmosphere. They facilitate gaseous exchanges of carbon dioxide, oxygen, and water vapor. Most water loss in crops occurs via transpiration from the stomata[166]. Breeding efforts have shown drought adaptation in rice was facilitated in part by reductions of stomatal density[167]. Thus, opportunity exists, especially in non-adapted cultivars, to fine tune stomatal density reductions.

The developmental biology of stomata has been studied extensively in myriad species[168–171]. Despite similar sequence identities, EPIDERMAL PATTERNING FACTORs (EPFs) and EPF-LIKE proteins (EPFLs) mediate opposing downstream stomatal development responses. *EPF1* and *EPF2* function as negative regulators expressed in stomatal-lineage cells. In contrast, *EPFL9*, also known as *STOMAGEN*, is

a positive regulator of stomatal development that is dynamically expressed in the mesophyll[14,172–177]. These mobile peptides regulate cell fate transitions and cell divisions to ensure proper spacing and number of stomata[172,174–178].

EPFL9 is composed of three distinct regions: an N-terminal signal peptide region, a pro-peptide region, and a C-terminal cysteine-rich active peptide region[172,179]. The full-length peptide is processed in vivo to yield a 45-residue C-terminal active peptide[172,178]. The active peptide encoded by *EPFL9* possesses the conserved cysteine residues of *EPF1* and *EPF2* and binds the same *ERECTA* (ER)-family receptors and co-receptor TOO MANY MOUTHS (*TMM*) in *Arabidopsis thaliana*[172,174,178,180]. A knockout of *EPFL9* in rice using CRISPR/Cas9 and CRISPR/Cpf1 yielded an eight-fold reduction in abaxial stomatal density in the IR64 background[14] Likewise, a knockout of *EPFL9-1* in *Vitis vinifera* reduced stomatal density significantly[181].

Interestingly, *EPFL9* appears to have undergone a duplication event in grasses[180]. Transgenic overexpression of rice *STOMAGEN* and its duplicate, previously named *EPFL9-2*, in *A. thaliana* revealed a shared, though attenuated, function of *EPFL9-2* as a positive regulator of stomatal development when ectopically expressed[182]. Ectopic expression of *Brachypodium distachyon* and *Triticum aestivum* (wheat) *STOMAGEN* and *STOMAGEN* paralogs in *A. thaliana* resulted in similar stomatal density increases[183]. In contrast, overexpression of negative regulators of stomatal development reduced stomatal density. Stomatal density reductions improved water use efficiency in *A. thaliana*, wheat, barley, and rice[15,184–188]. However, all stomatal density reductions achieved by overexpressing negative regulators of stomatal development to any extent also reduced stomatal conductance and carbon assimilation under physiologically relevant light conditions[184,185,188]. For example, rice lines overexpressing *EPF1* to reduce stomatal densities exhibited lower stomatal conductance and carbon assimilation at all light conditions that exceeded 1,000 μmol photons m^{-2} s[15]. These reductions may be detrimental as stomatal conductance has been shown to be essential for crop productivity[189–196]. In rice specifically, higher stomatal conductance has been associated with greater rates of leaf photosynthesis[190,192,197]. Also, of note, all previously tested strategies relied on the use of constitutive transgenic overexpression, lacking the tissue specificity and gene dosage that may regulate stomatal density phenotypes *in vivo*.

Gene editing of rice *STOMAGEN* and its duplicate were used as an alternative, non-transgenic, approach to tune stomatal density. Gene duplication events can provide genetic material for functional novelty[198], but may also disrupt optimal levels of gene expression, requiring significant evolutionary time to stabilize[199]. Gene editing of paralogs can provide a potent and straightforward mechanism to generate desirable variation in a wide array of traits. For example, knockout alleles of individual genes in a set of paralogs underlying disease resistance and yield in tomato and maize, respectively, has demonstrated the utility of paralog gene editing for generating desirable trait variation[36,200].

Here, we report novel characterization of the rice *STOMAGEN* paralog *EPFL9-2*, subsequently referred to as *EPFL10*, and its relationship to stomatal development. Furthermore, we explore the effect of the reductions in stomatal density in *stomagen* and *epfl10* on stomatal conductance, carbon assimilation, thermal regulation, water conservation, and yield in varying water regimes. We describe the use of paralog editing for achieving desirable variation in traits of interest and the implications of this work on future gene-editing strategies for improved water-use efficiency.

2.2 Methods

2.2.1 Plant Growth conditions:

Rice cultivar Nipponbare (*O. sativa* ssp. japonica) seeds were germinated and grown for 8 days in a petri dish with 20 mL of water in a Conviron growth chamber at 28°C for day-length periods of 16 hours in 100 μmol photons m-2s-1 of light and 80% relative humidity. Seedlings were transferred to a soil mixture comprised of equal parts turface (https://www.turface.com/products/infield-conditioners/mvp) and sunshine mix #4 (http://www.sungro.com/professional-products/fafard/).

Germinated seedlings used for stomatal phenotyping and growth chamber physiological assays were transferred to 10 cm, 0.75 L McConkey tech square pots and placed in growth chambers at 28°C for day-length periods of 16 hours in 400 μmol photons m-2s-1 of light and 80% relative humidity
.

Plants designated for yield trials, greenhouse physiological assays, and stomatal aperture measurements were moved to the greenhouse with temperature setpoints of 27°C day/22°C night at ambient light conditions in February 2020 with day lengths of 12 hours in 15.2 cm, 1.8 L pots. All plants were fertilized with 125 mL of 1% w/v iron solution one-week post-transplant. 1 L of 5% w/v JR Peter's Blue 20-20-20 fertilizer (https://www.jrpeters.com/) was added to each flat at 3- and 11-weeks post-germination.

2.2.2 Yield and water regimes:

Adapted from Caine et. al[15], grain and biomass yield in three watering regimes were tested: well-watered, vegetative drought, and reproductive drought. Well-watered flats were kept flooded for the entirety of the growth period. Vegetative drought was imposed by removing all water from flats containing pots for 7 days starting on day 28 after germination and for 9 days at day 56 after germination. In reproductive drought, water was removed from flats for 4 days at day 98 when panicles were undergoing grain filling. All grain and aboveground biomass from well-watered, vegetative and reproductive drought plants were harvested after 167 days, 177 days, and 181 days, respectively. Biomass measurements were completed on samples dried at 60°C for three days prior to weighing.

2.2.3 Generation of edited lines:

Guides for targeting *EPFL10* and *STOMAGEN* were selected to minimize off-target effects and maximize on-target efficiency in the first exon of the coding region. Guide

sequences were selected using CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/). Forward and reverse strand guide sequences with relevant sticky ends amenable for Golden Gate cloning were ordered from Integrated DNA Technology (IDTdna.com). Equal volumes of 10 mM primers were annealed at room temperature. Golden Gate cloning was used to insert guides into the PeGM entry clone containing the tracrRNA and U3 promoter. LR clonase reactions were used to insert the entry clone into destination vectors for biolistic transformation and *Agrobacterium*-mediated transformation. *epfl10* lines were produced via *Agrobacterium*-mediated transformation and *stomagen* lines via biolistic transformation. The vector maps are available as Supplemental Figures 7,8.

2.2.4 Plant material and culture of explants:
Mature seeds of rice (*O. sativa* L. japonica cv. Nipponbare) were de-hulled, and surface-sterilized for 20 min in 20% (v/v) commercial bleach (5.25% sodium hypochlorite) and 1% of Tween 20. Three washes in sterile water were used to remove residual bleach from seeds. De-hulled seeds were placed on callus induction medium (CIM) medium [N6 salts and vitamins[201], 30 g/L maltose, 0.1 g/L myo-inositol, 0.3 g/L casein enzymatic hydrolysate, 0.5 g/L L-proline, 0.5 g/L L-glutamine, 2.5 mg/L 2,4-D, 0.2 mg/L BAP, 5 mM $CuSO_4$, 3.5 g/L Phytagel, pH 5.8] and incubated in the dark at 28° °C to initiate callus induction. Six- to 8-week-old embryogenic calli were used as targets for transformation.

***2.2.5 Agrobacterium*-mediated transformation:**
Embryogenic calli were dried for 30 min prior to incubation with an *Agrobacterium tumefaciens* EHA105 suspension (OD_{600nm} = 0.1) carrying the binary vector for editing rice *EPFL10*. After a 30 min incubation, the *Agrobacterium* suspension was removed. Calli were then placed on sterile filter paper, transferred to co-cultivation medium [N6 salts and vitamins, 30 g/L maltose, 10 g/L glucose, 0.1 g/L myo-inositol, 0.3 g/L casein enzymatic hydrolysate, 0.5 g/L L-proline, 0.5 g/L L-glutamine, 2 mg/L 2,4-D, 0.5 mg/L thiamine, 100 mM acetosyringone, 3.5 g/L Phytagel, pH 5.2] and incubated in the dark at 21 ºC for 3 days. After co-cultivation, calli were transferred to resting medium [N6 salts and vitamins, 30 g/L maltose, 0.1 g/L myo-inositol, 0.3 g/L casein enzymatic hydrolysate, 0.5 g/L L-proline, 0.5 g/L L-glutamine, 2 mg/L 2,4-D, 0.5 mg/L thiamine, 100 mg/L timentin, 3.5 g/L Phytagel, pH 5.8] and incubated in the dark at 28ºC for 7 days. Calli were then transferred to selection medium [CIM plus 250 mg/L cefotaxime and 50 mg/L hygromycin B] and allowed to proliferate in the dark at 28 ºC for 14 days. Well-proliferating tissues were transferred to CIM containing 75 mg/l hygromycin B. The remaining tissues were subcultured at 3-to-4-week intervals on fresh selection medium. When a sufficient amount (about 1.5 cm in diameter) of the putatively transformed tissues was obtained, they were transferred to regeneration medium [MS salts and vitamins[202], 30 g/L sucrose, 30 g/L sorbitol, 0.5 mg/L NAA, 1 mg/L BAP, 150 mg/L cefotaxime] containing 40 mg/L hygromycin B and incubated at 26 °C, 16-h light, 90 µmol photons m^{-2} s^{-1}. When regenerated plantlets reached at least 1 cm in height, they were transferred to rooting medium [MS salts and vitamins, 20 g/L sucrose, 1 g/L myo-inositol, 150 mg/L cefotaxime] containing 20 mg/L hygromycin B and incubated at 26 ºC under conditions of 16-h light (150 µmol photons m^{-2} s^{-1}) and 8-h dark until roots were

established and leaves touched the Phytatray™ II lid (Sigma-Aldrich, St. Louis, MO, USA). Plantlets were then transferred to soil.

2.2.6 Biolistic-mediated transformation:
Embryogenic callus tissue pieces (3–4 mm) were transferred for osmotic pretreatment to CIM medium containing mannitol and sorbitol (0.2 M each). Four hours after treatment with osmoticum, tissues were bombarded as previously described[203] with modifications. Two mg of gold particles (0.6 µm), coated with 5 mg of plasmid DNA for editing rice STOMAGEN were divided equally among 10 macro-carriers and used for bombardment with a Bio-Rad PDS-1000 He biolistic device (Bio-Rad, Hercules, CA, USA) at 650 psi. Sixteen to 18 h after bombardment, tissues were placed on osmotic-free CIM and incubated at 28 °C under dim light (10-30 µmol photons m^{-2} s^{-1}, 16-h light). After 7 days, tissues were transferred to a selection medium (CIM containing 50 mg/l hygromycin B) and grown using the same procedure as described above, without timentin or cefotaxime supplemented in the media.

2.2.7 Validation of edits:
T_0 plants targeted for edits in Os*EPFL10* and Os*STOMAGEN* were evaluated using PCR to amplify the region of interest. PCR products were Sanger sequenced. Sequence data was analyzed using the Synthego ICE tool (https://ice.synthego.com/#/) to detect alleles present[204]. Only lines with homozygous frame-shift mutations were retained for downstream experiments. Azygous T_2 plants were used for experimental data collection to minimize somaclonal variation, which may have accumulated during tissue culture[205,206].

2.2.8 Phenotyping stomatal density, size, and aperture:
Stomatal densities were recorded from epidermal impressions of leaves using nail polish peels[190]. Stomatal densities of eight biological replicates of each leaf were taken from the widest section of fully expanded leaves. Images were taken using a Leica DM5000 B epifluorescent microscope at 10x magnification. Three images were collected per stomatal impression and density per image was averaged. The number of stomata in a single stomatal band were counted and the area of each band was measured[207]. Stomatal densities were calculated by dividing stomatal counts by stomal band area (mm^{-2}). Stomatal densities of fifth leaf abaxial tissues were assayed for each allele of *epfl10* and *stomagen*. All subsequent phenotyping was conducted on plants of pooled alleles.

Epidermal peels of 21-day old plants were produced using a razor blade on the adaxial leaf to remove tissues above the abaxial epidermal layer. Images of individual stomata at 100x magnification were captured. Guard cell length was measured using ImageJ. 35 individual stomata from five biological replicates of each genotype were measured.

Stomatal aperture measurements were generated using epidermal peels of flag leaves from 85-day old plants. Leaves were harvested at 1:00 p.m. and peels were generated immediately. Epidermal peels were then fixed by submerging in 4% formaldehyde for 30

seconds using a method adapted by Eisele et al.[208]. Images of 20 individual stomata from six biological replicates of each genotype were measured.

Confocal microscopy images were captured using a Zeiss LSM 710 on epidermal peels of each genotype stained with propidium iodide for 40 seconds and immediately washed in water. Images were processed using Bitplane's Imaris.

2.2.9 Quantifying OsSTOMAGEN and OsEPFL10 transcript abundance:
Total RNA was extracted from seedlings with the Qiagen Total RNAeasy Plant Kit at three developmental stages: eight days after germination, 15 days after germination from the basal 2.5cm above the leaf sheath of the fifth expanding leaf, and from the fully expanded leaf of 21-day-old leaves. RNA quality was validated on an agarose gel prior to reverse transcription using the QuantiTect™ reverse transcription kit to generate first-strand cDNA. Quantitative reverse transcription PCR was performed using FAST SYBR on Applied Biosystem's QuantStudio 3 thermocycler. Relative expression levels were calculated by normalizing to the rice *UBQ5* housekeeping gene (LOC_Os01g22490)[209]. Primers used for qPCR listed in Supplemental Table 1. Relative log fold expression was calculated using the $2^{-\Delta\Delta CT}$ method using OsSTOMAGEN in adult leaves as the control group.

2.2.10 Determining methylation profile of genes of interest:
Methylation profiles of rice genes of interest were viewed using the Plant Methylation Database (https://epigenome.genetics.uga.edu/PlantMethylome/)[210,211]. Snapshots of CHH and CHG methylation 1.5 kb upstream of the start codon and 1.5 downstream of the stop codon were taken. The methylation data was collected on 3-week-old rice leaf tissues.

2.2.11 Evolutionary analysis of STOMAGEN paralogs:
Single-copy orthologs were searched using BUSCO v4.0.6[212] and the viridiplantae_odb10 database for the species used in this study (Table S1). 82 orthologous groups present in at least 23 species were individually aligned with MAFFT v7.487 (--maxiterate 1000 --globalpair)[213]. All multiple sequence alignments were concatenated, trimmed with TrimAl v1.4.rev15 (-gt 0.2)[214]and then used to infer a species tree with FastTree v2.1.10[215]. The copy number variations of the *STOMAGEN* family was determined by searching for the stomagen domain (PF16851) from the protein annotation sets with hmmsearch v3.3[216,217] or from the genomes with exonerate v2.2.0[218] if genome annotations are absent. To understand the sequence variations of STOMAGEN and *EPFL10* orthologs at the species and family level, we collected non-redundant *Oryza* or Poaceae species that have single copies of *STOMAGEN* or *EPFL10*. The stomagen domain of *STOMAGEN* or *EPFL10* orthologs was aligned with MAFFT, and the filtered alignment was used to compute normalized Shannon's entropy. Gaps were ignored.

2.2.12 Photosynthesis and stomatal conductance assays:

Light response curves were generated using a LI6800 infrared gas analyzer (LI-COR, Lincoln, NE, USA) with chamber conditions set to: leaf temperature 25°C; flow rate 500 μmol s^{-1}; water vapor pressure deficit 1.8 kPA ; and CO_2 concentration of sample 400 μmol mol^{-1}. Light intensity was first increased to 2000 μmol photons $m^{-2}s^{-1}$, and with steady-state waiting times of 5 to 10 minutes, subsequently decreased to 1500, 1200, 1000, 750, 500, 300, 200, 100, and 50 μmol photons $m^{-2}s^{-1}$ m s light. Light was composed of at least 90% red light and at maximum 40 μmol photons m^{-2} s^{-1} blue light to match equipment specifications. Measurements were taken on fully expanded fifth leaves of 32-day-old plants grown in the greenhouse. Anatomical g_{smax} was calculated using the double end-corrected version of the Franks and Farquhar[219] equation from Dow et al.[220] as described in Caine et. al[15].

Physiological assays in Figures 3a, 3c were conducted on fully expanded leaf 5 of 21-day old plants. Stomatal conductance and CO_2 assimilation data for Fig 3C was captured using an infrared gas analyzer (LI6400XT, LI-COR, Lincoln, NE, USA) with chamber conditions set to light intensity 1000 μmol photons m^{-2} s^{-1} (90% red light, 10% blue light); leaf temperature 27°C; flow rate 500 μmol s^{-1}; relative humidity 40%; and CO_2 concentration of sample 400 μmol mol^{-1}.

2.2.13 Thermal imaging:
Thermal images were captured using a FLIR E8-XT Infrared Camera (FLIR-DIRECT, Wilmington, NC, USA). Images of well-watered and vegetative drought plants were taken 65 days after germination on the last day of the vegetative drought treatment. Images of reproductive drought plants were captured 102 days post-germination on the last day of the reproductive drought treatment. Images were captured between 1:00 p.m. and 2:00 p.m. to represent the effects of transpiration-mediated cooling during the hottest part of the day. Images were processed using FLIR Thermal Studio. Leaf temperatures of 4-6 leaves per biological replicate were quantified.

2.2.14 Water loss:
Two plants of identical genotype were placed in a 10”x10” flat and were covered with aluminum foil to minimize evaporation from soil. Non-plant evaporation was estimated by measuring daily water loss from covered flats containing pots without plants. These control flats were distributed within the greenhouse assay area to reflect environmental variation. Daily water loss of each flat was calculated by taking the difference of flats with plants and the average of the flats without. Eight replicate flats for each genotype were measured daily from 70-77 days after germination. Pots remained well watered during the course of this experiment. Water loss was recorded, and flats were replenished, daily.

2.2.15 Graphs and statistics:
All graphs were produced using the ggplot2 package in R studio[221]. All statistics were calculated in R-studio using Tukey honest significant difference (HSD) post-hoc tests.

2.3 Results

2.3.1 Evolution and regulation of STOMAGEN and EPFL10 suggests functional differences of paralogs

Previous work has suggested the Os*STOMAGEN* paralog Os*EPFL10* (LOC_Os08g41360) may function as a weak, positive regulator of stomatal development[182,183], having arisen from a putative duplication event in the most recent common ancestor of the Poaceae species[180]. We examined evolutionary divergence of *STOMAGEN* and *EPFL10* that may explain such functional differences. The two paralogs have accumulated divergent point mutations. In rice, homology cannot be detected between *STOMAGEN* and *EPFL10* across the N-terminal signal peptides and pro-peptide domain. The functional 45 C-terminal sequences show 73% sequence identity with 12 non-synonymous substitutions (Fig. 1C). These positions were mapped onto the existing complex structure of *TMM*, *ERL1* and *EPF1* (Fig. 1D), assuming the binding of STOMAGEN and EPFL10 may be similar to *EPF1*. Many positions highlighted with non-synonymous substitutions were in proximity to the receptors, possibly suggesting that the paralogs may have different binding affinity to the receptors.

The phylogenetic tree also indicated that the two orthologous groups may have experienced differential selective pressure (Fig. 1A). To gain better insights, we calculated sequence variations of the *STOMAGEN* and *EPFL10* orthologs with greater numbers of sequences collected from the *Oryza* species and the Poaceae family members (Fig. 1C; Table S1; Table S2). Consistent with the phylogeny, both species- and family-level sequence diversity was higher in *EPFL10* orthologs, indicating relatively rapid divergence across the species. On the contrary, STOMAGEN orthologs have maintained a high level of sequence conservation across the species and family, suggesting their sequence evolution is strictly constrained. Furthermore, regulatory variations existed between *STOMAGEN* and *EPFL10*. Repressive CHG and CHH methylation marks vicinal to the *EPFL10* coding region suggested regulatory restriction, while this was not observed for *STOMAGEN*. Concordantly, we observed that *STOMAGEN* mRNA abundance also greatly exceeded *EPFL10* expression in leaf base tissues where stomatal development occurs and *STOMAGEN* and *EPFL10* expression is greatest (Fig. 1B; Supplemental Table S1). There is minimal expression of either transcript in adult leaf where stomatal complexes are fully matured (Fig 1B). Collectively, the sequence evolution and regulation of *STOMAGEN* and *EPFL10* suggested possibly differential evolution and usage of the paralogs. This suggested that *EPFL10* may be a better candidate to fine-tune stomatal development without abolishing the role of *STOMAGEN*, the primary positive regulator of stomatal development.

2.3.2 Stomatal density and morphology are altered in knockout lines

To clarify the functional role of Os*STOMAGEN* and Os*EFPL10* in regulating stomatal density *in vivo*, single and double mutant lines were generated in rice (*Oryza sativa* cv. Nipponbare). CRISPR/Cas9-mediated knockout of Os*STOMAGEN* and Os*EPFL10* was achieved by targeting guides to the first exon of each gene to disrupt the open reading

frame (Fig. 1E and 1F). Two unique homozygous knockout alleles were generated in Os*EPFL10* and in Os*STOMAGEN* in the T_0 generation using a single guide sequence adjacent to a PAM motif (Fig. 1E, 1F).

epfl10 exhibited reductions in stomatal densities which represented 80% of wild-type densities, whereas *STOMAGEN* possessed only 25% of wild-type densities in the fifth fully expanded adult leaf (Fig. 2A, Fig. S1). Similar proportions of stomatal density reductions in mutants were measured in flag leaves and their adaxial leaf surfaces (Fig. 2B and 2C). Stomatal length was measured to determine if there was a relationship between stomatal density reductions and size increases in the Nipponbare background. The mean guard cell length of stomata in *stomagen* were longer relative to *epfl10* and wild type (Fig. 2D). Representative confocal microscopy captured stomatal density relationships in a single biological replicate of each genotype (Fig. 2E). The stomatal density of stomagen *epfl10* double mutants could not be distinguished from *stomagen* and were thus excluded from future physiological analyses (Fig. S6). Collectively, our data suggest that Os*EPFL10* plays a functional role in rice stomatal formation, but the loss of Os*STOMAGEN* is epistatic to *EPFL10*-mediated stomatal development.

2.3.3 Stomatal density reductions are concomitant with gas exchange reductions in *stomagen* but not *epfl10*

The assayed genotypes revealed anatomical level differences in stomatal densities and length. To determine if these developmental differences corresponded to alterations in carbon assimilation (A_n) and stomatal conductance (g_s), the efficiency of gas-exchange was measured across genotypes in response to increasing incident light.

At ambient CO_2 (400 ppm), *epfl10* maintained wild-type levels of A_n and g_s, whereas *stomagen* exhibited reduced gas exchange capacity in light response curves relative to *epfl10* and wild type at all light intensities greater than 1,000 µmol photons m^{-2} s^{-1} (Fig. 3A and 3B). In an independent cohort of plants assayed at saturating light (1,000 µmol photons m^{-2} s^{-1}), *stomagen* steady-state A_n and g_s were lower relative to wild-type and *epfl10* grown in growth chambers (Fig.S3, S4). Similar reductions of A_n and g_s in *stomagen* lines were recapitulated in greenhouse measurements at 1,000 µmol photons m^{-2} s^{-1} (Fig. S6). However, under vegetative drought *stomagen* did not exhibit lowered levels of carbon assimilation at 1,000 µmol photons m^{-2} s^{-1}, despite reduced levels of stomatal conductance (Fig. S5c-d).

Measurements of stomatal apertures on the abaxial side of flag leaves indicated that *epfl10* lines maintained a larger stomatal aperture than the wild type, and *stomagen* lines exhibited an even greater aperture (Fig. 3C and 3D). Thus, *EPFL10* and *STOMAGEN* maintained even greater levels of stomatal conductance per individual stoma mediated by a larger aperture in well-watered conditions (Fig. 3E). Increased stomatal aperture at reductions in stomatal density of 25% but not 80% were able to sustain wild-type levels of steady-state A_n and g_s.

2.3.4. Decreasing stomatal density increases ratio of theoretical stomatal conductance to operating stomatal conductance

Despite similar operating stomatal conductance, *epfl10* and WT significantly differ in their theoretical maximum stomatal conductance. This is in contrast to *stomagen* which had both significantly lower operating and anatomical stomatal conductance. Thus, reductions in stomatal density result in a stomatal conductance that operates at a greater proportion of its theoretical maximum for both *stomagen* and *epfl10*, though the latter is able to compensate to sustain WT gas exchange (Fig 2A, 2B).

2.3.5 *EPFL10* maintains wild-type thermoregulation and yield while increasing water conservation

Measurements of A_n and g_s and g_{smax} revealed differences in the regulation of gas exchange among wild type, *epfl10*, and *stomagen*. To further resolve the lifetime implications of these edits, we assessed differences in thermoregulatory, yield, and water conservation capacities.

Thermal imaging was used to assess evaporative cooling in lines with altered stomata. In well-watered conditions, *stomagen* lines were warmer on average than wild-type, whereas *epfl10* leaf temperatures were wild-type like or cooler (Fig. 4A). No difference in leaf temperature was detected across genotypes during vegetative or reproductive drought. To test the impact of reduced stomatal densities on water conservation, daily water loss was measured over the course of a week beginning with 70 days after germination. Despite differences in thermoregulation, *stomagen* and *epfl10* both conserved greater volumes of water in a week by 48 mL and 83 mL, respectively (Fig. 4D).

epfl10 exhibited a range of phenotypes that suggested an increased fitness under drought via moderate stomatal density reductions. This phenotype contrasted with *stomagen*, which may be less optimal for high-yielding production due to large reductions in A_n and g_s. To assess the impacts of stomatal modifications on potential crop performance, yield trials were conducted using three watering regimes in the greenhouse. However, in well-watered, vegetative drought, and 4-day reproductive drought conditions, there was no significant difference in grain yield or aboveground biomass among genotypes (Fig. 4B, C).

