UNDERGRADUATE RESEARCH IN NATURAL AND CLINICAL SCIENCE AND TECHNOLOGY (URNCST) JOURNAL
Read more URNCST