2.3.6. Paralogs of STOMAGEN in other species may also be targets for tuning stomatal density

The potential of paralog editing for tuning stomatal density, as demonstrated in rice *epfl10* lines, can be extended to other crop species. Further phylogenetic investigation of the duplication of STOMAGEN among angiosperms revealed an additional putative family-level STOMAGEN duplication in the Asteraceae (Fig. 5A). Species level duplications were noted in carrot (*Daucus carota)*, date palm (*Phoenix dactylifera)*,

Balbis banana (*Musa balbisiana)*, and *Carex littledalei* based on the species included in this study.

Similar to rice *STOMAGEN* and *EPFL10*, the paralogs of *STOMAGEN* in carrot and sunflower are differentially expressed in tissues most relevant to gas exchange suggesting regulatory divergence after the duplication event (Fig 5B, C). Unlike the rice *STOMAGEN* duplicates, both sunflower and carrot paralogs exhibit less relative sequence variation (Fig. 5D, E). Regardless of functional divergence, duplicated *STOMAGEN* copies may provide genetic material for optimization of expression in organisms where multiple copies exist.

2.4 Discussion

Drought is the most severe and widespread environmental stressor in South and Southeast Asia[222]. Application of gene editing in crops for climate change could serve as a potent mechanism for realizing actual technology transfer to growers. Few gene editing applications for addressing abiotic stressors have been reported to date[223]. Preceding transgenic manipulations of epidermal patterning factors generated rice with improved water conservation and maintenance of yields in greenhouse conditions; however, these lines exhibited reductions in A_n and g_s[15,184,185]. Exploration of the rice *STOMAGEN* paralog, Os*EPFL10*, was undertaken as a means of generating novel, transgene-free variation in rice stomatal density. The evolutionary and regulatory features of *EPFL10* suggested it as a promising target for engineering drought resilience.

Variation in stomatal density introduced by single-gene knockouts of Os*EPFL10* and Os*STOMAGEN* provided a basis for further physiological exploration. A double knockout of Os*EPFL10* and Os*STOMAGEN* was also generated. *stomagen epfl10* double mutants phenocopy *stomagen* in stomatal density, suggesting that loss of *STOMAGEN* may be epistatic to *EPFL10*. However, the stomatal density reductions of *stomagen* lines are so severe that STOMAGEN knockout alleles may mask the subtle contribution of *EPFL10* towards stomatal density. Further investigation of *EPFL10* and *STOMAGEN* interactions are needed to clarify the relationship of these genes.

Stomatal conductance and photosynthesis of *stomagen* but not *epfl10* were lower at all light intensities greater than 1,000 μmol photons m^{-2} s^{-1} relative to wild-type. In field conditions, photosynthetic flux densities regularly exceed 1,000 μmol photons m^{-2} s^{-1} at midday, coinciding with the hottest parts of the day[224]. Sustaining photosynthesis may work synergistically with optimizing thermoregulation as *stomagen* exhibits reduced gas exchange simultaneous with warmer temperatures in well-watered conditions whereas *epfl10* maintained wild-type levels of thermoregulations (Fig. 3A, 3B, 4A). In accordance with anticipated global warming caused by climate change, maintained or improved thermoregulation will be vital for crop agronomic performance[158].

Larger stomatal apertures were measured in *stomagen* and *epfl10* lines relative to wild type (Fig. 3D). Pore area adjustments in *stomagen* lines were unable to physiologically

compensate for large reductions in stomatal densities, unlike *epfl10* lines, which maintained wild-type levels of A_n and g_s (Fig. 3A, B). The theoretical maximum stomatal conductance of grass stomata greatly exceeds the measured stomatal conductance[15,196]. *stomagen* maintained high levels of specific stomatal conductance concurrent albeit with low overall A_n and g_s (Fig. 3A, B,E). High levels of specific stomatal conductance in *stomagen* reveal that engineering greater stomatal conductance in a reduced density background, may be a promising mechanism for maintaining photosynthetic capacities simultaneous with water-use efficiency[196,225].

No detectable differences were found in greenhouse grain or biomass yield among the reported genotypes under periods of vegetative or reproductive drought. However, there is a reasonable basis to prioritize maintenance of wild-type gas exchange for maximizing yield. Other literature suggests that high levels of leaf photosynthesis and stomatal conductance are linked to higher yields among C3 crops[190–194,226], and it is possible that more severe drought stress or in-field validation may resolve differences between genotypes.

Independent of biomass and yield, *epfl10* provides a potent demonstration of the capacity to reduce stomatal densities and increase soil water conservation without concomitant reductions in gas exchange essential for optimal crop performance at steady-state conditions. Stomatal conductance measurements are an instantaneous measurement of plants taken at steady state. However, dynamic environmental conditions exist in greenhouse conditions where water conservation measurements were made. Periods in this dynamic range where stomata may have been closed, such as at nighttime, or less open, like periods of cloud cover, may have contributed the capacity of *epfl10* to conserve more water than WT despite statistically similar stomatal conductance. Transpiration via closed stomata or less open stomata is a non-negligible mechanism of water loss that is frequently overlooked and that may, in-part, explain water loss phenotypes [227]. Furthermore, stomatal density alone does not account for gas exchange entirely. Stomatal kinetics in dynamic environments has been shown to drive differences in conductance[228,229].

It is noteworthy that *stomagen* lines conserved water to a similar extent as *epfl10* despite having much greater reductions in stomatal density. Larger stomata typically exhibit slower dynamic responses[230]. Slower rates of environmental response in fluctuating light or environmental conditions may account for a non-linear relationship between stomatal density and water conservation. Our data indicate that fewer stomata with larger apertures offer comparable water conservation properties as lines possessing greater numbers of stomata with smaller apertures. It is still unclear how more severe and field-relevant drought stresses may affect water loss and assimilation when specific stomatal conductance is limiting.

Knockout of the Os*STOMAGEN* paralog Os*EPFL10* builds on other work that has shown that null alleles of paralogs can achieve desirable phenotypic outcomes[36,200]. Rice *EPFL10* had reduced expression levels in relevant tissues and significant peptide level differences from *STOMAGEN*, reinforcing ex-situ evidence that Os*EPFL10* may

have evolved a weaker overall function in stomatal development (Fig. 1). A similar exploration of *STOMAGEN* duplicates in other organisms was used to identify other candidate species for paralog editing. *STOMAGEN* copy II in sunflower and *STOMAGEN* copy I in carrot also each exhibit lower expression that may substantiate these genes as targets for moderate reductions in stomatal density similar to *EPFL10* in rice. A previous report identified two copies of *STOMAGEN* in a collection of grape genotypes using high-quality published genomes and verifying by PCR. Advances in sequencing such as genome and pangenome availability may reveal other putative duplications at the species or cultivar level. Gene editing of paralogs can provide a convenient gene-editing target for quantitative variation in traits like stomatal density.

Peptide variation of the *STOMAGEN* duplicate, *EPFL10*, in rice exceeded that of carrot and sunflower (Fig 5). It is enticing to consider a scenario where the significant sequence variation underlying Os*EPFL10* at the family and genus level may evidence selection against functional redundancy of *STOMAGEN* (Fig. 1C). Thus, both decreases in expression, observed in all duplicates, and significant peptide variation, observed in rice *EPFL10*, may underlie functional natural variation that knocked down stomatal density to restore gene dosage imbalances incurred by stochastic *STOMAGEN* duplication. Further functional characterization of diverse *STOMAGEN* duplicates is necessary to resolve their role and costs to plant fitness. Paralog editing of stomatal density and other traits may be a promising avenue to accelerate the production of climate-adapted varieties without the constraints of transgenic germplasm.

To this aim, we report here modest stomatal density reductions in *epfl10* mutants with no associated decreases in stomatal conductance or carbon assimilation. *epfl10* lines maintained wild-type physiological capacities of stomatal conductance, carbon assimilation, thermoregulation, and yield while also conserving more water than wild type. These attributes could contribute to improved climate resilience in current and future conditions where water is limiting, and temperatures are increased. Field-based investigations of *epfl10* and *stomagen* will further resolve the agronomic utility of these edited rice lines.

Figure 1:

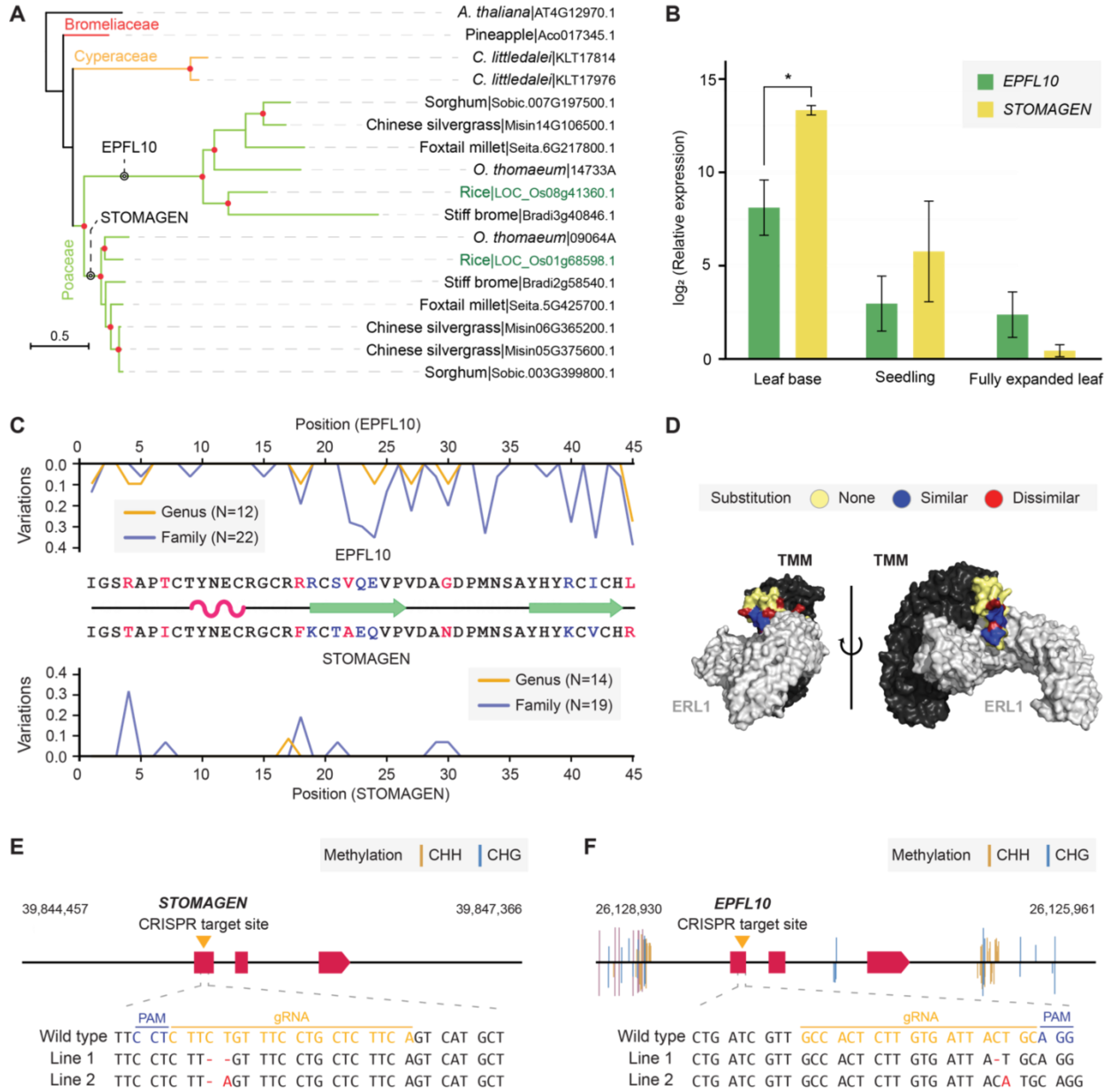


Figure 1| Evolution and regulation of *STOMAGEN* and *EPFL10*

A, Gene trees of the STOMAGEN family members in Poales. *Arabidopsis thaliana* was used as an outgroup. **B**, qRT-PCR determined expression levels of EPFL10 and STOMAGEN in fully expanded leaf, leaf base, and seedling. Bars represent means and error bars represent standard deviation from the mean. The asterisk indicates a significant difference between the means (P<0.05). **C**, Genus and family-level sequence variations of STOMAGEN and EPFL10 orthologs. The sequence variations represent normalized Shannon's entropy, with 0 being no variations and 1 being complete variations. The secondary structure annotation originates from the experimentally determined structure of STOMAGEN (PDB: 2LIY)[179] 26. The colored residues highlight variable positions between rice STOMAGEN and EPFL10. Substitutions to similar and dissimilar amino acids based on BLOSUM62 are indicated with blue and red. Alpha helix is represented by pink and beta sheets by green. **D**, The variable positions between STOMAGEN and EPFL10 mapped to the experimentally determined complex structure of EPF1, ERL1 (in gray) and TMM (in black) (PDB: 5XJO)[231] 9. Peptide sequence variations between EPFL10 and STOMAGEN with similar or dissimilar substitutions are indicated in blue and red, respectively. Complex is visualized from two orientations. **E** and **F**, The gene models of (E) STOMAGEN and (F) EPFL10 with the location of the CRISPR/Cas9 target sites indicated by the yellow triangle. Repressive CHH and CHG methylation marks are indicated for 1.5 kb up- and down- stream of the coding regions. Methylation data was collected from 3-week-old leaf tissues. The two unique homozygous edits generated in STOMAGEN and EPFL10 by CRISPR/Cas9 are shown in red with the PAM annotated in blue and guide sequence in yellow.

Figure 2:

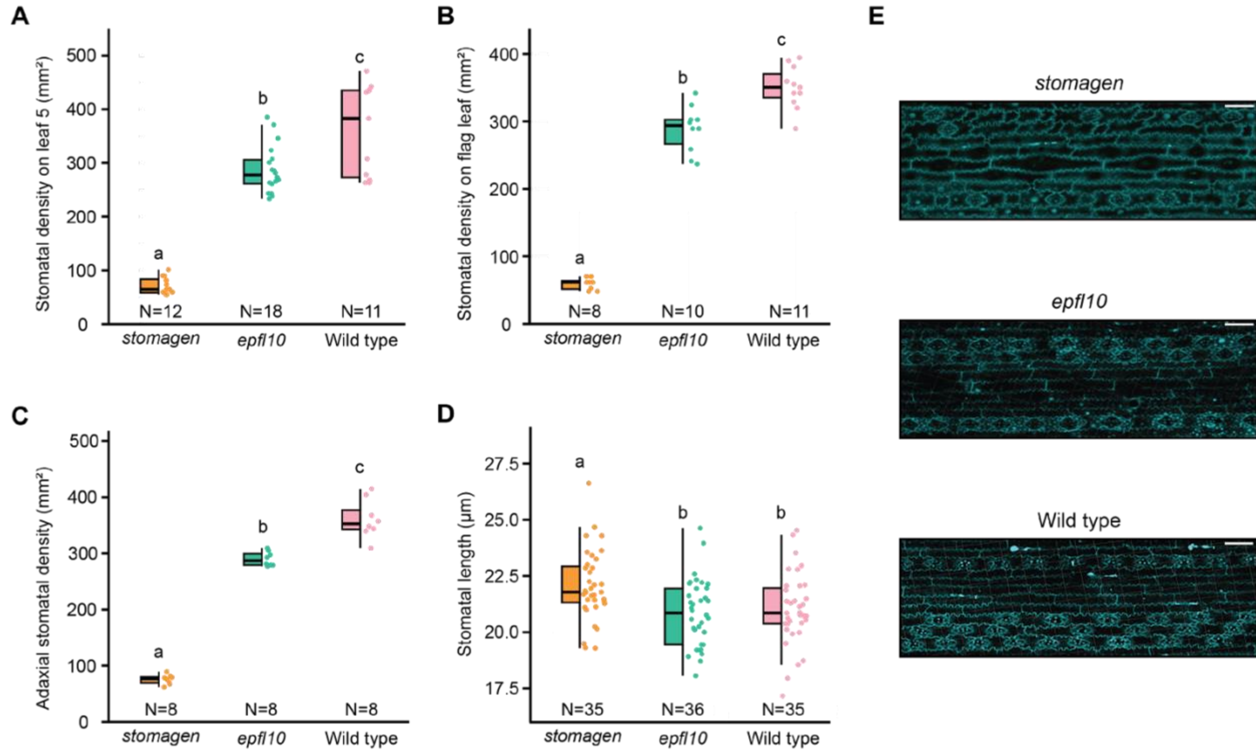


Figure 2| EPFL10 is a weak positive regulator of stomatal development in Nipponbare (*Oryza sativa* spp. Japonica)**.**

A-**C**, Stomatal density of *EPFL10, STOMAGEN,* and wild type. **A**, Stomatal density of the fifth fully expanded true leaf. Stomatal densities of 21-day old plants were measured. **B**, Stomatal density of the flag leaf on the primary tiller during grain filling. Flag leaves of 55-day old plants were measured. **C**, Adaxial stomatal densities of the fifth fully expanded true leaf on 21-day old plants. **D**, Stomatal length of *EPFL10, STOMAGEN,* and wild type. **E**, Representative confocal images of *EPFL10, STOMAGEN,* and wild type. Images were taken of 55-day-old leaves stained with propidium iodide. **A**-**D**, Graphs are box-and-whisker plots where the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Letters indicate a significant difference between means ($P<0.05$, one-way ANOVA Tukey HSD post-hoc test).

Figure 3:

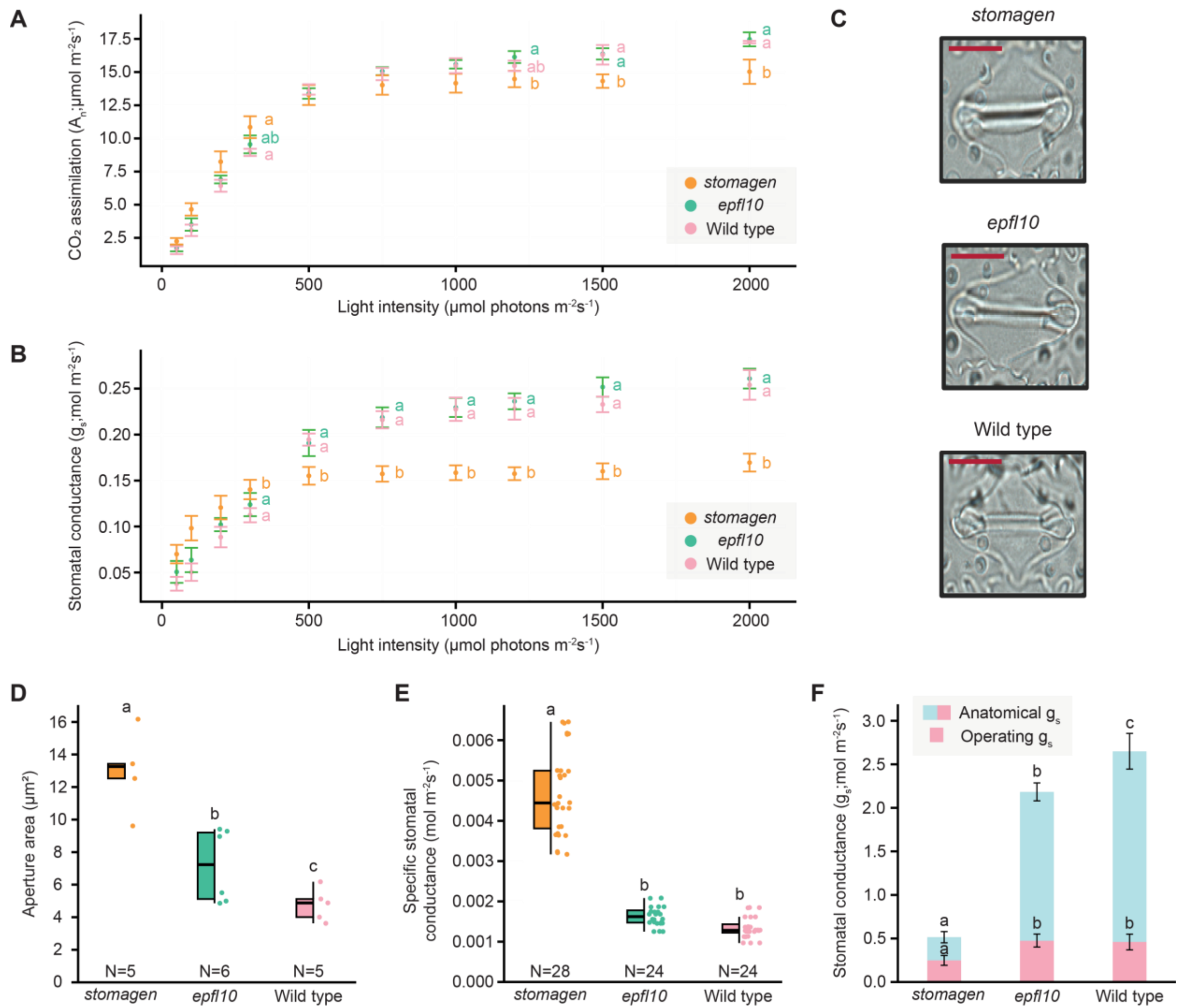


Figure 3| Gas exchange measurements and stomatal pore area measurements in reduced stomatal density backgrounds in Nipponbare (*Oryza sativa* spp. Japonica).

Carbon assimilation (A) and stomatal conductance (B) measurements of *STOMAGEN, EPFL10,* and wild-type across a range of light intensities: 100, 200, 300, 500, 650, 1000, 1200, 1500, and 2000 µmol photons m^{-2} s^{-1} in 32-day-old plants grown in the greenhouse. **(C)** Representative images of stomatal pore size variations for *EPFL10, STOMAGEN,* and wild type. **D**, Stomatal aperture of *EPFL10, STOMAGEN,* and wild type. **E**, Specific stomatal conductance of *EPFL10, STOMAGEN,* and wild type. Specific conductance values were calculated by dividing stomatal conductance by the average stomatal density of the probe area of the respective genotype. **F**, Anatomical g_{smax} was calculated for each genotype. Operating g_s at 1,500 µmol photons m^{-2} s^{-1} is reported in pink. The percentage of operating to anatomical g_s is reported above the pink bar for each genotype. **A** and **B,** dots represent means and error bars are standard deviations from the mean. **C and E** in the box-and-whisker plots, the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Letters indicate a significant difference between means (P<0.05, one-way ANOVA Tukey HSD post-hoc test). **F** is a barplot where mean is represented with error bars showing standard error of the mean. Letters indicate a significant difference between means (P<0.05, one-way ANOVA Tukey HSD post-hoc test).

Figure 4:

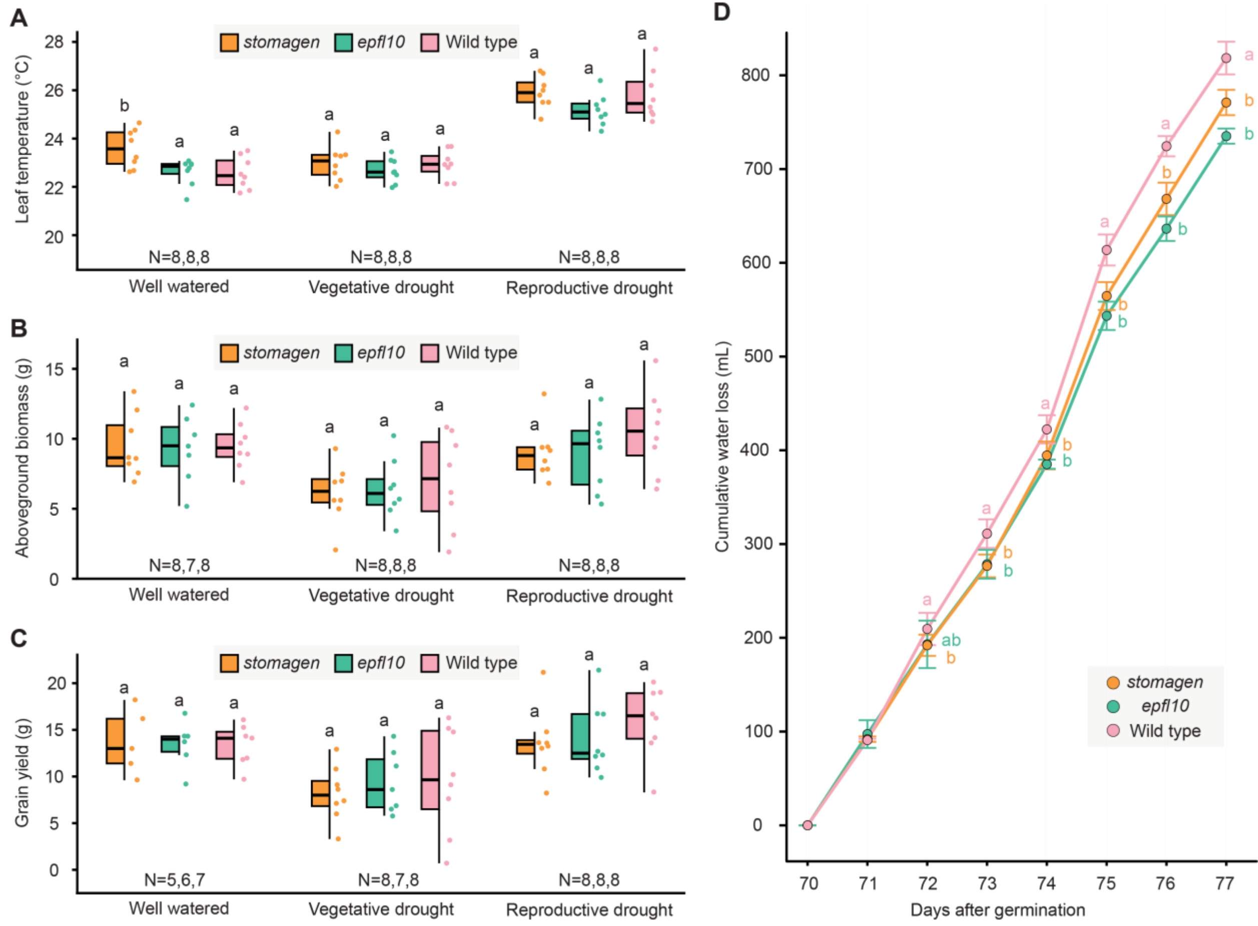


Figure 4| Stomatal density reductions influence thermoregulation and water conservation but not greenhouse yield in Nipponbare (*Oryza sativa* spp. Japonica). **A**, Leaf temperatures of *STOMAGEN, EPFL10*, and wild-type in well-watered, vegetative drought, and reproductive drought. **B**, Aboveground biomass of *STOMAGEN, EPFL10*, and wild-type in well-watered, vegetative drought, and reproductive drought. **C**, Grain yield of *STOMAGEN, EPFL10*, and wild-type in well-watered, vegetative drought, and reproductive drought. **A**-**C**, Graphs are box-and-whisker plots where the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. **D**, Cumulative water loss of *STOMAGEN, EPFL10,* and wild type from days 70-77 after germination. Pots remained well watered during the course of this experiment. Flats were replenished after daily water loss recordings. The dots represent means and error bars are standard errors from the mean. Letters indicate a significant difference between means ($P<0.05$, one-way ANOVA Tukey HSD post-hoc test).
Figure 5:

Figure 5

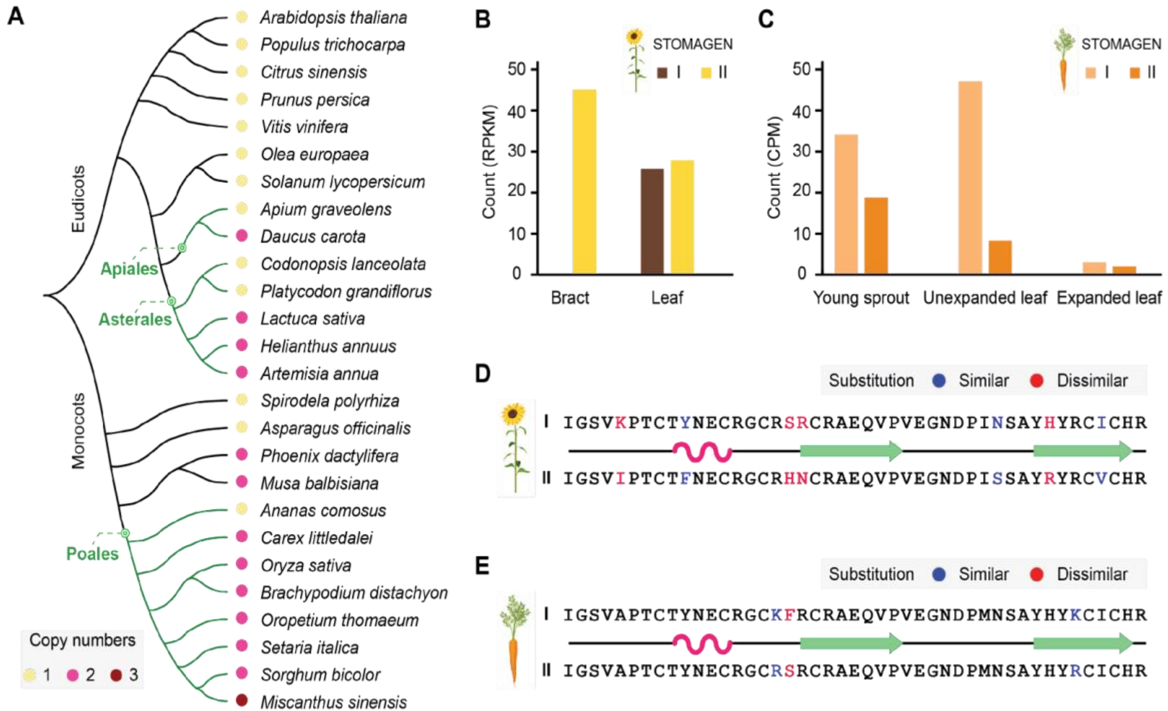


Figure 5| Multiple STOMAGEN duplication events in angiosperms provide new editing targets. **A**, A dendrogram of angiosperm species and copy number variations of STOMAGEN family members. STOMAGEN copy number indicated by colors in legend. **B** and **C**, Expression patterns of the paralogous STOMAGENs in (B) sunflower (*Helianthus anuus*) and (C) carrot (*Daucus carota)* in tissues assayed most relevant to gas exchange. **D** and **E**, The C-terminal active STOMAGEN sequences with non-synonymous substitutions highlighted for the paralogs in sunflower (D) and carrot (E). Substitutions to similar and dissimilar amino acids based on BLOSUM62 are indicated with blue and red The secondary structure annotation originates from the experimentally determined solved structure of STOMAGEN (PDB: 2LIY) [26]. Alpha helix is represented by pink and beta sheets by green.

Supplemental Figure 1

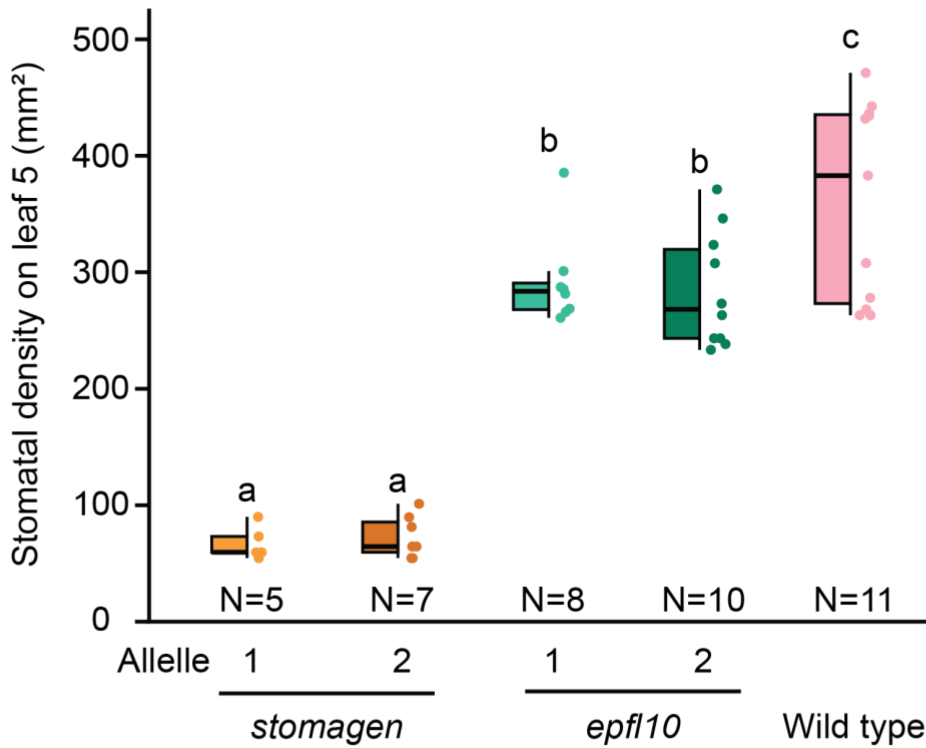


Figure S1| Stomatal density of the fifth fully expanded true leaf. Stomatal density measurements of each allele of *stomagen; epfl10;* and WILD TYPE taken on 21-day old plants grown in the growth chamber. Allele numbers correspond to the alleles described in Figure 1E,F. In this box-and-whisker plot the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Letters indicate a significant difference between means ($P<0.05$, one-way ANOVA Tukey HSD post-hoc test).

Supplemental Figure S2

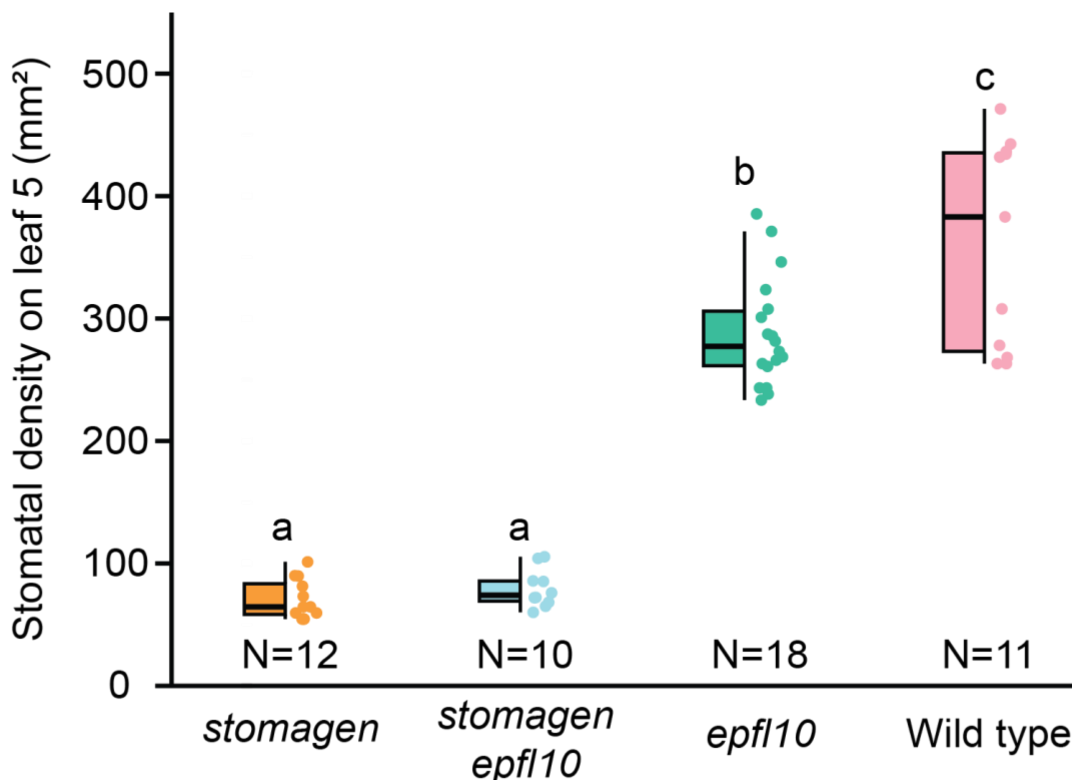


Figure S2| Stomatal density of the fifth fully expanded true leaf. Stomatal density measurements of *stomagen, epfl10; stomagen; epfl10;* and WILD TYPE taken on 21-day old plants grown in the growth chamber. In this box-and-whisker plot the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Letters indicate a significant difference between means ($P<0.05$, one-way ANOVA Tukey HSD post-hoc test). Measurements were taken on full expanded leaf 5 of 28-day-old plants grown in well-watered conditions plants using an infrared gas analyzer (LI6800XT, LI-COR, Lincoln, NE, USA) with chamber conditions set to: light intensity 1000 µmol photons m^{-2} s^{-1} (90% red light, 10% blue light); leaf temperature 27°C; flow rate 500 µmol s^{-1}; relative humidity 40%; and CO_2 concentration of sample 400 µmol mol^{-1}.

Supplemental Figure 3

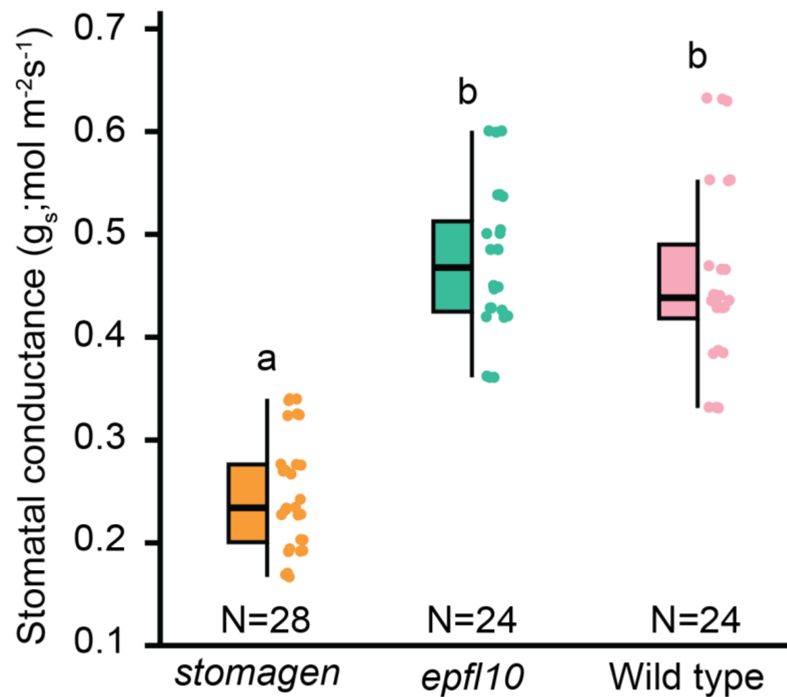


Figure S3| Stomatal conductance measurements of *stomagen*, *epfl10*, and wild type at 1000 µmol photons m-2s-1 grown in the growth chamber. In this box-and-whisker plots the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Letters indicate a significant difference between groups.

Supplemental Figure 4

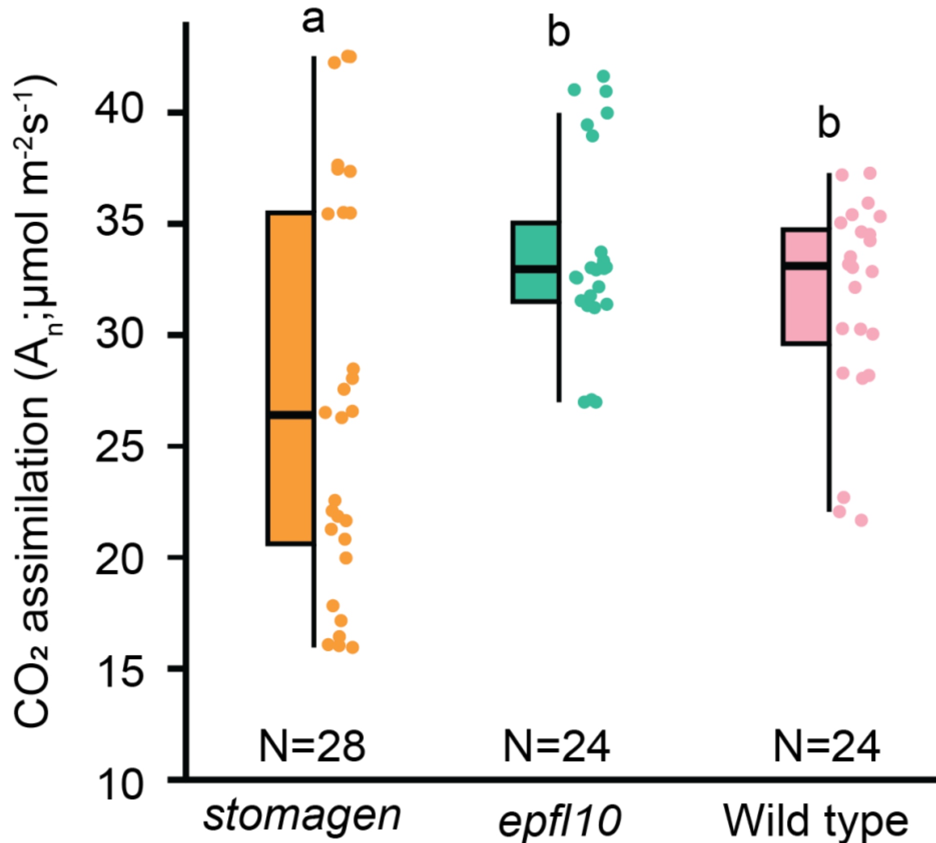


Figure S4| Carbon assimilation measurements of *stomagen*, *epfl10*, and wild type at 1000 µmol photons m-2s-1 grown in the growth chamber. In this box-and-whisker plot the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Letters indicate a significant difference between means ($P<0.05$, one-way ANOVA Tukey HSD post-hoc test). Measurements were taken on full expanded leaf 5 of 28-day-old plants grown in well-watered conditions plants using an infrared gas analyzer (LI6800XT, LI-COR, Lincoln, NE, USA) with chamber conditions set to: light intensity 1000 µmol photons m-2s-1 (90% red light, 10% blue light); leaf temperature 27°C; flow rate 500 µmol s^{-1}; relative humidity 40%; and CO_2 concentration of sample 400 µmol mol^{-1}.

Supplemental Figure 5

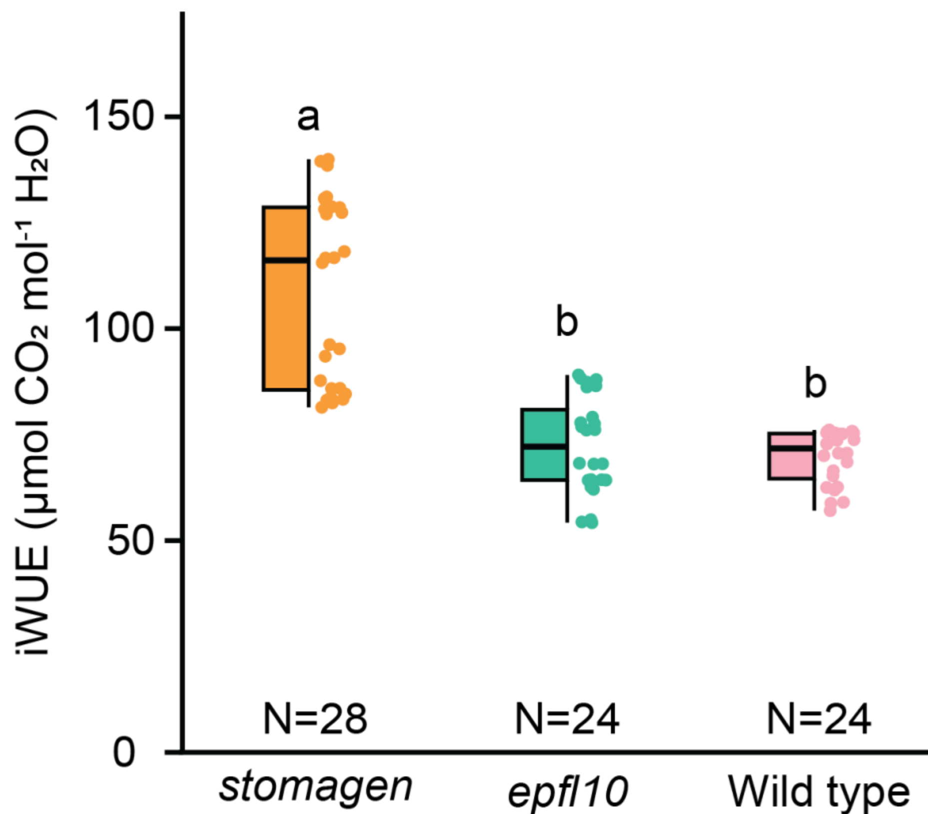


Figure S5| Intrinsic water-use efficiency of *epfl10* and *stomagen* lines in Nipponbare (*Oryza sativa* spp. Japonica) grown in the growth chamber. In this box-and-whisker plots the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Letters indicate a significant difference between means ($P<0.05$, one-way ANOVA Tukey HSD post-hoc test). Measurements were taken on full expanded leaf 5 of 28-day-old plants grown in well-watered conditions. Intrinsic water use efficiency (iWUE) is calculated by dividing photosynthesis by stomatal conductance for each biological replicate.

Supplemental Figure 6

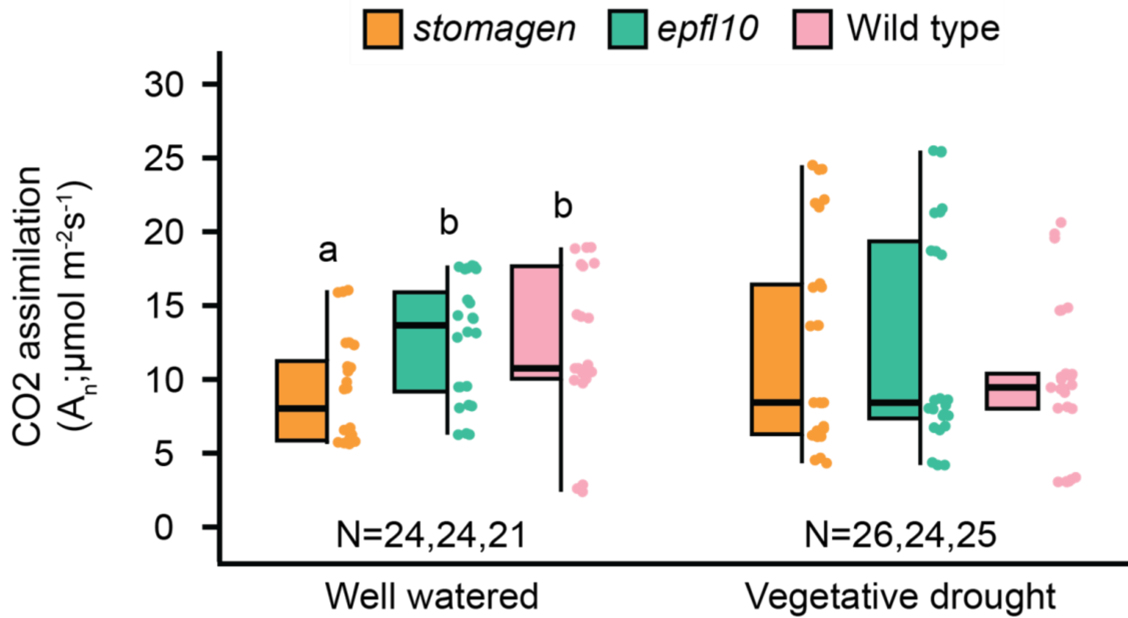


Figure S6| Measurements of carbon assimilation *epfl10* and *stomagen* lines in Nipponbare (*Oryza sativa* spp. Japonica) grown in the greenhouse under two watering regimes: well-watered, and vegetative drought. In this box-and-whisker plot the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Letters indicate a significant difference between means ($P<0.05$, one-way ANOVA Tukey HSD post-hoc test). Measurements were taken on full expanded leaf 5 of 28-day-old plants grown in well-watered conditions and vegetative drought.

Supplemental Figure 7

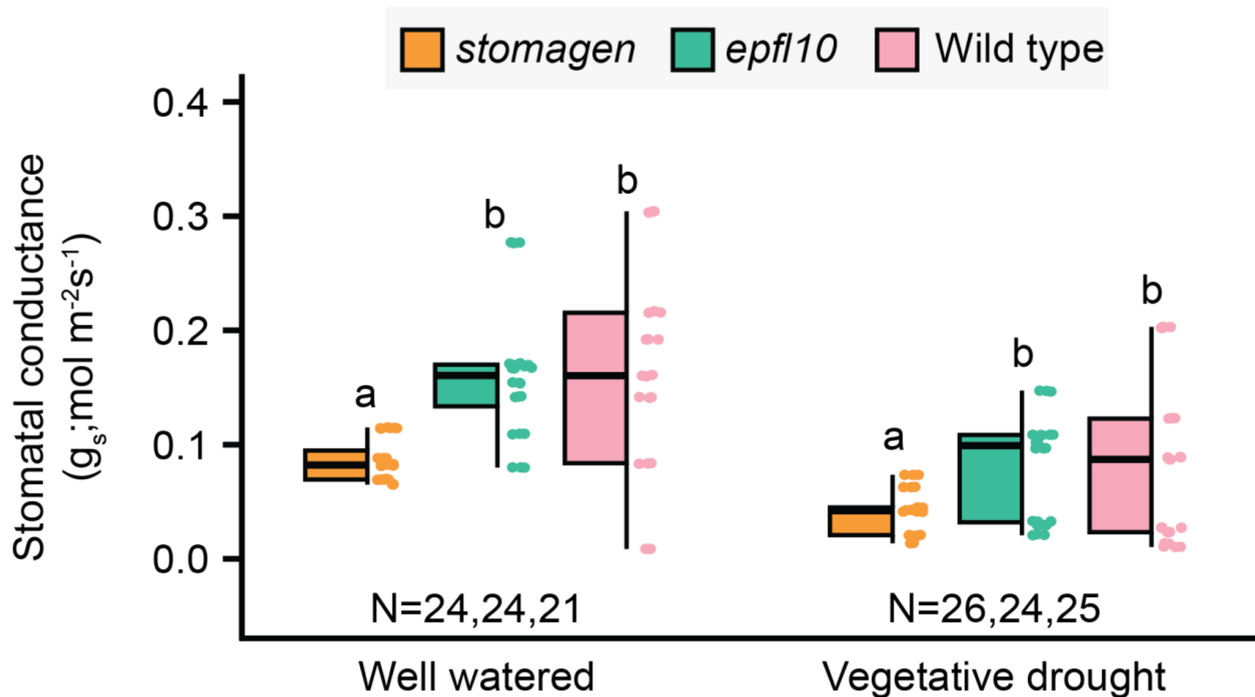


Figure S7| Measurements of stomatal conductance of *epfl10* and *stomagen* lines in Nipponbare (*Oryza sativa* spp. Japonica) grown in the greenhouse under two watering regimes: well-watered, and vegetative drought. In this box-and-whisker plot the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Outliers are represented by black dots. Letters indicate a significant difference between means ($P<0.05$, one-way ANOVA Tukey HSD post-hoc test).

Supplemental Figure 8

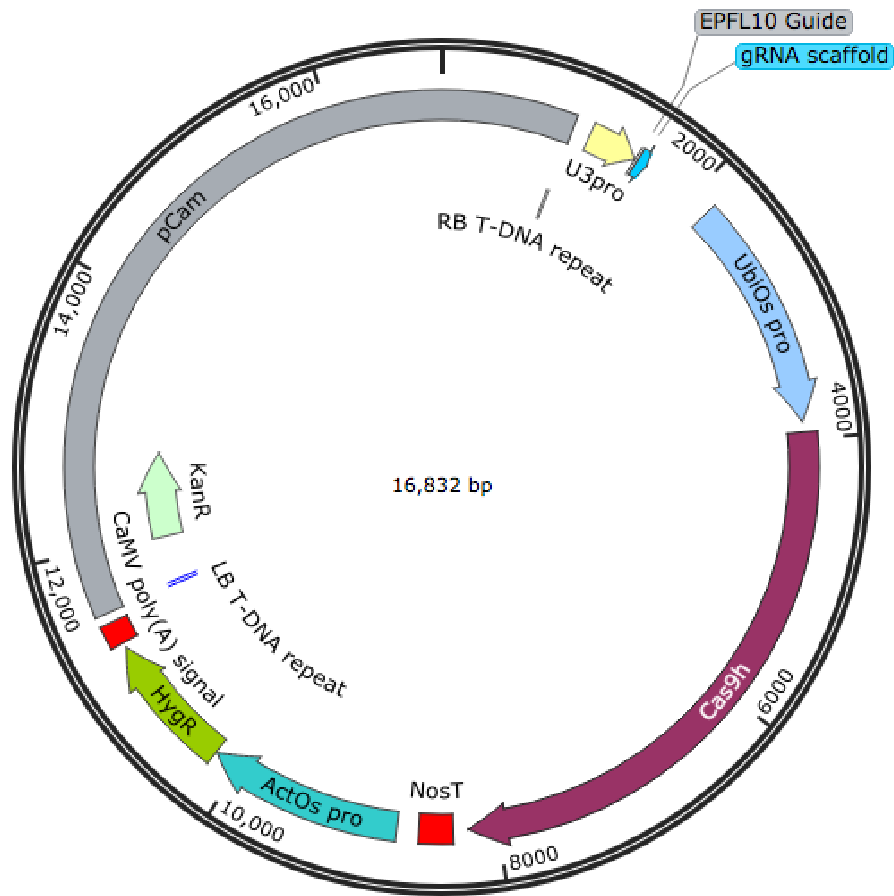


Figure S8| Representative plasmid map of binary gene editing vector used to generate *OsEPFL10* mutations. Full plasmid sequence is available in supplemental information.

Supplemental Figure 9

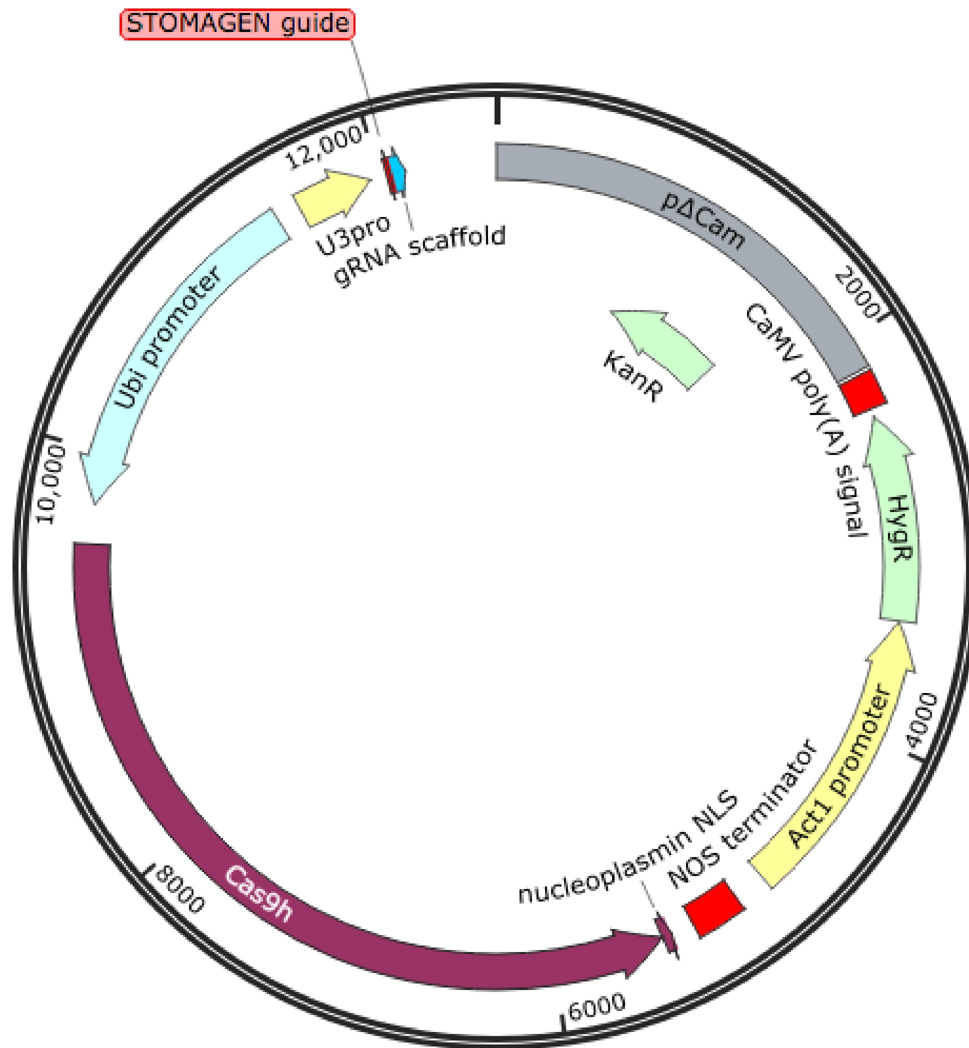


Figure S9| Representative plasmid map of gene editing vector used to generate *OsSTOMAGEN* mutations. Full plasmid sequence is available in supplemental information.

Supplemental Table 1

Sequence (5' to 3')	Application
GCCTACGTGTGTGTGTATTGC	Genotyping *epfl10*
GAGCAGCAGCAGCTAAAACG	
ATAGTCTCCAGCATTTGCTCCC	Genotyping *stomagen*
CTGATGCAAAGGGGTACCTGAG	
ACCACTTCGACCGCCACTACT	UBQ5 housekeeping gene qPCR
ACGCCTAAGCCTGCTGGTT	
CTCCCCGTGCTTCTCCTTCTGATG	EPFL10 qPCR
GGTGGTAACAAGTGTACGTCTGCCC	
GCTCGTTGCAATCAAGGGCA	STOMAGEN qPCR
GCAGCCTCTCCTTGTTTAGAAC	

Chapter 3

Quantitative trait engineering in rice towards generating stomatal morphological variation adapted to broad and dynamic environments

Gene editing in plant species driven by the introduction of CRISPR/Cas tools has been leveraged to generate variation in a breadth of traits [223]. The vast majority of edits targeted to improving climate change resilience have been achieved through gene knockouts [223]. In some cases, a knockout-based approach can provide sufficient trait variation simultaneous with minimal pleiotropic effects to be useful [223,232]. This is especially true in cases where paralogs exist [200,232,233]. However, this approach is limited by a small pool of genes whose null phenotypes are not detrimental to overall plant fitness.

Editing *cis*-regulatory elements is an attractive, alternative, gene editing approach. Targeting mutations to regulatory regions can fine tune expression of genes towards more subtle modifications to plant phenotypes with fewer pleiotropic effects relative to coding sequence mutations [77,233,234]. Quantitative variation in traits of interest can be generated by unique promoter alleles. Whereas knockout based approaches in coding sequence editing can be much more limited in possible phenotypic variation. Beyond the capacity for crop improvement, cis-regulatory element editing can help provide insights into the complex and obscure process of transcriptional regulation.

As such, promoter editing is emerging as a widely, albeit nascent, tool for generating plant phenotypic diversity. Efforts in plant promoter engineering often leverage a set of bioinformatic approaches to design guides in regions putatively significant for regulation of expression, including but not limited to analyzing ATAC-seq, CHIP-seq, transcription factor binding site analysis, and conserved non-coding sequence datasets [77,234,235]. Sequence conservation of non-coding sequences has become an increasingly relevant and prioritized parameter for rational guide design [234,235]. It is hypothesized that conserved promoter regions may be more important for the regulation of expression relative to more divergent promoter sequences. Edits to conserved non-coding sequences (CNS) within species or among family members have been shown to impart phenotypic effects. CNSs have been identified across plant families; however, the phenotypic outcomes of editing these CNSs across plant families have yet to be discretely determined [234].

Leveraging these existing methods, we sought to generate functional guides for the promoter of *OsSTOMAGEN* to enable the production of quantitative variation in stomatal density. Rice production landscapes encompass a broad range of environmental conditions including, but not limited to, radically variable water availability and differing pathogen pressure [163,164]. Cultivated varieties of rice display a range of optimized stomatal densities and morphologies corresponding to production environment [167,191]. We thus pursued promoter editing as mechanism to fine-tune

stomatal densities for increasing compatibility of a single rice cultivar to extensive potential rice production systems.

Beyond the capacity for crop improvement, cis-regulatory element editing was used to interrogate the structure-function relationships of stomatal density and physiology. Stomatal density significantly influences other stomatal morphological traits, including gas exchange, water use efficiency, and drought tolerance in rice [15,167,232,236,237]. Some studies that demonstrate these influences have been limited by the evaluation of only a few stomatal density variants, however, preventing description of clear trends between stomatal density and other traits [15,232]. Other studies are limited instead by hugely variable genetic backgrounds that prevent stomatal density from being investigated separately from the effects of other genetic determinants [167,237]. One case of multiple stomatal density variants in a single genetic background has been published [236]. However, the range of stomatal density variation reported in this study was very limited, only encompassing 17 to 35% reductions in four unique stomatal density lines. Furthermore, the mapping population was established by random mutagenesis, wherein the mutations underlying stomatal density variation are heterogenous.

For promoter-edited plants to have utility in field conditions, the potential pleiotropic impacts of edits must be minimized. *cis*-regulatory regions are partly responsible for mediating plant responses to dynamic environmental conditions. It is known that stomatal density is an environmentally responsive trait. Environmental conditions such as carbon dioxide concentrations, light intensity, drought stress, and pathogens can induce stomatal density reprogramming [238–240]. Maintaining capacity for stomatal density reprogramming will thus be important in some environments for optimizing productivity.

Our work applies a bioinformatic toolkit to designing guides for producing quantitative trait variation in rice stomatal density. The transcriptional and phenotypic outcomes of cis-regulatory editing of the promoter of *OsSTOMAGEN* are measured. The quantitative trait variation established is subsequently leveraged to consider the relationship of stomatal density to gas exchange in a near-isogenic population. Finally, we consider stomatal density reprogramming under abiotic stress to assess the impacts of cis-regulatory element editing on environmental response. We apply these findings towards a framework to understand how approaches in promoter editing can be used to established quantitative trait variation towards broad and dynamic environments.

3.2 Methods

3.2.1 Plant Growth conditions:

Rice cultivar Nipponbare (*O. sativa* ssp. japonica) seeds were germinated and grown for 8 days in a petri dish with 20 mL of water in a Conviron growth chamber at 28°C for day-length periods of 16 hours in 100 µmol photons m^{-2} s^{-1} of light and 80% relative humidity. Seedlings were transferred to a soil mixture comprised of equal parts turface

(https://www.turface.com/products/infield-conditioners/mvp) and sunshine mix #4 (http://www.sungro.com/professional-products/fafard/).
Germinated seedlings used for stomatal phenotyping and growth chamber physiological assays were transferred to 10 cm, 0.75 L McConkey tech square pots and placed in growth chambers at 28°C for day-length periods of 16 hours in 400 µmol photons m^{-2} s^{-1} of light and 80% relative humidity.

Plants designated for greenhouse measurements were placed in setpoints of 27°C day/22°C night at ambient light conditions in with day lengths of 12 hours in 15.2 cm, 1.8 L pots. All plants were fertilized with 125 mL of 1% w/v iron solution one-week post-transplant. 1 L of 5% w/v JR Peter's Blue 20-20-20 fertilizer (https://www.jrpeters.com/) was added to each flat at 3- and 11-weeks post-germination.

3.2.2 Identifying putative transcription factor binding sites, conserved non-coding sequences, regions of open chromatin, and H3K27ac DNA interaction regions

Transcription factor binding sites were identified using the total available transcription factor binding site motifs in JASPAR Plant Core [241]. The region 2,000 base pairs upstream of the translation start site was queried for the existence of transcription factor binding sites using a 99% score threshold.

Conserved non-coding sequences within the Poaceae family were identified using mVista [242]. The sequences of the 2kb region upstream of the translation start site of the homolog of *OsSTOMAGEN* from *Brachypodium distachyon*, *Hordeum vulgare*, *Setaria italica,* and *Zea mays* was retrieved from Phytozome. A duplication of *OsSTOMAGEN* in Poaceae required the use of the gene tree generated by Phytozome to identify the appropriate copy of *STOMAGEN* within each species assayed. All *STOMAGEN* copies also had the highest percent identity compared to *OsSTOMAGEN* relative to other paralogs of *STOMAGEN* in species assayed.

ATAC-seq and Chip-seq data of *OsSTOMAGEN* was extracted from publicly available RiceENCODE database (http://glab.hzau.edu.cn/RiceENCODE/pages/Browser.html) [243]. Open chromatin is known to be variable among varying tissue types [244]. Expression of *STOMAGEN* in *Arabidopsis thaliana* occurs in the mesophyll, extending this knowledge to rice, data from rice mesophyll cells was prioritized. Chip-seq data used in this study was generated using an antibody to H3K27ac, a histone typically associated with enhancer elements in promoters [244].

3.2.2 Generation of edited lines:

Guides for targeting the region upstream of *STOMAGEN* were selected to in accordance with bioinformatic analysis in Figure 1. Forward and reverse strand guide sequences with relevant sticky ends amenable for Golden Gate cloning were ordered from Integrated DNA Technology. Guide sequences are listed in Table S1. Guides are arranged in order from most distal to proximal of Os*STOMAGEN* transcription start site. Equal volumes of 10 mM primers were annealed at room temperature. Golden Gate

cloning was used to insert all eight guides simultaneously into the entry clone containing the tracrRNA and U3 promoter. LR clonase reactions were used to insert the entry clone into destination vectors for biolistic transformation and *Agrobacterium*-mediated transformation.

The guide for targeting the coding sequence of *OsSTOMAGEN* was selected to minimize off-targeting, using CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/). Forward and reverse strand guide sequences with relevant sticky ends amenable for Golden Gate cloning were ordered from Integrated DNA Technology (IDTdna.com). Equal volumes of 10 mM primers were annealed at room temperature. Golden Gate cloning was used to insert guides into the PeGM entry clone containing the tracrRNA and U3 promoter. LR clonase reactions were used to insert the entry clone into destination vectors for b *Agrobacterium*-mediated transformation.

3.2.3 Plant material and culture of explants:
Mature seeds of rice (*O. sativa* L. japonica cv. Kitaake) were de-hulled, and surface-sterilized for 20 min in 20% (v/v) commercial bleach (5.25% sodium hypochlorite) and 1% of Tween 20. Three washes in sterile water were used to remove residual bleach from seeds. De-hulled seeds were placed on callus induction medium (CIM) medium [N6 salts and vitamins[201], 30 g/L maltose, 0.1 g/L myo-inositol, 0.3 g/L casein enzymatic hydrolysate, 0.5 g/L L-proline, 0.5 g/L L-glutamine, 2.5 mg/L 2,4-D, 0.2 mg/L BAP, 5 mM $CuSO_4$, 3.5 g/L Phytagel, pH 5.8] and incubated in the dark at 28° °C to initiate callus induction. Six- to 8-week-old embryogenic calli were used as targets for transformation.

3.2.4 *Agrobacterium*-mediated transformation:
Embryogenic calli were dried for 30 min prior to incubation with an *Agrobacterium tumefaciens* EHA105 suspension (OD_{600nm} = 0.1) carrying the binary vector for editing rice STOMAGEN promoter. After a 30 min incubation, the *Agrobacterium* suspension was removed. Calli were then placed on sterile filter paper, transferred to co-cultivation medium [N6 salts and vitamins, 30 g/L maltose, 10 g/L glucose, 0.1 g/L myo-inositol, 0.3 g/L casein enzymatic hydrolysate, 0.5 g/L L-proline, 0.5 g/L L-glutamine, 2 mg/L 2,4-D, 0.5 mg/L thiamine, 100 mM acetosyringone, 3.5 g/L Phytagel, pH 5.2] and incubated in the dark at 21 °C for 3 days. After co-cultivation, calli were transferred to resting medium [N6 salts and vitamins, 30 g/L maltose, 0.1 g/L myo-inositol, 0.3 g/L casein enzymatic hydrolysate, 0.5 g/L L-proline, 0.5 g/L L-glutamine, 2 mg/L 2,4-D, 0.5 mg/L thiamine, 100 mg/L timentin, 3.5 g/L Phytagel, pH 5.8] and incubated in the dark at 28 ºC for 7 days. Calli were then transferred to selection medium [CIM plus 250 mg/L cefotaxime and 50 mg/L hygromycin B] and allowed to proliferate in the dark at 28 ºC for 14 days. Well-proliferating tissues were transferred to CIM containing 75 mg/l hygromycin B. The remaining tissues were subcultured at 3 to 4 week intervals on fresh selection medium. When a sufficient amount (about 1.5 cm in diameter) of the putatively transformed tissues was obtained, they were transferred to regeneration medium [MS salts and vitamins[202], 30 g/L sucrose, 30 g/L sorbitol, 0.5 mg/L NAA, 1 mg/L BAP, 150 mg/L cefotaxime] containing 40 mg/L hygromycin B and incubated at 26 ºC, 16-h light, 90 µmol photons m^{-2} s^{-1}. When regenerated plantlets reached at least 1 cm in height, they

were transferred to rooting medium [MS salts and vitamins, 20 g/L sucrose, 1 g/L myo-inositol, 150 mg/L cefotaxime] containing 20 mg/L hygromycin B and incubated at 26 ^{o}C under conditions of 16-h light (150 µmol photons m^{-2} s^{-1}) and 8-h dark until roots were established and leaves touched the Phytatray™ II lid (Sigma-Aldrich, St. Louis, MO, USA). Plantlets were then transferred to soil.

3.2.5 Validation of edits:

T_0 plants targeted for edits in Os*STOMAGEN* promoter were evaluated using PCR to amplify the entire 2kb region upstream of the translation start site. PCR products were visualized on an agarose gel to verify successful amplification. To account for the possibility of heterozygous promoter mutations PCR products were subcloned into Zero Blunt™ TOPO™ (ThermoFisher, Waltham, MA) and heat shocked transformed into competent E. coli, which were plated on LB plates containing kanamycin selection. Fifteen colonies were from each plate were selected, grown in liquid culture overnight, miniprepped, and sent for sequencing with primers listed in Table 1. Seeds from T_0 plants with heterozygous promoter mutations were genotyped using the same method in the T_1 generation to isolate homozygous promoter mutations. Azygous T_2 plants were used for experimental data collection to minimize somaclonal variation, which may have accumulated during tissue culture[205,206].

Edits in the coding sequence of *OsSTOMAGEN* were evaluated using PCR to amplify the region of interest (Table S2). PCR products were Sanger sequenced. Sequence data was analyzed using the Synthego ICE tool (https://ice.synthego.com/#/) to detect alleles present[204]. Only lines with homozygous frame-shift mutations were retained for downstream experiments

3.2.6 Phenotyping stomatal density and size

Stomatal densities were recorded from epidermal impressions of leaves using nail polish peels[190]. Stomatal densities of eight biological replicates of each leaf were taken from the widest section of 32-day-old fully expanded leaves. Images were taken using a Leica DM5000 B epifluorescent microscope at 10x magnification. Three images were collected per stomatal impression and density per image was averaged. The number of stomata in a single stomatal band were counted and the area of each band was measured[207]. Stomatal densities were calculated by dividing stomatal counts by stomal band area (mm^{-2}). Stomatal densities of fifth leaf abaxial tissues were assayed for each allele.

Stomatal density of leaves that developed during vegetative drought was measured using the same methods described above. The vegetative drought was applied by removing all water from the flats. Emerging leaves from the three largest tillers were marked four days after drought initiation. Drought was applied for a total of 7 days prior to rehydrating. Marked leaves were allowed to expand fully prior to making epidermal impressions.

Epidermal peels of 40-day old plants were produced using a razor blade on the adaxial leaf to remove tissues above the abaxial epidermal layer. Images of individual stomata at 100x magnification were captured. Guard cell length was measured using ImageJ. 30 individual stomata collected from a total of five biological replicates of each genotype were measured.

3.2.7 Quantifying OsSTOMAGEN transcript abundance:
Total RNA was extracted from plants with the Qiagen Total RNAeasy Plant Kit at three developmental stages: five days after germination, from the basal 2.5 cm of the youngest developing leaf on 32-day old plants, and 2.5 cm from the leaf tip of the flag leaf of the primary tiller from 64-day old plants for each promoter allele and wild type. For comparisons of transcript abundance in vegetative drought total RNA was also extracted from the basal 2.5 cm of the youngest developing leaves of 32-day old plants after a 5-day vegetative drought stress was imposed. Flats of plants to be droughted were drained of all water five days prior to a sampling

RNA was reverse transcribed using the QuantiTect™ reverse transcription kit to generate first-strand cDNA. Quantitative reverse transcription PCR was performed using FAST SYBR on Applied Biosystem's QuantStudio 3 thermocycler. Relative expression levels were calculated by normalizing to the average of rice housekeeping genes, *UBQ5* housekeeping (LOC_Os01g22490) and *eEF-1A* (LOC_Os03g08020). [209]. Primers used for qPCR listed in Supporting Table 1. Relative log fold expression was calculated using the $2^{-\Delta\Delta CT}$ method using "Do my qpcr" web tool [245]. For flag leaves, seedlings, and developing leaves all comparisons are made to wild type. For comparisons of well-watered to vegetative drought developing leaves all expression is relative to wild type well-watered. For genotype specific comparisons across tissue types, comparisons were made to flag leaves.

3.2.8 Photosynthesis and stomatal conductance assays:

Physiological assays in Figure 3 were conducted on fully expanded leaf 5 of 32-day old plants. Data for Figure 3 was captured using an infrared gas analyzer (LI6800XT, LI-COR, Lincoln, NE, USA) with chamber conditions set to: light intensity 1500 μmol photons m^{2} s^{-1} (90% red light, 10% blue light); leaf temperature 25°C; flow rate 500 μmol s^{-1}; relative humidity 50%; and CO_2 concentration of sample 400 μmol. Each biological replicate was allowed to acclimate for 15 minutes prior to data collection. Leaves from which gas exchange data was captured were marked and subsequently phenotyped for stomatal density.

Measurements of gas exchange on vegetatively droughted plants were conducted using the methods described above. Drought was applied by draining flats of plants entirely. Gas exchange measurements were taken on the fifth day of drought.

Light response curves were generated using a LI6800 infrared gas analyzer (LI-COR, Lincoln, NE, USA) with chamber conditions set to: leaf temperature 25°C; flow rate 500 µmol s^{-1}; relative humidity 50%; and CO_2 concentration of sample 400 µmol mol^{-1}. Light intensity was first increased to 2000 µmol photons m^2 s^{-1}. Plants were given a 20 minute acclimation period prior to sequential decreasing light intensity to 1750, 1500, 1200, 1000, 750, 500, 300, 200, 100, and 50 µmol photons m^2 s^{-1} light with a steady-state waiting times of minimum 10 minutes and 15 minutes maximum between light. Light was composed of at least 90% red light and at maximum 40 µmol photons m^2 s^{-1} blue light to match equipment specifications. Measurements were taken on fully expanded fifth leaves of 32-day-old plants grown in the greenhouse.

3.2.9 Deriving stomatal limitation, J_{max}, and V_{cmax} from A/Ci curves

Curves of carbon assimilation rates relative to substomatal CO_2 concentrations were produced using the Dynamic Assimilation Technique ™ (https://www.licor.com/env/support/LI-6800/topics/dynamic-assimilation-technique.html) using a LI6800 infrared gas analyzer (LI-COR, Lincoln, NE, USA) [246]. Chamber parameters were set to: leaf temperature 25°C; flow rate 500 µmol s^{-1}; relative humidity 50%; light intensity 1500 µmol photons m^2 s^{-1}; fan speed 10,000 rpm. CO_2 concentration was varied from 10 to 1800 ppm with a ramp rate of 150 ppm per minute. Data was logged every four seconds. Data from a total of four biological replicates per genotype were collected.

A curve relating carbon assimilation to substomatal carbon dioxide concentration (C_i) was fit to each biological replicate using the "Plantecophys" package in R Studio for all values of carbon assimilation greater than zero [247]. A C_i threshold of 700 ppm was used for modeling the curves relevant to the regions where stomatal limitation occurs.

Stomatal limitations were calculated using the differential method [248]. Briefly, the carbon assimilation rate predicted by the model when Ci equals the ambient carbon dioxide concentration set at 370 ppm was subtracted from the carbon assimilation rate predicted by the model when Ci = 370ppm. This difference was divided by the carbon assimilation rate predicted by the model when Ci = 370ppm and multiplied by 100 to represent the percentage of stomatal limitation. Stomatal limitations were calculated independently for each biological replicate.

A-Ci curves were also fit for the entire dynamic range of C_i values in order to estimate J_{max} and V_{cmax}. All default parameters were used to model A-Ci curves.

3.2.10 Graphs and statistics:

Plots in Figures 1 and 5 were generated using Adobe Illustrator 24.1. Plots in Figures 2,3, and 4 were produced using Prism 9.5. Statistics were computed in R studio. Comparisons of multiple groups were conducted using ANOVA and Tukey's honest significant difference (HSD) post-hoc tests.

3.3 Results

3.3.1 Bioinformatic analyses identify discrete promoter regions for editing

Overlaying putative transcription factor binding sites, conserved non-coding sequences (CNS) within Poaceae, ATAC-seq and Chip-seq data helped reveal discrete regions ideal for targeted by gene editing (Figure 1). These signatures exhibited the greatest overlap near to the translation start site but outside of the transcription start site. The greatest conservation among Poaceae family members, ATAC-seq, and Chip-seq peaks are all aligned. The greatest conservation to rice non-coding sequences is observed for *Brachypodium distachyon* relative to other family members, consistent with its this species sharing the most recent common ancestor with *O. sativa*. Some putative transcription factor binding sites align with other bioinformatic signatures. However, this indicator was the least harmonious with other bioinformatic data assayed.

Guides selected for targeting the 5' upstream region of *OsSTOMAGEN* are indicated by blue triangles in Figure 1e. The guide nearest to the translation start site is within the peak region of the ATAC-seq, Chip-Seq, and CNS. All guides selected correspond to at least one signature detected in the bioinformatic analysis, prioritizing regions where ATAC-seq, Chip-seq, and CNSs peaks coincided.

A construct containing the eight rationally designed guides was subsequently used to transform rice (*Oryza sativa* cv. Kitaake) and generate six unique promoter alleles (Figure 1f). All alleles generated indicate editing outcomes consistent with simultaneous activity of multiple guides. Some alleles also contain indels at specific guide sites suggesting partially asynchronous guide activity as well. A single guide construct was used to generate coding sequence mutations in the first exon of *OsSTOMAGEN* (Supplemental Figure 1).

The genetic diversity generated by targeting the 5' upstream region of *OsSTOMAGEN* and its coding sequence were subsequently assayed for corresponding phenotypic diversity.

3.3.2 *OsSTOMAGEN* promoter alleles alter stomatal density and morphology

The phenotypic implications of *OsSTOMAGEN* promoter and coding sequence mutations were assessed firstly by measuring stomatal density. The stomatal density of *stomagen* in *Oryza sativa* cv. Kitaake exhibited an 80% reduction of wild type stomatal density, consistent with knockout phenotypes observed in cv. Nipponbare and cv. IR64 [14,232](Figure 2a, Supplemental Figure 2). The panel of promoter-edited alleles exhibited a broad diversity of stomatal densities including lines with densities lower than— and greater than — wild type (Figure 1a, Supplemental Figure 2). Two promoter alleles exhibit a phenotypic gain-of-function while the remaining four alleles exhibited stomatal density phenotypes intermediate to wild-type and *stomagen*. The ranking of stomatal

densities remained the same in greenhouse and growth chamber conditions (Figure 2a, Supplemental Figure 2).

In addition to density, guard cell length was measured as a proxy for stomatal size. Variation in stomatal size was also observed in the panel of *OsSTOMAGEN* alleles assayed (Figure 2b). A negative, linear, correlation of stomatal density and size was established (Figure 2c). However, the strength of this liner relationship was diminished primarily by two promoter alleles, 4 and 5. The stomatal size of allele 4 was greater than expected, whereas the stomatal size of allele 5 was smaller. Broad quantitative variation in stomatal density and size was successfully established by coding sequence and promoter editing.

3.3.3 *OsSTOMAGEN* transcript abundance assayed across multiple tissue types does not correspond to stomatal density phenotypes

We next determine the relationship of expression and phenotype in our promoter-allele panel by assessing transcript abundance of *OsSTOMAGEN* across many tissue types. *OsSTOMAGEN* transcript abundance was measured in each promoter allele relative to wild type in flag leaves, seedlings, and developing leaves (Fig 2d,e,f). In developing leaves where *OsSTOMAGEN* exhibits the greatest expression in wild type (Supplemental Figure 3), there is no obvious co-linearity of stomatal density and expression level. A similar lack of an association between expression and phenotype is observed in seedlings and flag leaves (Fig 2e and 2f).

Variation in expression level is observed among promoter alleles, however. The ratio of *OsSTOMAGEN* present within each tissue type differs in each promoter allele relative to wild type (Supplemental Figure 3). Promoter editing of *OsSTOMAGEN* yields differences in transcriptional regulation; however the differences measured at the developmental timepoints may not necessarily be directly related to stomatal morphological variation. Despite an unclear transcriptional mechanism for producing stomatal morphological variation, the diversity in stomatal characteristics generated through promoter editing were ideal for further evaluation of to the relationships between stomatal density and gas exchange physiology.

3.3.4 Near-isogenic panel of stomatal variants reveals relationship of density to gas exchange physiology

The anatomical diversity of stomatal density generated by editing the promoter and coding sequence of *OsSTOMAGEN* in the same genetic background was primed for further assessments of physiology. The relationship of stomatal density to carbon assimilation, stomatal conductance, intrinsic water-use efficiency, ΦPSII, J_{max} and V_{cmax} were established leveraging the diversity inherent to the panel (Figure 3, Supplemental Figure 4, Supplemental Figure 5). A strong, positive, linear association between stomatal density and carbon assimilation, stomatal density and stomatal conductance, and stomatal density and ΦPSII at a light intensity of 1500 μmol photons m^{-2} s^{-1} are reported (Figure 3a,b,e and Supplemental Figure 4). Likewise, a strong, negative,

linear association between stomatal density and intrinsic water-use efficiency was established (Figure 3c). Stomatal limitation, derived from A/C_i curves, in *stomagen* far exceeded other promoter alleles (Figure 3d, Supplemental Figure 5). Limited variation in stomatal limitation was observed among promoter alleles, however. Additionally, measurements of J_{max} and V_{cmax} derived from A-Ci curves indicate some differences among alleles.

In order to establish clear associations of anatomy to physiology under vegetative drought stress, the response of stomatal conductance in stomatal morphological variants was assayed. The variation in stomatal conductance of stomatal morphological variants in well-watered conditions is nearly eliminated after imposing vegetative drought (Figure 3e, f). No significant differences among any promoter alleles were detected (Figure 3f). The ratio of the mean stomatal conductance in vegetative drought relative to well-watered conditions did vary among stomatal density lines. Lines with densities that exceeded wild type exhibited greater phenotypic range in response to diverse watering regimes (Figure 3f). Variable physiological responses to vegetative drought among promoter allelic variants were observed

3.3.5 Capacity for stomatal density reprogramming in response to abiotic stress is reduced among some panel members

Whereas *stomagen* maintained a near consistent overall stomatal conductance regardless of drought status, other stomatal morphological variants exhibited greater stomatal conductance responsiveness. A similar assay to observe longer-term developmental responsiveness to vegetative drought was also undertaken. A moderate vegetative drought applied to *Oryza sativa* cv. Kitaake induced stomatal density increases in the wild type background (Figure 4a). This finding is consistent with previous studies where a vegetative drought was applied to a grass species [239,249]. *stomagen*, however, exhibited a very limited stomatal density reprogramming response after drought, indicating the essential role of the *OsSTOMAGEN* pathway in mediating stomatal density reprogramming in abiotic stress (Figure 4a).

Most *OsSTOMAGEN* promoter alleles exhibited a near wild type increase in stomatal density after abiotic stress (Figure 4a). However, in alleles 1 and 6, the capacity for drought responsiveness stomatal density reprogramming was slightly diminished (Figure 4a). Dynamic environmental responses may thus be affected by promoter editing. Explorations of *OsSTOMAGEN* transcript abundance after abiotic stress treatment were undertaken to discern the effect of promoter edits on expression in a dynamic environment.

A transcriptional increase of *OsSTOMAGEN* under vegetative drought of wild type consistent with an increase in stomatal density was observed (Figure 4b). Likewise, promoter allele 1 also exhibited an increase in *OsSTOMAGEN* transcript abundance, despite exhibiting limited stomatal density reprogramming. No *OsSTOMAGEN* expression increases after vegetative drought were detected in any other promoter

allele. Consistent with previous *OsSTOMAGEN* expression data, limited co-linearity between expression levels and phenotype in response to vegetative drought, despite a consistent stomatal density increase among most promoter alleles (Figure 4b, Supplemental Figure 6).

3.4 Discussion

Editing cis-regulatory regions of genes of interest can generate quantitative variation. In this case, application of this approach to *OsSTOMAGEN*, a positive regulator of stomatal density, facilitated the production of an array of promoter alleles representing 70% to 120% of wild type stomatal density in the greenhouse. A multiplexed guide design approach leveraging publicly available bioinformatic tools enabled the selection of guides that had a substantial impact on phenotypic outcomes. While this rational guide design approach has been markedly effective in many studies, the relative contributions of editing specific promoter motifs, identified through a range of predictive indices, to phenotypes is not yet clear. Our approach nonetheless produced six distinct promoter alleles each corresponding to a unique stomatal density phenotype. A higher stomatal density was observed in two edited promoter alleles. To date, gain-of-functions mediated by promoter editing have been associated with large chromosomal rearrangements [250]. We report here, the first demonstrated phenotypic gain-of-function without a large chromosomal rearrangement. A unique attribute of these two alleles is a complete lack of editing at the guide site nearest to the transcription start site. This guide also happens to be located within the region with the most significant ATAC-seq, Chip-seq, and conserved non-coding sequence peaks relative to all other guides. It is possible that the region at this guide site mediates interactions that enhance expression. The maintenance of this guide site in combination with other cis-regulatory that may eliminate repressors of transcription could operate simultaneously to yield the phenotypes described.

The mechanism by which relatively small indels in the promoter of *OsSTOMAGEN* could generate misexpression was captured by assaying transcript abundance in various tissues. There were differences in *OsSTOMAGEN* expression levels among promoter alleles within various tissue types. Additionally, the ratio of expression among tissues types for each promoter allele differed from wild type. However, differences in stomatal density could not be directly attributed to expression levels observed. It is noteworthy that stomatal density phenotypes did not relate to expression levels for any allele in any tissue type assayed (Figure 2d,e,f). This finding is consistent with some previous applications of promoter editing where expression levels of targeted genes were not explanatory of phenotypic outcomes [77,235]. Whereas, in some promoter editing applications, altered expression levels are indeed consistent with phenotypes, our study, among others, finds no co-variation of expression levels with phenotypic outcomes in promoter edited alleles [77,200,235]. In examples where expression levels do not predict phenotypes, misregulation of expression of targeted genes may occur in highly discrete spatial or temporal zones such that capturing the transcriptionally relevant outcomes of promoter editing is challenging. A better understanding of when

and where transcription of genes is pertinent to development may enable expression to be used as a proxy for phenotype in applications of promoter editing. However, the implications of aberrant expression on phenotypic outcomes may still be hard to predict. Notably, expression of *OsSTOMAGEN* was lowest in wild-type flag leaf tissues collected from leaf tips. Stomatal development occurs longitudinally across the leaf axis, such that stomatal complexes are fully developed in leaf tips corresponding to low relative expression of *OsSTOMAGEN* (Supplemental Figure 3) [180]. Promoter editing, in this case, caused metabolic flux to be directed towards transcribing *OsSTOMAGEN* in a developmental context where its function is likely limited. Promoter editing was nonetheless able to alter to the transcript abundance of the targeted gene and mediate the generation of stomatal density variation in rice.

Beyond stomatal density, promoter alleles of *OsSTOMAGEN* had implications on stomatal size. There is a well-documented inverse relationship of stomatal density and stomatal size across many species including rice [15,232,251–253]. To a large extent this trend remained true among the panel of promoter alleles with two notable exceptions. Alleles 4 and 5 both deviated significantly from the expected relationship of size to density. Developmentally, this may allude to a previously suggested role of OsSTOMAGEN in mediating stomatal size in addition to density [254].

Promoter editing successfully induced morphological variation in rice stomata. This variation was leveraged to establish relationships of stomatal density to carbon assimilation and stomatal conductance. Strong, positive, linear relationships of stomatal density with stomatal conductance and with carbon assimilation were established, consistent with previous reports [15,191,232,236,255]. CRISPR/Cas9 mediated production of *OsSTOMAGEN* promoter alleles enabled the establishment of discrete structure-function relationships in near-isogenic background. The generated CRISPR-NILs (cNILs) provide a major advantage in overcoming previous limitations of mapping populations of natural variation for understanding how stomatal density relates to gas exchange. cNILs are able to mitigate the confounding influence of genetic heterogeneity and the challenge of minimal diversity that limited previous explorations of how stomatal density relates to gas exchange. Induced variation is morphological parameters beyond stomatal density such as stomatal size or mesophyll conductance may potentially influence the physiological parameters measured. Regardless, promoter editing may be broadly applied to elucidate the role of variation in a specific trait in an array of phenotypic outcomes. One could imagine leveraging this approach for improved understandings of the relationships of root lengths to drought tolerance, plant height to yield, or trichome density to pathogen resistance, among many other possibilities.

The cNIL panel established in this study was exploited further for evaluations of physiological to stomatal morphological alterations in vegetative drought. A strong, positive, linear relationship of stomatal density to stomatal conductance and carbon assimilation was also present in this environmental condition. The physiological plasticity of higher density lines exceeded lower density lines as indicated by lower ratios of stomatal conductance in vegetative drought relative to well-watered conditions. cNILs with stomatal densities approaching the phenotype of the null coding sequence

mutant were underrepresented in our promoter allele panel. Generating additional promoter alleles or crossing the null allele to the existing promoter alleles already could expand the phenotypic diversity of the cNIL panel and associated strength of the derived structure-function relationships

Beyond capturing differing physiological plasticity of promoter alleles in vegetative drought, we sought to establish an understanding of the implications of promoter editing on developmental plasticity. Environmental cues, including drought stress, are known to cause stomatal density reprogramming [239,249,256]. We establish here an additional demonstration of stomatal density reprogramming in a grass (*Oryza sativa* cv. Kitaake) to moderate vegetative abiotic stress. Consistent with a previous report of stomatal density shifts in drought stress, we find moderate increases in stomatal density in the applied drought regime. Stomatal closure induced by drought stress may result in less evapotranspirative cooling, leading to warmer leaf temperatures and overall greater heat stress [15,232]. Stomatal density increases may be a heat stress response to increase rates of cooling[239,249]. Some promoter alleles exhibited a lowered abiotic stress responsive capacity.

Modifying cis-regulatory elements may hinder integration of environmental cues in environmentally responsive traits. It is well understood that cis-regulatory elements and their corresponding transcription factors modulate transcription of responsive genes when triggered by environmental stimuli [244,257]. We demonstrate here, for the first time, the developmental implications of promoter editing on trait variation in dynamic environments. In this case, stomatal density reprogramming capacity during abiotic stress was lessened in select alleles. Applications of promoter editing for improved crop improvement must be wary of pleiotropy of edits in dynamic environments inherent to nearly all production landscapes. Promoter editing has been leveraged as a tool to prevent upregulation of susceptibility loci during pathogen invasion [29]. Dampening environmental response by gene editing cis-regulatory elements may also be a desirable approach for other traits achievable by promoter editing.

Taken together, our data offer an improved understanding of the opportunities of cis-regulatory element editing in generating quantitative trait variation for broad and dynamic environments (Figure 5). Stomatal morphology variants generated by promoter editing can be matched with the relevant production landscape best suited. Elite cultivars confined to narrow geographies due to limitations in a discrete set of traits could theoretically be optimized by promoter editing for compatibility to a much broader set of environments. Compatibility with dynamic environments may be compromised however when pleiotropic effects of cis-regulatory element editing is considered. Our work offers an expansion of promoter editing as a tool for: generating phenotypic gain-of-function without large chromosomal rearrangements, establishing near isogenic panels for assessments of discrete structure-function relationships, and generating expansive trait diversity compatible with similarly expansive environments.

Figure 1:

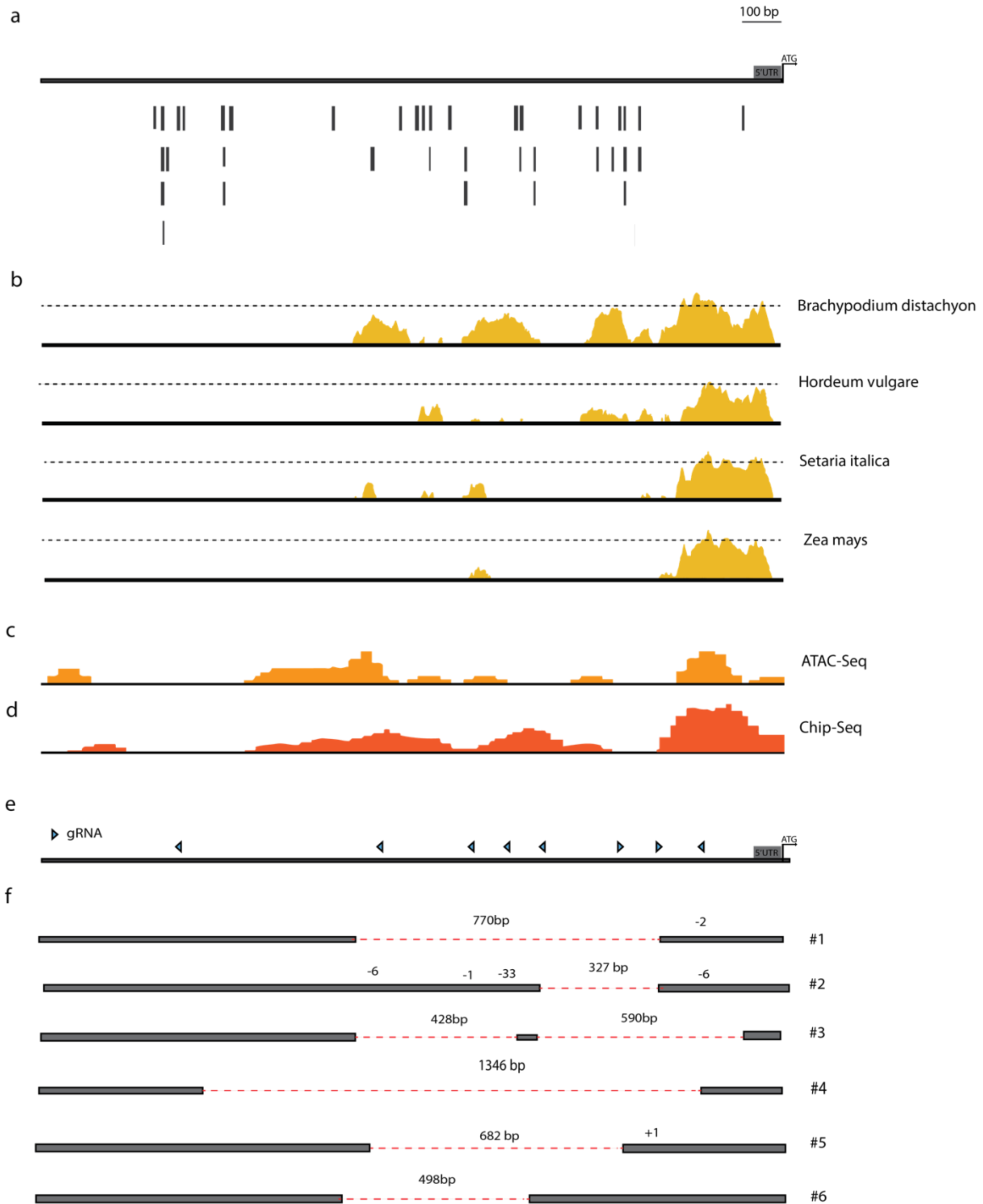


Figure 1| Rational guide design approach for targeting the promoter of *OsSTOMAGEN*

Bioinformatic analysis facilitated the design of eight guides for multiplexed CRISPR/Cas9 mediated editing of *OsSTOMAGEN* promoter. (a) Putative transcription factor binding sites using a 99% identity threshold annotated with reference to translation start site (b) mVISTA plot displaying conserved non-coding sequences among evolutionarily dispersed Poaceae family members. The dashed line represents a 75% conserved threshold. Peaks displayed represent regions of minimum 50% conservation. Poaceae family members are arranged from nearest evolutionary relationship to most distant (c) ATAC-seq data extracted from RiceENCODE is shown for the promoter region of *OsSTOMAGEN* (d) Chip-Seq data on the histone, H3K27ac, extracted from RiceENCODE database. (e) A summary of the positions and orientations of the guide sequences used to target the promoter of *OsSTOMAGEN* each blue triangle represents an individual guide, with the triangle pointing towards the 3' PAM site. (f) An overview of each unique allele generated. Large deletions are represented by red dashed lines and indels at each guide site are denoted by (+) to indicate insertions or (-) for deletions alongside a number representing total quantity of base pairs associated with each indel. Each allele is labeled with a unique number.

Figure 2:

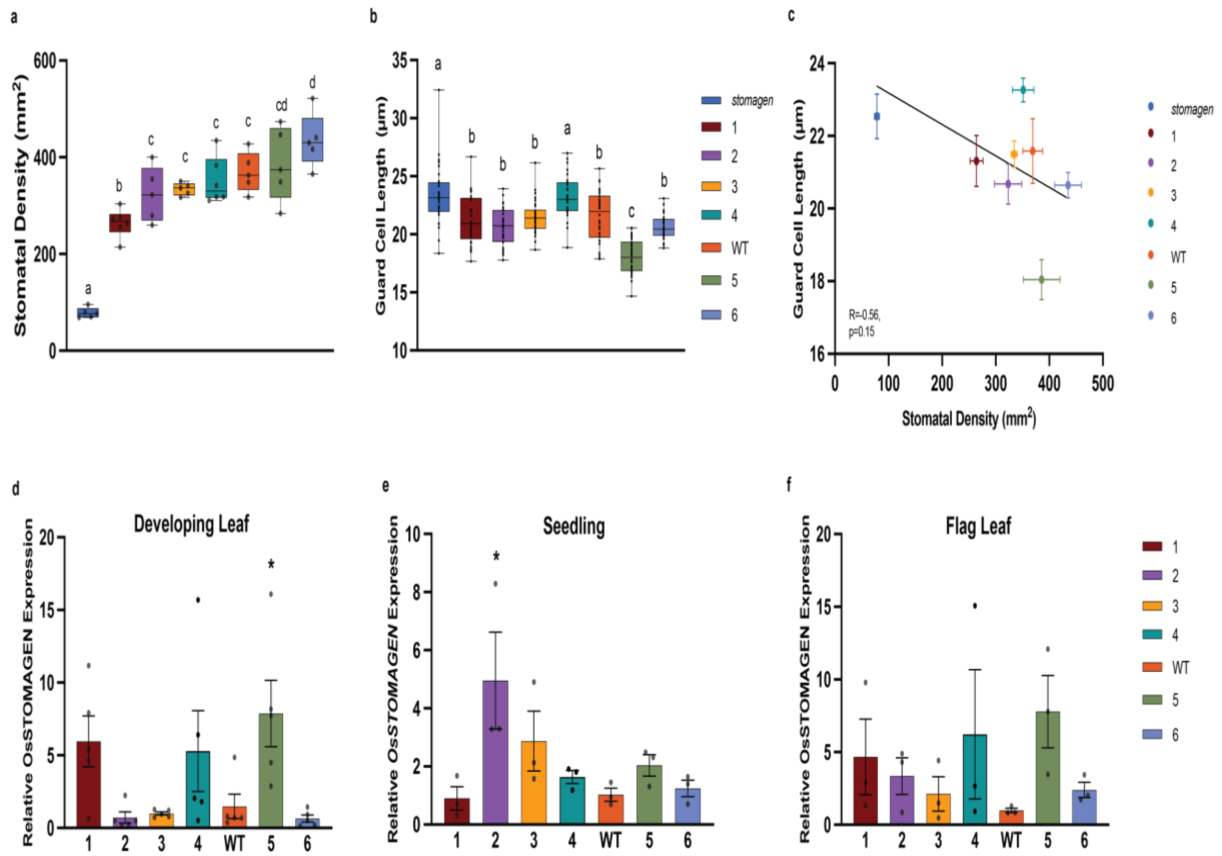


Figure 2 | Stomatal -density, -morphological, and *OsSTOMAGEN* transcript abundance variation among promoter alleles

(a)Box-and-whisker plot of the stomatal density of each allele assayed (b) Box-and-whisker plots of guard cell length of each allele assayed. In the box-and-whisker plot, the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Each dot represents a biological replicate. Letters indicate a significant difference between means ($P<0.05$, one-way ANOVA Tukey HSD post-hoc test). (c) The linear correlation between stomatal density and guard cell length. Dots represent the mean of each variable and error bars represent the standard error of the mean. The correlation coefficient (R) and p-value are reported on the plot. Barplots of normalized relative expression of *OsSTOMAGEN* of each allele in (d) developing leaves (e) seedlings and (f) fully expanded flag leaves, normalized to WT expression in each tissue type. Barplots mean is represented with error bars showing standard error of the mean. Asterisks represent a significant difference in expression relative to wild type ($P<0.05$, one-way ANOVA Tukey HSD post-hoc test).

Figure 3:

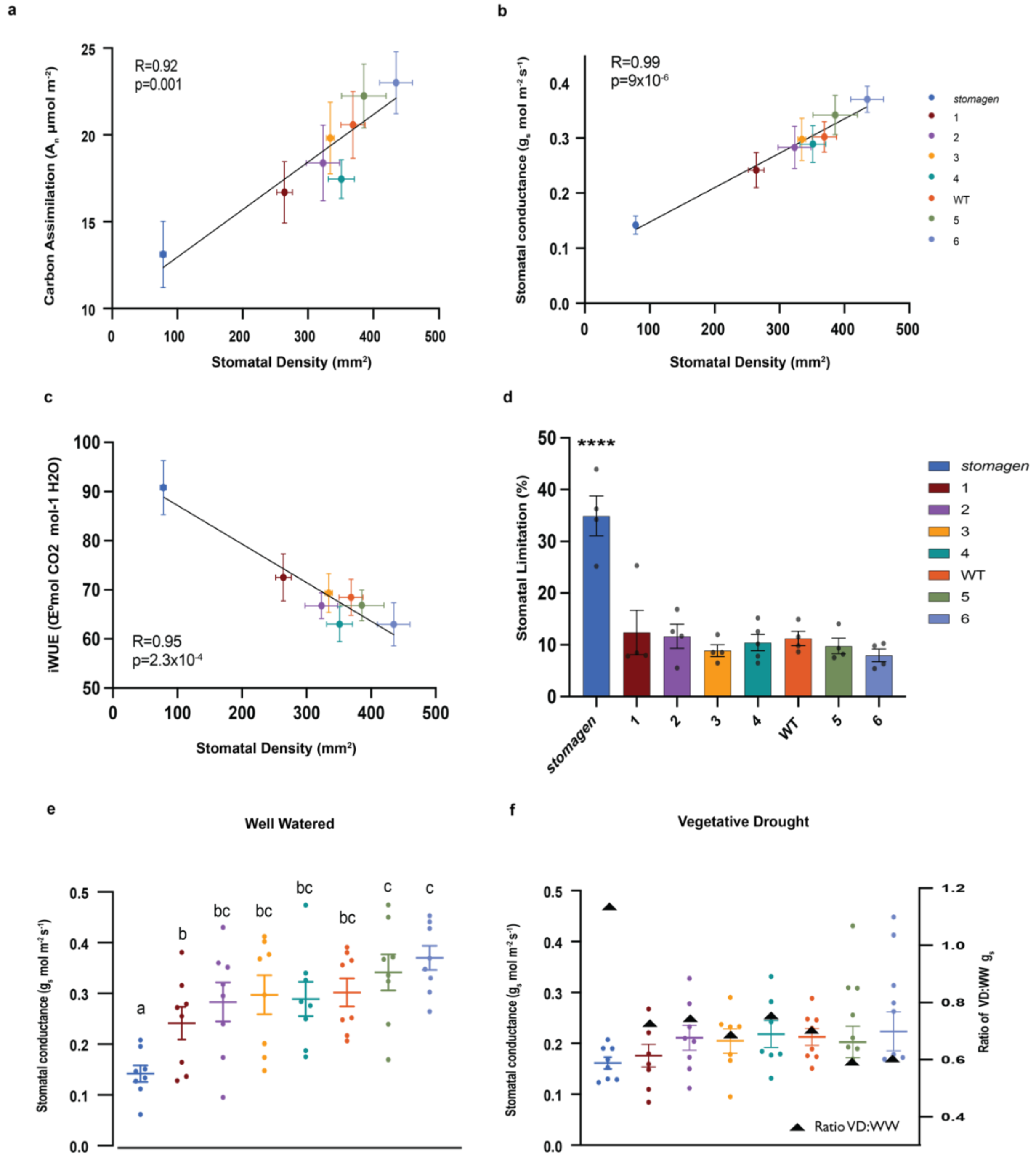


Figure 3 | Stomatal morphological variation corresponds to gas exchange variation in a near-isogenic panel

(a) Linear regression of stomatal density and (a) carbon assimilation and (b) stomatal conductance and (c) intrinsic water-use efficiency. Correlation coefficient (R) and p-value of each correlation are noted in each panel. Mean and standard error of the mean are reported. (d) A barplot of stomatal limitation percentages derived from A/C_i curves generated on biological replicates of each allele showing mean and standard error of the mean. Dotplot of stomatal conductance in (e) well-watered and (f) vegetative drought conditions in each allele assayed. Each dot represents a biological replicate with bars indicating mean and standard error of the mean. In (e) black triangles represent the ratio of the mean stomatal conductance values of vegetative drought relative to well-watered In each plot each genotype is represented by eight biological replicates. In plot (e) unique letters represent statistical significance between groups. In plot (d) **** represents a p-value <0.0001.

Figure 4:

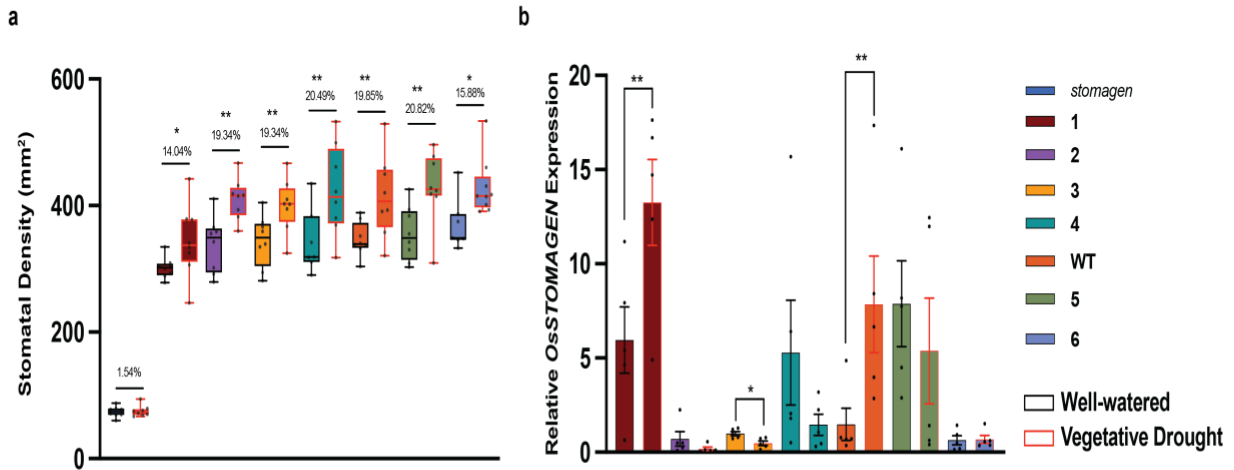


Figure 4| Stomatal developmental and *OsSTOMAGEN* transcriptional responses of alleles to vegetative drought

(a) Stomatal density reprograming in response to vegetative drought. The percent increase of vegetative drought stomatal density relative to well-watered is reported above each genotype. Each dot represents a biological replicate. (b) Barplots of relative *OsSTOMAGEN* expression in each allele in well-watered and vegetative drought. In the box-and-whisker plot, the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Barplot shows means and error bars represent standard error of the mean. In (a) and (b) black and red outlines of represents well-watered and vegetative drought, respectively. * represents a p-value <0.1, >0.05, and ** represents a p-value <0.05 (Student's t-test).

Figure 5:

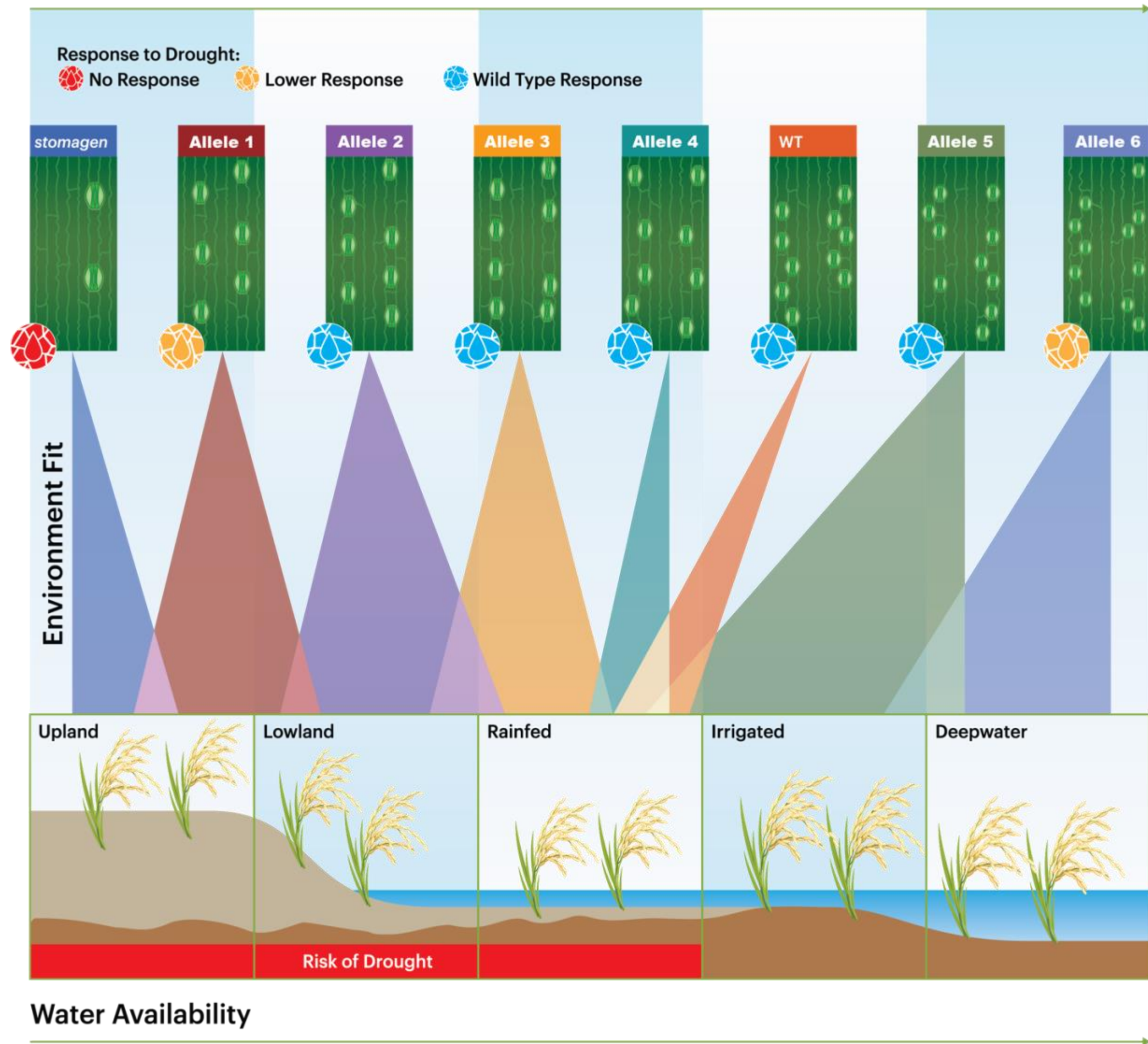
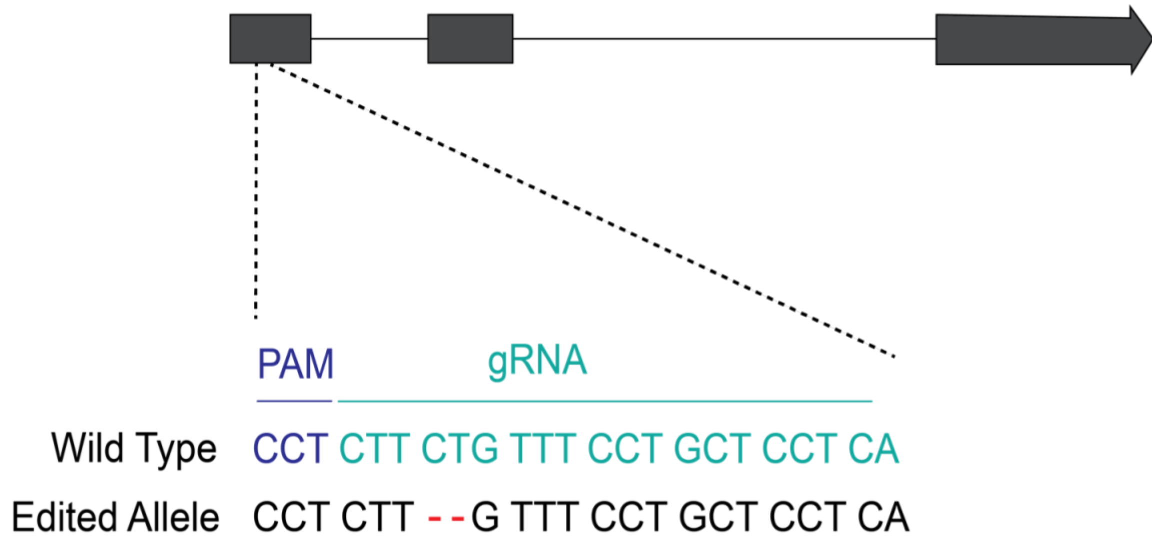


Figure 5 | Stomagen density variation can be matched to broad and dynamic production environments

Rice is produced in diverse environments with varying water availabilities and fluctuations. Variation generated by gene editing can be matched to variation in environments. Each stomatal variant is represented by a graphic of stomatal density and size. Colored spotlights indicated potential environmental range of each allele according to stomatal characteristics including stomatal density and responsiveness to abiotic stress represented by red, yellow, and blue water droplet icons.

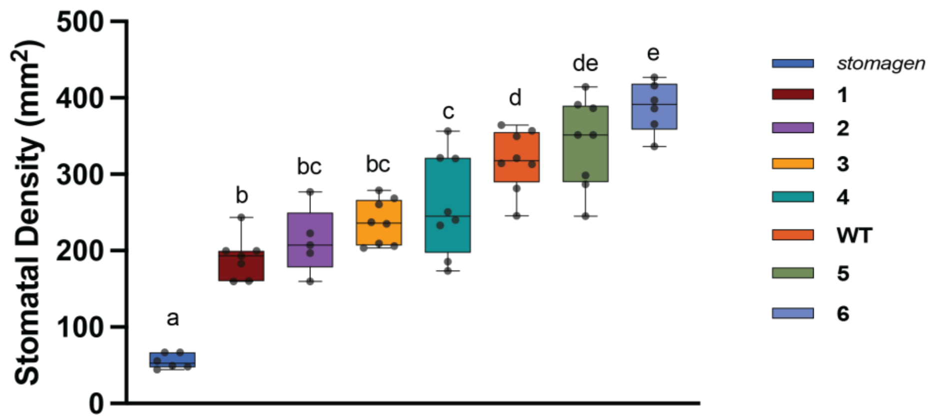
Supplemental Figure 1:



Supplemental Figure 1| Genotype of edited *stomagen* allele

The gene model of *OsSTOMAGEN* with the location of the CRISPR/Cas9 guide RNA indicated in blue. The unique edits generated by CRISPR/Cas9 are shown in red.

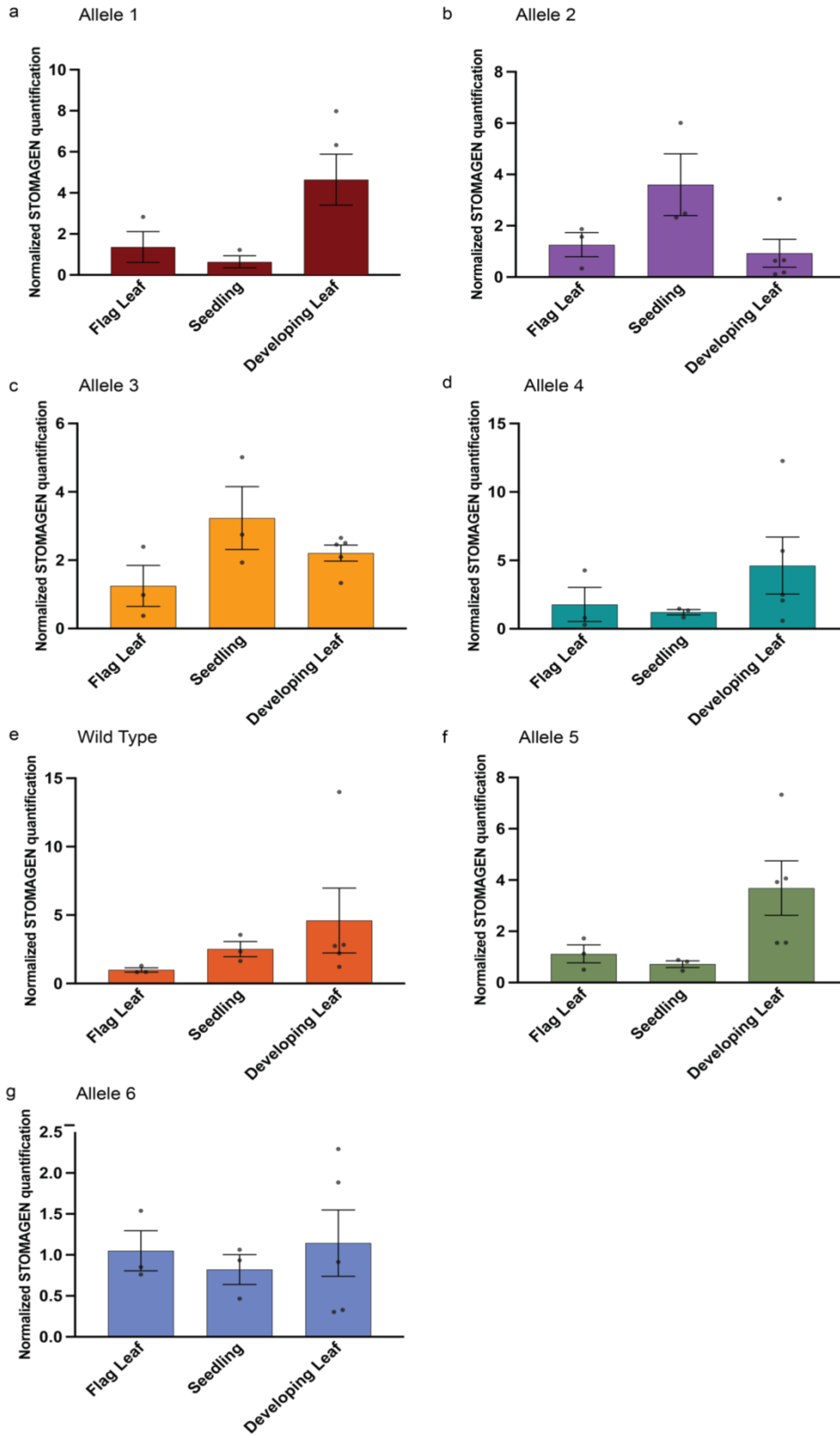
Supplemental Figure 2:



Supplemental Figure 2| Stomatal density variation of promoter alleles grown in growth chamber

a) Box-and-whisker plot of the stomatal density of each allele assayed. In the box-and-whisker plot, the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Each dot represents a biological replicate. Letters indicate a significant difference between means ($P<0.05$, one-way ANOVA Tukey HSD post-hoc test). Plans were grown in chambers at 28 °C for day-length periods of 16 h in 400 μmol photons/m^2/s of light and 80% relative humidity.

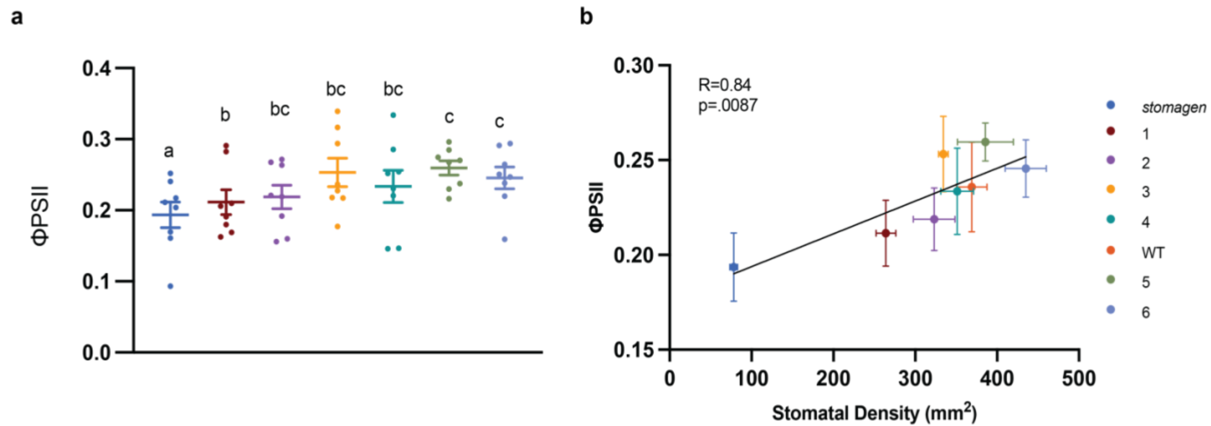
Supplemental Figure 3:



Supplemental Figure 3| Relative Expression of *OsSTOMAGEN* in varying tissues within each promoter allele

A comparison of *OsSTOMAGEN* transcript abundance among flag leaves, seedlings, and developing leaves in (a) Allele 1 (b) Allele 2 (c) Allele 3 (d) Allele 4 (e) Wild type (f) Allele 5 (g) Allele 6. In each genotype values are relative to flag leaf expression and normalized to the average of two housekeeping genes. Barplot shows means and error bars represent standard error of the mean

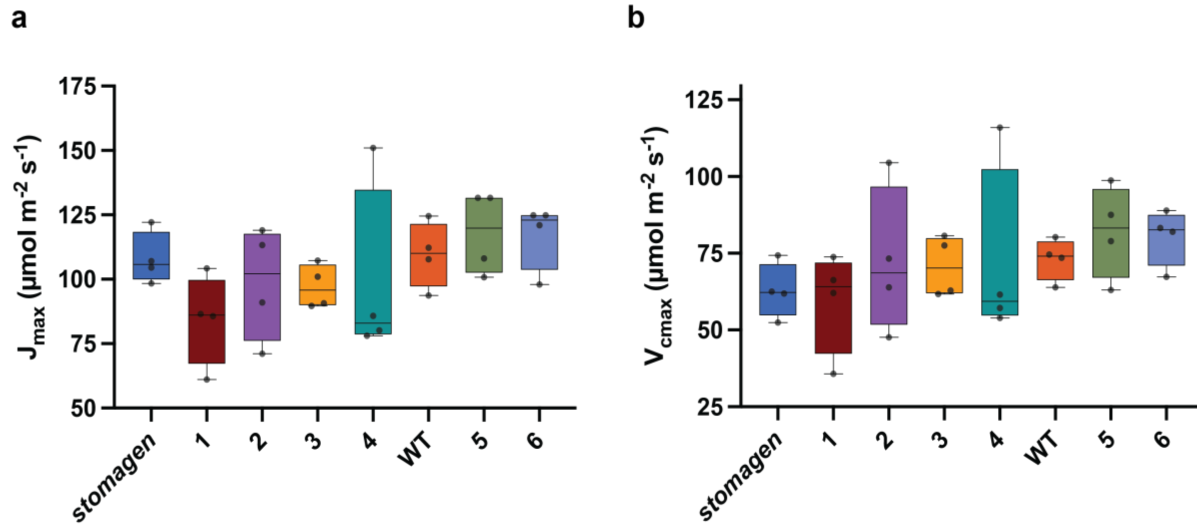
Supplemental Figure 4:



Supplemental Figure 4| ΦPSII responds to stomatal variation

(a) Dotplot of ΦPSII measured on each allele. Each dot represents a biological replicate with bars indicating mean and standard error of the mean. Letters indicate a significant difference between means (P<0.05, one-way ANOVA Tukey HSD post-hoc test). (b) Linear regression of stomatal density and ΦPSII. Correlation coefficient (R) and p-value of each correlation are noted in the panel.

Supplemental Figure 5



Supplemental Figure 5| J_{max} and V_{cmax} of *OsSTOMAGEN* alleles

Box-and-whisker plot of (a) J_{max} and (b) V_{cmax} derived from A-Ci curves using Plantecophys package in R studio. In the box-and-whisker plot, the center horizontal indicates the median, upper and lower edges of the box are the upper and lower quartiles and whiskers extend to the maximum and minimum values within 1.5 interquartile ranges. Each dot represents a biological replicate Each allele is represented by four biological replicates.

Supplemental Figure 6:

a

stomagen

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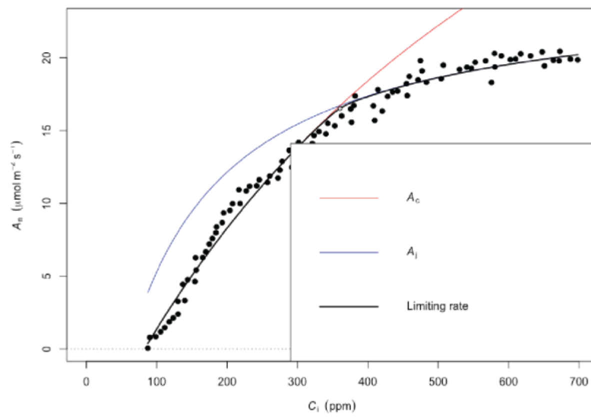

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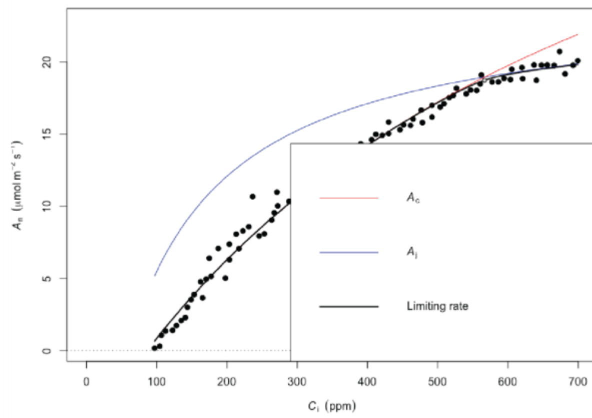

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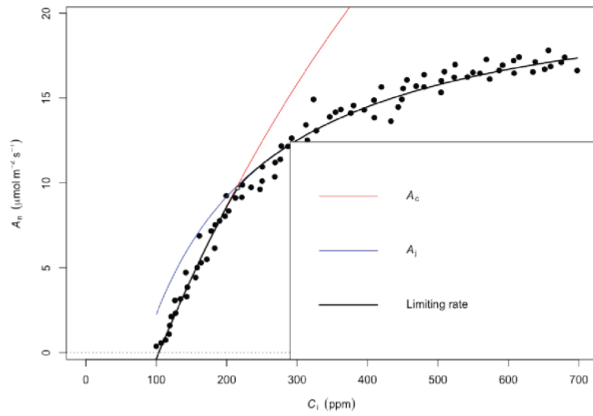

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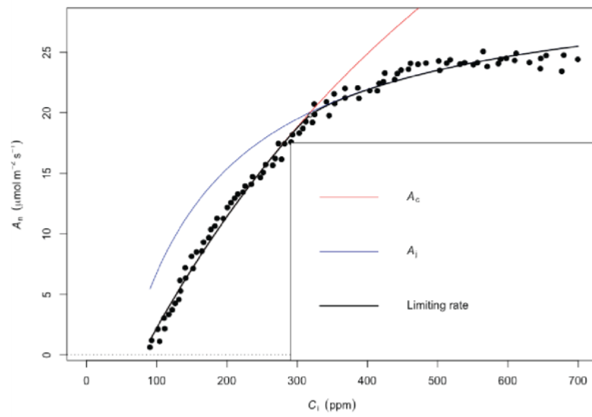

b

Allele 1

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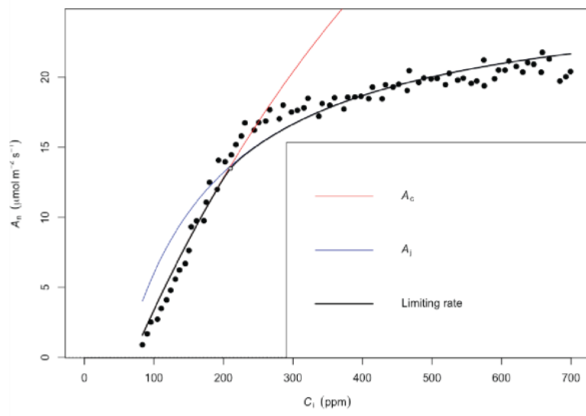

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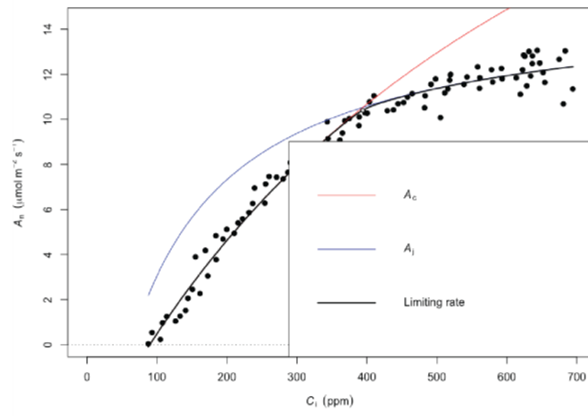

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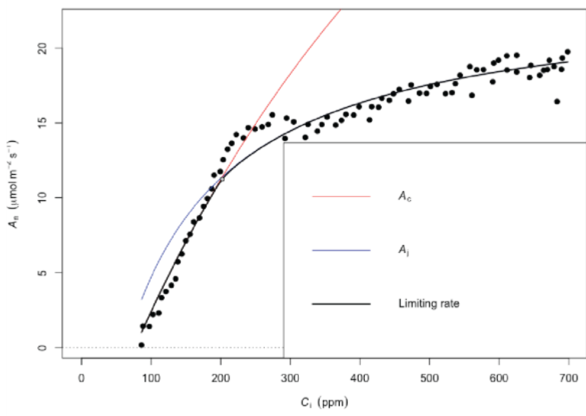

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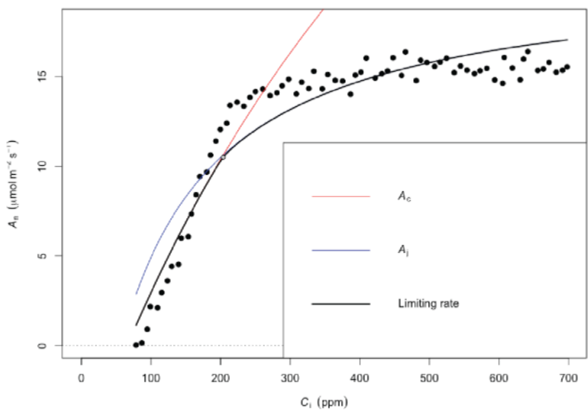

c

Allele 2

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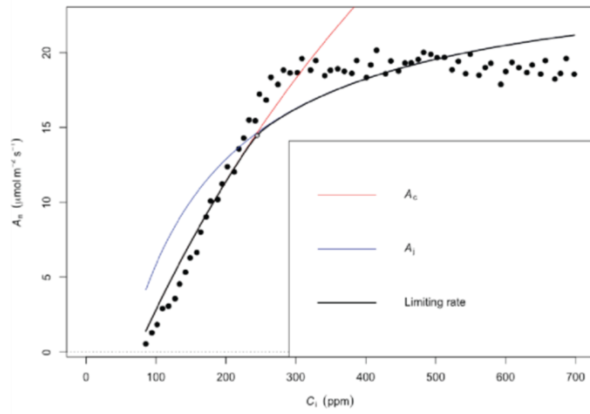

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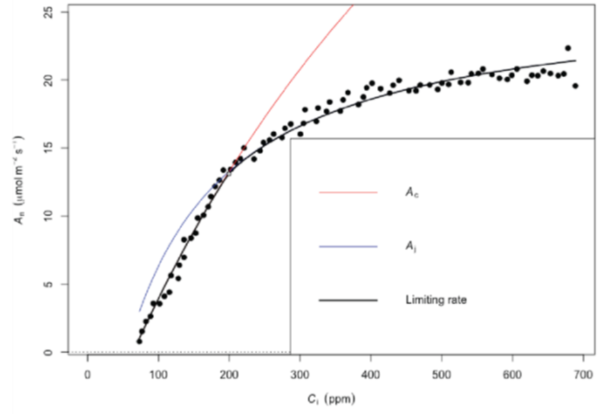

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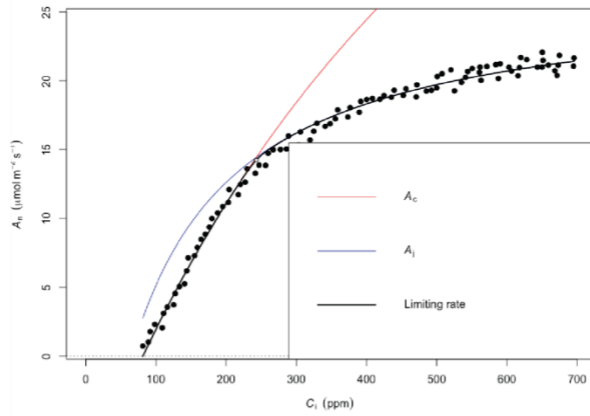

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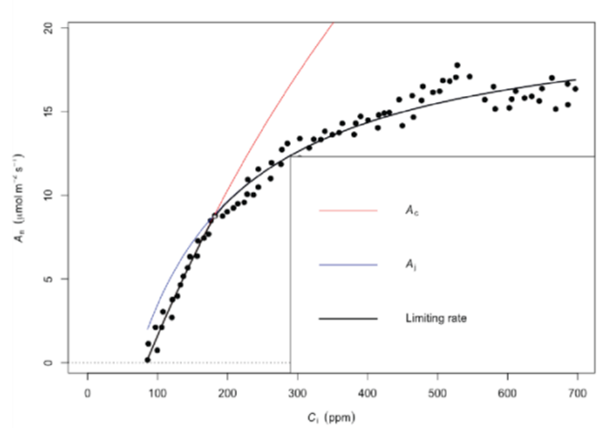

d

Allele 3

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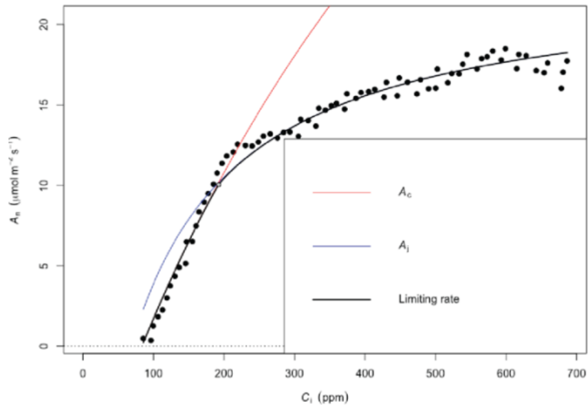

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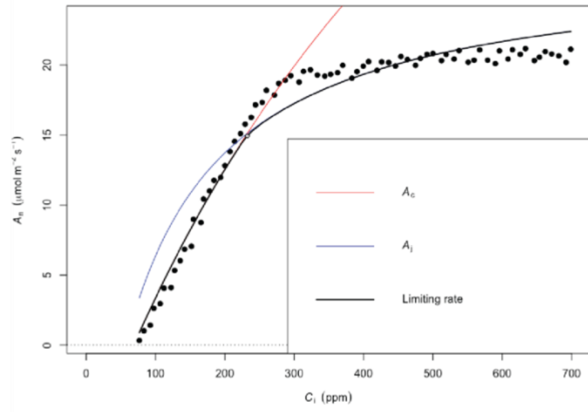

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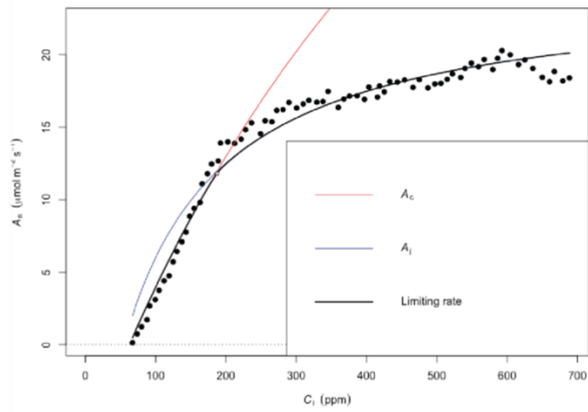

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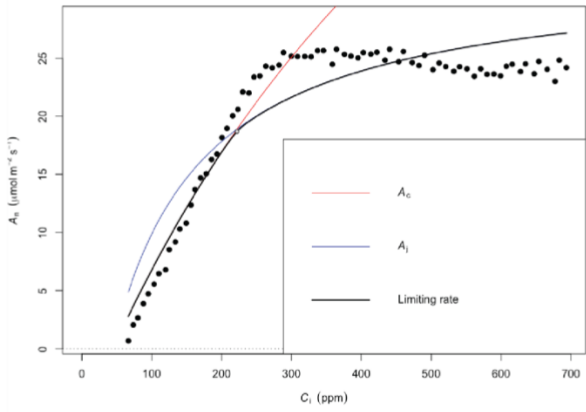

e

Allele 4

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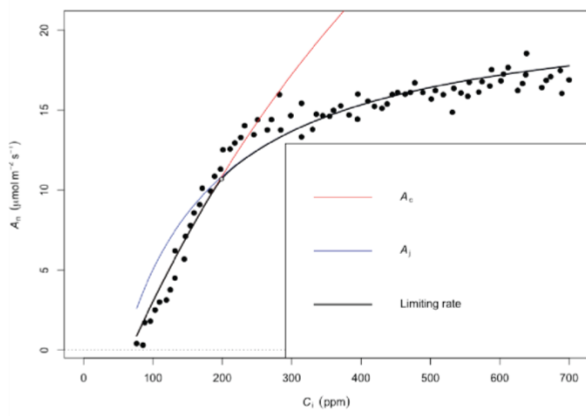

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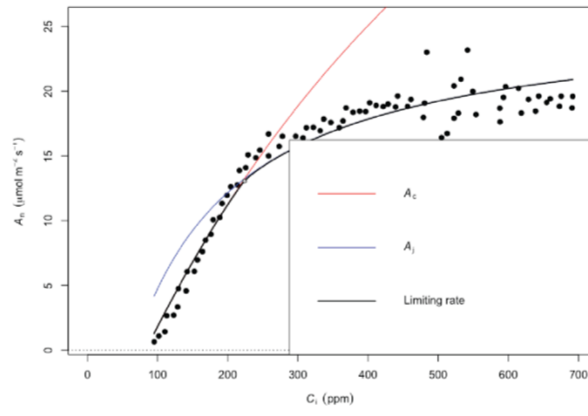

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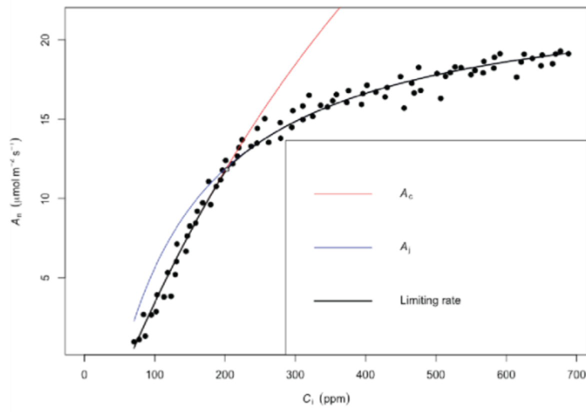

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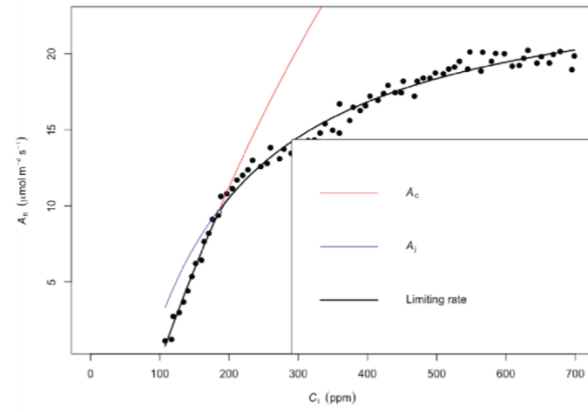

f

Wild type

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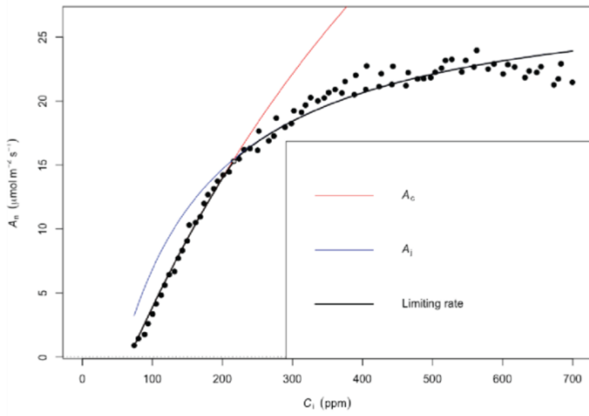

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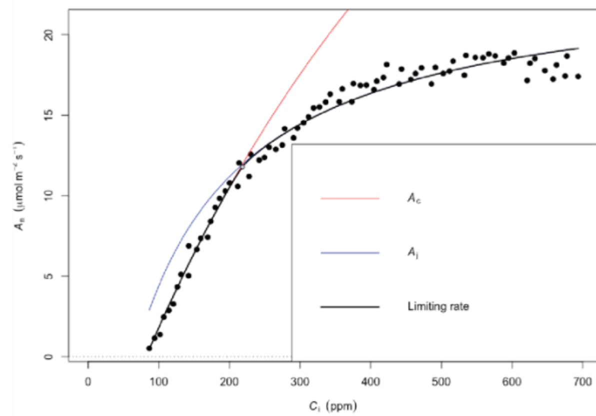

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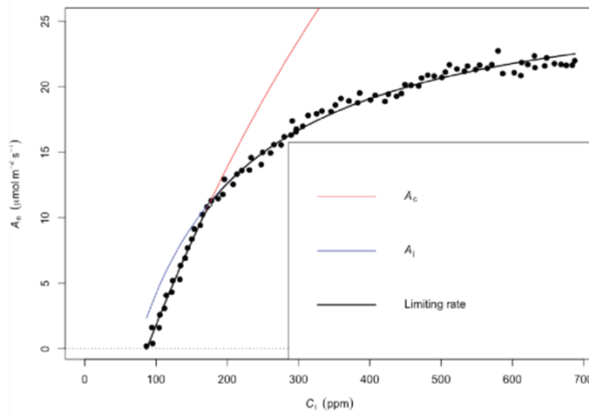

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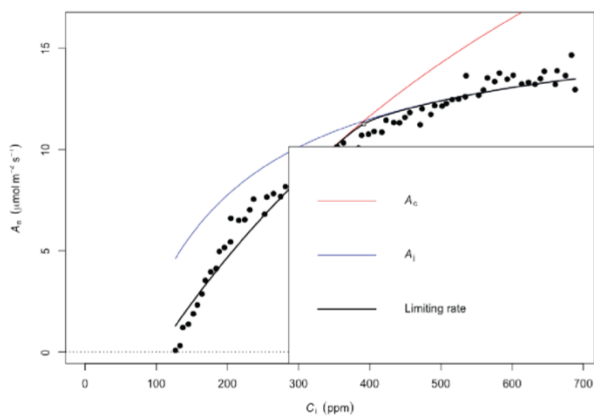

g

Allele 5

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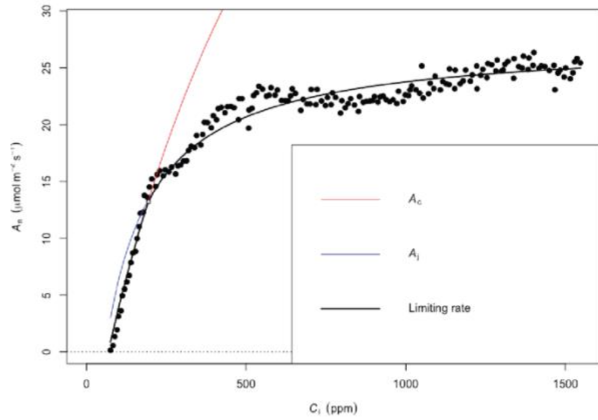

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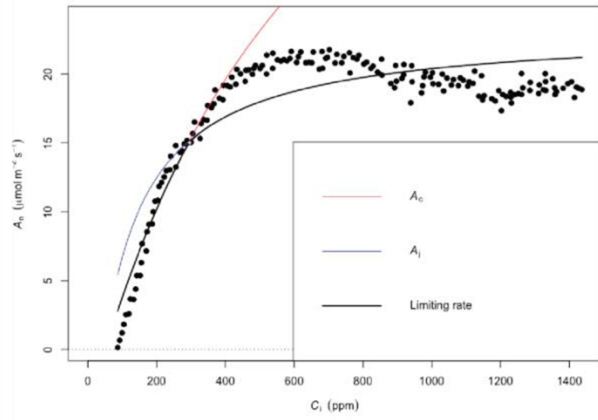

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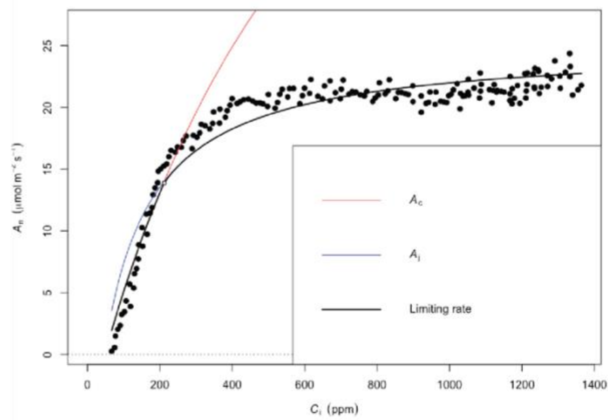

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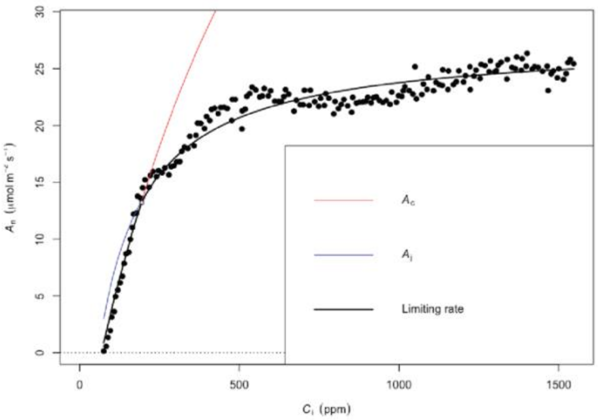

h

Allele 6

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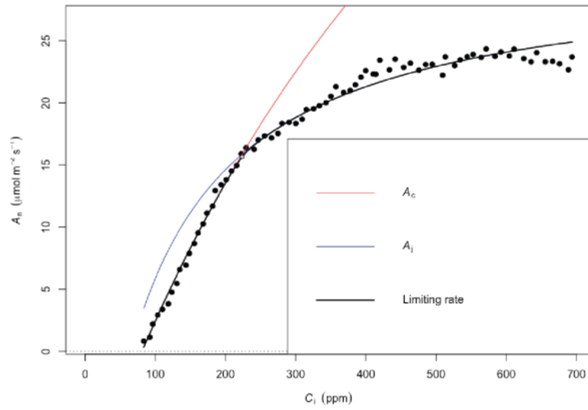

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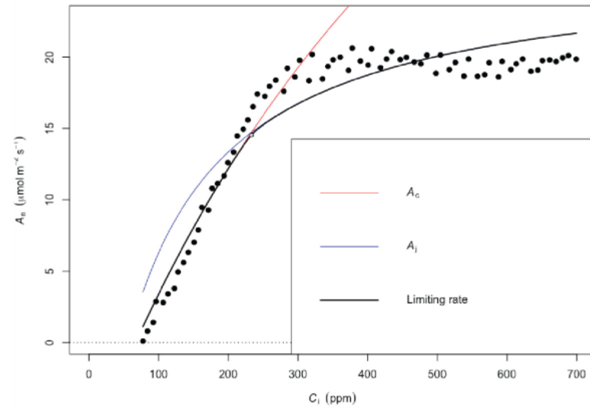

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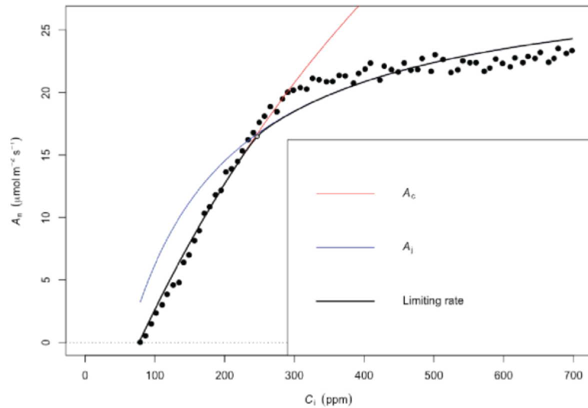
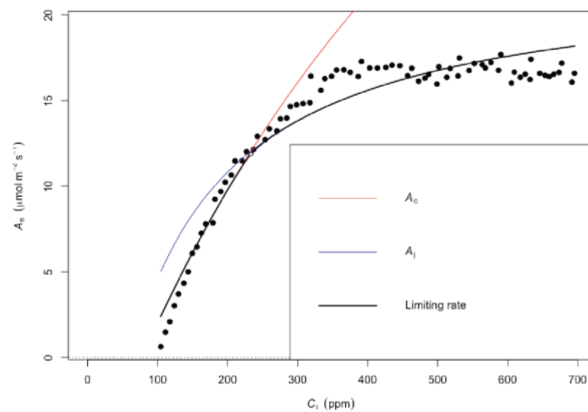


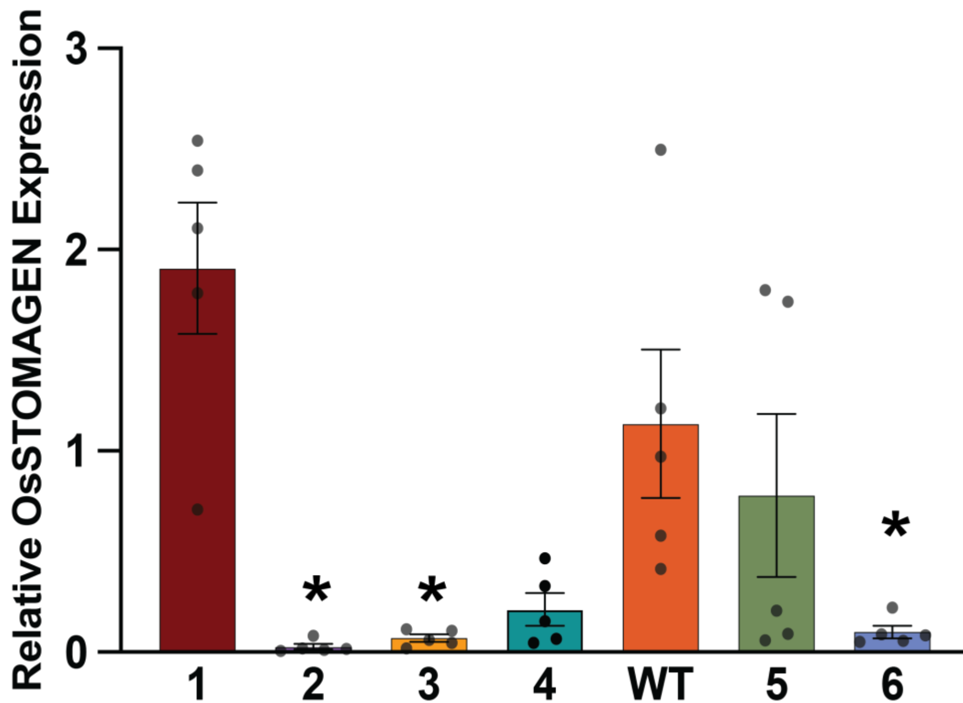
Replicate 4:



Supplemental Figure 6| A-Ci curves

The A-Ci curve fit to each biological replicate of (a) *OsSTOMAGEN* (b) Allele 1 (c) Allele 2 (d) Allele 3 (e) Allele 4 (f) Wild type (g) Allele 5 (h) Allele 6. Curves were fit to all Ci <700ppm for which assimilation values >0 using the ‘plantecophys’ package in Rstudio [247].

Supplemental Figure 7:



Supplemental Figure 7| *OsSTOMAGEN* expression after vegetative drought among promoter alleles

A comparison of *OsSTOMAGEN* transcript abundance among promoter alleles in developing leaves after vegetative drought. In each genotype values are relative to wild type expression and normalized to the average of two housekeeping genes. Barplot shows means and error bars represent standard error of the mean. Asterisks represent a significant difference in expression relative to wild type ($P<0.05$, one-way ANOVA Tukey HSD post-hoc test).

Table S1: Primer sequences

Sequence (5' to 3')	Application
TAACCTTGAGTTAGATCCAGTGAAGCAAC	Amplifying and subcloning *OsSTOMAGEN* promoter
AACCCTTCTTCAAACAAATGGATAGAGAATGG	
ATAGTCTCCAGCATTTGCTCCC	Amplifying *OsSTOMAGEN* coding sequence
CTGATGCAAAGGGGTACCTGAG	
ACCACTTCGACCGCCACTACT	Os*UBQ5* qPCR primers
ACGCCTAAGCCTGCTGGTT	
TTTCACTCTTGGTGTGAAGCAGAT	Os*eEF-1A* qPCR primers
GACTTCCTTCACGATTTCATCGTAA	
GCTCGTTGCAATCAAGGGCA	Os*STOMAGEN* qPCR primers
GCAGCCTCTCCTTGTTTAGAAC	

Table S2: Guide sequences arranged from distal through proximal to translation start site

TAAAATGTATTTAAAGCTTG
TTTGCACGCAATGAAGCATT
ATCTTGCAGAAAGCAATTGA
ACTTACCGCCTGTTACACGA
TGGGCGAGAAAGCAATGAGA
GTAGAACAAAAAGAACAAAG
GCAGAAGAGCACATGTATAA
CAGTGTTGTATAGCGAGAAG

Table S3: Summary of linear regressions

Regression of stomatal density by	Correlation Coefficient	p-value	Equation of the line of best fit
Guard cell length	-0.56	0.15	y=24-0.0087x
Carbon assimilation	0.92	0.001	y=10+0.027x
Stomatal Conductance	0.98	0.000009	y=0.085+0.00062x
Intrinsic water-use efficiency	-0.95	0.00023	y=95-0.079x
ΦPSII	0.84	0.0087	y=0.18+0.00017x

Chapter Four

Transporter editing in cassava validates local production of cyanogenic glucosides in, and export from cassava roots

4.1 Introduction

Applications of gene editing for improvement of rice, a major global staple crop, towards improved drought tolerance have shown some success but are nascent. Rice, along with maize and wheat comprise a vast proportion of total global calories consumed [258]. However, this dependence a limited set of crops has also limited dietary diversity, resulting in widespread micronutrient deficiencies [116]. Applications of gene editing to crops beyond rice are thus essential in safeguarding against the impositions of climate change and providing sufficient nutrition.

Cassava (*Manihot esculenta)* is a globally important staple crop, particularly in sub-Saharan Africa. Its widespread consumption may be attributed to its hardiness. Relative to other staple crops such as maize, wheat, and rice, cassava produces more energy per unit area in periods of drought and in marginal soils [259]. Furthermore, cassava roots can be stored below ground for extended periods of time prior to harvest, enabling greater management flexibility for producers [259]. Thus, cassava cultivation can safeguard against food insecurity especially as climate change imposes severe threats to agricultural productivity.

Despite its global importance, cassava accumulates human-toxic metabolites in the form of cyanogenic glucosides (CGs), chemical precursors to cyanide, which must be removed prior to safe human consumption [260]. During periods of environmental or sociopolitical stress, risk of improper cassava processing increases [260]. Chronic cyanide exposure as a result of insufficient processing can result in damage to the central nervous system, and in severe cases paralysis [260]. To mitigate the severe human health impacts of CGs, researchers have developed strategies to attenuate their accumulation in cassava. CG levels have been successfully reduced by gene editing to knockout CG biosynthesis genes [261,262].

Some evidence indicates that acyanogenic varieties generated through gene editing suffered from greater herbivory in greenhouse conditions, which may have implications for yield [262].

4.2 Methods

Guide Design and Plant Transformation

Target sequences were identified in *Manes.15G180400* using the AM560-2 reference assembly v8.1 and CRISPR-P 2.0 online software. Agrobacterium-mediated transformation was utilized to deliver CRISPR-Cas9 gene editing tools into friable embryogenic calli (FEC) of cassava accession 60444. Subsequently, plants were

regenerated following the protocol described by Taylor *et al* and Chauhan *et al* [263,264]. CRISPR constructs were transformed into homogenous FEC via *Agrobacterium* strain AGL1. Regenerated plants were maintained in Phytatrays II (Sigma-Aldrich) and subcultured every three weeks.

Soil Transfer

After approximately three weeks, rooted plantlets were transferred to soil. Fertilizer was applied during soil transfer by soaking premixed potting mix in aqueous solution per preparation in Gomez et al. 2023. After transferring to soil, plants were watered until thoroughly moistened. Low domes of 2” height were placed over pocket trays with drainage holes to allow evaporation. Trays were placed on misting bench atop a heating pad set at 80°F. Humidity was controlled at 100% and a white cloth with opacity 40% was placed atop trays [261].

After two weeks on the mist bench, the 6” domes replaced the vented 2” covers. After an additional week, plants were removed from the mist bench and placed in greenhouse room 127B experiencing ambient light and natural day length. Plants were reorganized to randomize location on the bench and spaced 6 plants to a 28 count pocket tray. Plants were fertilized and hand watered according to the protocol in Gomez et al., 2023.

Genotyping Methods

Two leaf punches were collected from the third fully expanded leaf and placed in a 2 ml microcentrifuge tube with 3 mm glass beads and frozen with liquid nitrogen. Samples were pulverized in the Mini Beadbeater (Biospec Products, Inc.) and DNA was extracted via modified CTAB protocol (Gomez et al., 2023). Incubation period at 65°C was extended to 20 minutes, after ethanol application to the pellet, tubes were left to evaporate in a 37°C incubator for up to three hours. The pellet was resuspended in 50µL water and left on the bench top to incubate overnight. Forward primer (5’ GGAGAACGGAAATGATCATGC 3’) and reverse primer (5’ TGAAGAGAGGAAGTACCAGAAAGG 3’) were designed to amplify the gRNA-targeted region in the *MeCGTR1* gene. PCR reactions were performed using Q5 2X Master Mix. Amplified products were sequenced and analyzed using Synthego ICE analysis [204]. Plants indicated as wild type in this assay contained no mutations at the guide site, but were transformed according to aforementioned methods.

Measurement of cyanide content in multiple cassava tissues

Two unique *cgtr1* alleles were assayed and compared to wild type control plants that also underwent tissue culture. Five biological replicates for each allele were selected for sampling. Plants were genotyped at the guide site using Sanger sequencing prior to the experiment. The first three plants from each line were simultaneously sampled for leaves and roots. The lowermost three leaves were removed to represent the bottom leaf data. The three uppermost leaves were likewise sampled for the top leaf data. All sample leaves were removed from the stem, sheared, and pulverized using a mortar and pestle. 100 mg of pulverized leaves was used for the picrate assay.

All roots larger than 1 cm in diameter were cleaned, dried, and cut into rounds with a kitchen mandolin set to the 1/4 mm setting. The periderm was removed, and root cross sections that preserved all layers were cut to weigh 100 mg.

A 4 cm sample of stem tissue located 8–12 cm above the soil level, was removed and manually stripped of its periderm. Samples were then pulverized, and 100 mg of the pulverized material was collected for subsequent picrate assay using a kit from the Australia National University Konzo Prevention Group (https://biology.anu.edu.au/research/resources-tools/konzo-kits). Vials were prepared with buffer paper and 1 ml of water. Individual vials were used to hold root and leaf samples, and indicator paper was added to each vial. Vials were sealed and left to incubate overnight or for at least 12 hours.

After incubation, indicator paper was added to 5 mL water and placed on a shaker to transfer pigment to water for 30 minutes. The absorbance of each pipette-mixed solution was measured according to protocol established in Gomez et al., 2023.

Girdling Experiment
Wild type and *cgtr1* plant stems were marked 10 cm from their bases. A ½ mm ring was cut into the outer layers to sever the phloem. Girdled plants were left in the greenhouse for 48 h. Two cm of stem tissue above and below the girdle point was sampled and assayed for cyanide using the same method described for stems.

Statistical analysis

Comparisons among the three groups in Figures 1a, Figure S1a–b, and Figure S3a, S3b were conducted using Tukey's post-hoc test. All other comparisons were made using Student’s t-test. Statistical analysis and figures were generated using Prism 9 version 9.5 (cite).

4.3 Results and Discussion

To substantiate the relationship between CGs and yield, we analyzed publicly available data from CassavaBase [265]. Data aggregated from Nigerian research trials show an association of greater CG levels with increased fresh storage root weight (Figure 1a, Supplemental Figure 1). This trend remained consistent when data were aggregated instead by two individual field sites in Nigeria. In both sites, higher levels of CGs were associated with greater yields (Supplemental Figure 1).

Previous literature has suggested that CGs move primarily are synthesized in the shoot apex and transporter to roots [266]. Interruption of this transport could potentially lower levels of CGs in roots. However, evidence of de novo biosynthesis of CGs in roots of cassava raises questions regarding the extent to which basipetal transport or de novo biosynthesis enriches cassava roots with CGs [266,267]. Jørgensen et al. validated a putative, high-affinity transporter of CGs derived from the cassava genome through

heterologous expression in *Xenopus laevis* oocytes. Only a single queried transcript, hereafter referred to as *MeCGTR1*, was found to have transport capacity of linamarin, the most abundant CG in cassava [268]. The expression profile of *MeCGTR1* is also consistent with its role as a transporter (Supplemental Figure 2) [269]. These findings provided a discrete target for gene editing to affect cassava CG transport. Leveraging the nascent ability to make CRISPR-Cas9-mediated edits in cassava, we generated knockouts of *MeCGTR1* (Figure 1b). Two unique alleles each resulting in an early stop codon were produced (Figure 1b).

Cyanide levels of wildtype (WT) and *cgtr1* lines were measured in roots, stems, top leaves, and bottom leaves using a picrate assay[261]. Lower levels of CGs were detected in top leaves and stems of *cgtr1* relative to WT, with no difference found between genotypes for roots or bottom leaves (Figure 1c–g). Bottom leaves had the lowest overall CG levels followed by roots, stems, and top leaves in WT (Supplemental Figure 3). Presence of CGs in stems is consistent with the reported phloematic transport of these metabolites. The very low detected levels of CGs in stems of knockout lines therefore provide evidence for the function of *MeCGTR1* as a systemic transporter of CGs (Figure 1f). CGs detected in stems of *cgtr1* lines may be a result of the activity of an alternative transporter. A putative paralog of Me*CGTR1*, *Manes.17G021100*, is a probable candidate gene.

cgtr1 lines would hypothetically have lower root CGs if CGs are transported basipetally. However, we detected no difference in cyanide levels between roots of WT and *cgtr1* lines. Notably, there was a reduction of detected cyanide in the top leaves of *cgtr1* lines (Figure 1d). The findings of the picrate assay suggest that *cgtr1* is indeed a systemic transporter of CGs in cassava, and begins to suggest an acropetal mode of CG flow as an alternative to previously established evidence of exclusively basipetal movement. A root-upwards mode of CG transport was further substantiated by a publicly available expression database captured from 3-month old cassava plants [269]. Expression of *CYP79D1* and *CYP79D2*, genes that encode the enzymes that catalyze the first committed step of cyanogenic glucoside synthesis, were found to be highest in fibrous roots by many fold relative to any shoot tissues (Supplemental Figure 2) [269]. We undertook a phloem girdling approach to provide further resolution of the directionality of CG movement. Incisions to the phloem to prevent movement of CGs were made and measurements of CGs above and below the incision zone were subsequently taken [266]. Overall lower levels of CGs were found in *cgtr1* lines, consistent with stem picrate assay data (Figure 1h). Comparisons of CG levels below to above the incision point were calculated. In WT plants ratios were greater than one, indicating an acropetal direction of CG movement (Figure 1i). A higher ratio was observed in WT relative to *cgtr1* lines, consistent with *MeCGTR1's* function as a transporter (Figure 1i).

Discussion

Efforts to improve the safety of cassava by editing cyanogenesis genes may be an effective approach in some contexts. Transporter editing as an alternative approach was considered in this work. Tissue-specific metabolite levels have been successfully

modulated by transporter engineering in other organisms [270]. Our work extends these findings to cassava and demonstrates the first *in vivo* validation of a systemic transporter in this crop. Leveraging the newly characterized function of *MeCGTR1* as a systemic transporter of CGs, we sought to probe the extent to which de novo biosynthesis of CGs contributes to total CG content in cassava storage roots relative to transport from shoot apex tissues.

Our work suggests that the primary derivation of cassava storage root CGs is root biosynthesis. It is possible that greater production of root CGs is induced by mutations in *MeCGTR1*, but unlikely, considering high root expression levels of biosynthesis genes in wild type plants. The reduction of top leaf CGs in edited lines suggests a root-to-shoot method of CG movement. Lower CG levels in top leaves of *cgtr1,* accumulation of CGs below the incision point of the phloem girdle, and high expression of biosynthesis genes in fibrous roots, all indicate an acropetal mode of CG movement. Multiple directions of CG movement are possible [270]. Shifts between basipetal and acropetal movements of CGs may be contingent upon developmental stage, environmental stress, or other conditions. It is also possible that a basipetal mode of transport may be confined to tissues vicinal to the shoot apex. It is known that CG levels in cassava storage roots increase during drought stress [260]. Further investigation is required to determine the source of elevated CGs in these conditions. In the case that basipetal shuttling is the primary mode of enrichment in this condition, *cgtr1* lines could prove an effective strategy for limiting storage root CG content.

Figure 1:

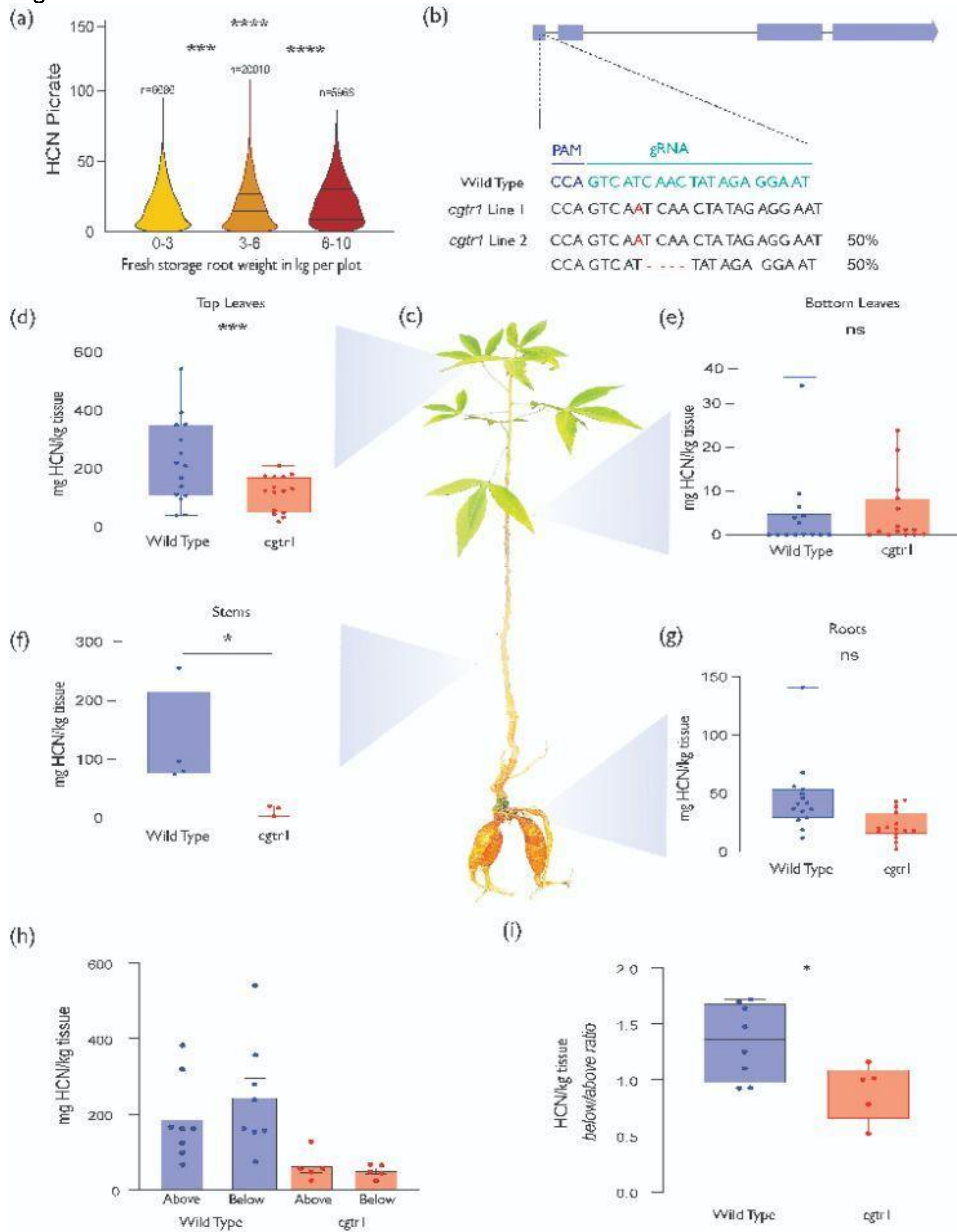
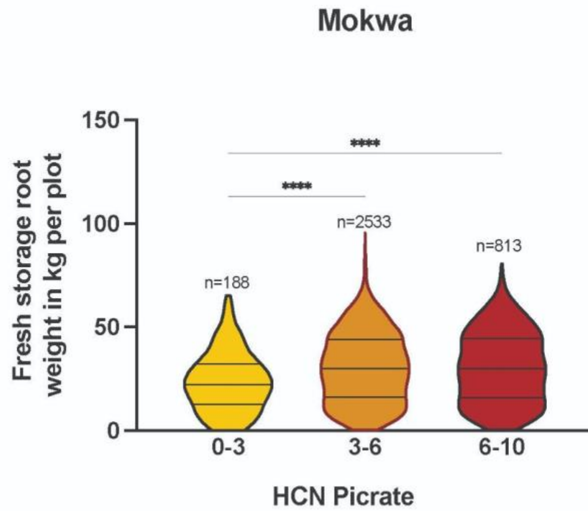


Figure 1| Local synthesis and export of cyanogenic glucosides from cassava tuberous roots

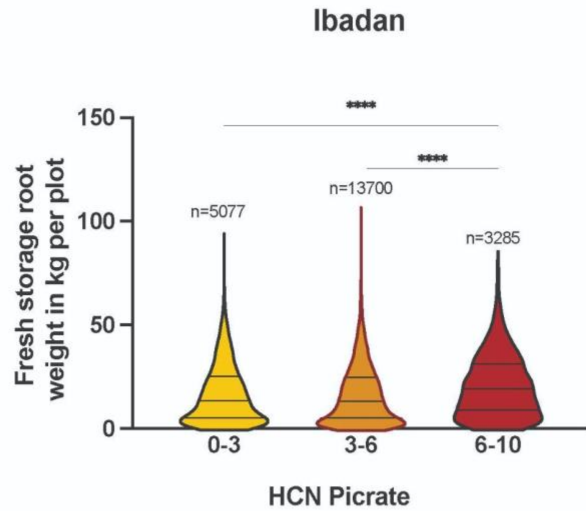
(a) Violin plots of cassava fresh storage root weight in kg compared to measured hydrogen cyanide (HCN) picrate level. Number of replicates is denoted above each plot violin. (b) The gene model of *MeCGTR1* with the location of the CRISPR-Cas9 guide RNA indicated in blue. The two unique edits generated by CRISPR-Cas9 are shown in red. (c) A representative cassava plant image. Measurements of mg cyanide per kg of tissue from picrate assay data comparing wild type to *cgtr1* on (d) top leaves (e) bottom leaves (f) stems (g) roots. (h) Measurements of mg HCN/kg tissue from picrate assay on girdled stems of wild type and *cgtr1*. Individual plots for measurements above and below girdle incision point in each genotype are presented. (i) Ratios of mg HCN/kg tissues below girdle incision zone: above girdle incision point for wild type and *cgtr1*. Plots in figures 1 d,e,f,g,h,i are box-and-whisker plots where the center horizontal indicates the median; upper and lower edges of the box are the upper and lower quartiles; and whiskers extend to the maximum and minimum values. Individual biological replicates are presented as points. $P < 0.1$ denoted by *, $P<0.05$ by **, $P<0.001$ denoted by ***, $P<0.0001$ denoted by ****.

Supplemental Figure 1

(a)



(b)



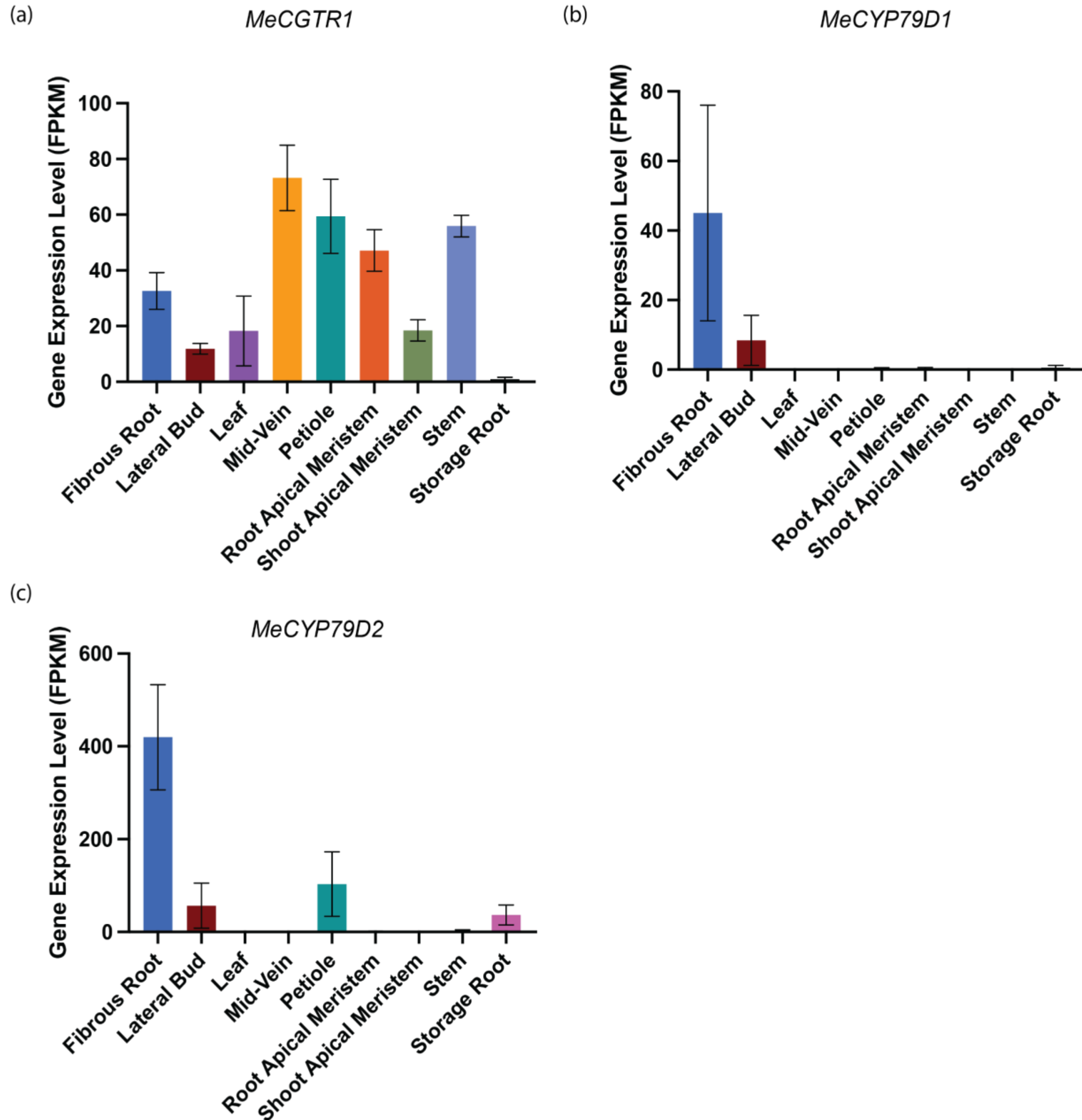
(c)

Field Trial Location	HCN picrate category [0-3, 3-6, 7-10]	Mean fresh storage root weight per plot, kg	Standard error of mean, fresh storage root weight per plot, kg
Mokwa	0-3	23.31	1.031
Mokwa	3-6	30.21	0.3363
Mokwa	6-10	30.37	0.5964
Ibadan	0-3	16.96	0.1971
Ibadan	3-6	16.41	0.1193
Ibadan	6--10	21.21	0.2668
Nigeria aggregated	0-3	17.63	0.1710
Nigeria aggregated	4-6	18.39	0.1054
Nigeria aggregated	7-10	21.38	0.1985

Supplemental Figure 1| Yield and cyanogenic glucoside levels in Nigerian field trials

Violin plots of cassava fresh storage root weight in kilograms compared to measured HCN picrate level in (a) Mokwa (b) Ibadan. Number of replicates is denoted above each plot violin. $P < 0.05$ denoted by *, $P<0.01$ by **, $P<0.001$ denoted by ***, $P<0.0001$ denoted by ****. (c) Table of means and standard error of the means of fresh storage root weight in Mokwa, Ibadan, and aggregated Nigerian data.

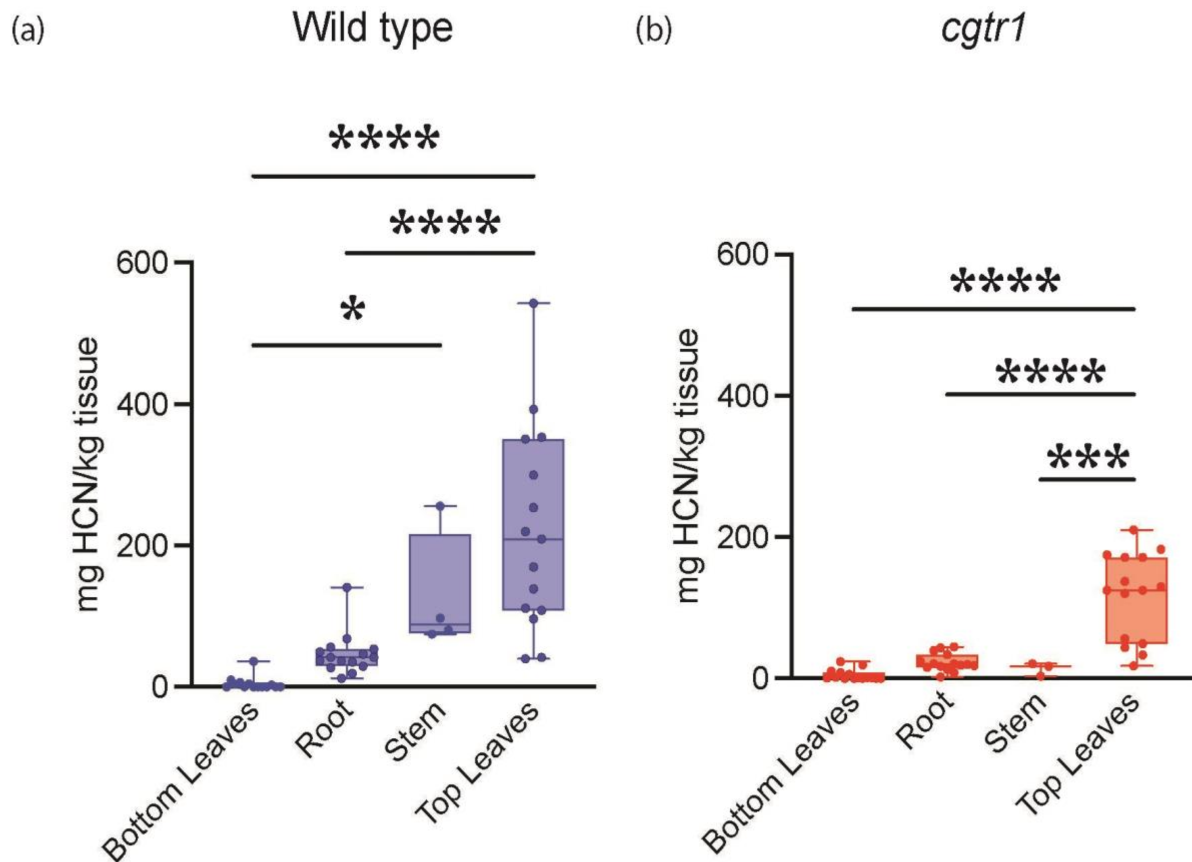
Supplemental Figure 2



Supplemental Figure 2| Expression levels of *MeCGTR1*, *CYP79D1*, and *CYP79D1* across cassava tissues

Expression levels of (a) *MeCGTR1* (b) *CYP79D1* and (c) *CYP79D2* in nine cassava (*Manihot esculenta* cv. TME 204) tissue types. Barplots show mean gene expression levels in FPKM and error bars are standard deviations.

Supplemental Figure 3



Supplemental Figure 3| Comparison of cyanide levels among tissues

Picrate assay data comparing (a) Wild type and (b) *cgtr1* tissue types. Box-and-whisker plots where the center horizontal indicates the median; upper and lower edges of the box are the upper and lower quartiles; and whiskers extend to the maximum and minimum values. Individual biological replicates are presented as points. P-values are shown as brackets above bars. P< 0.1 denoted by *, P<0.05 by **, P<0.001 denoted by ***, P<0.0001 denoted by ****.

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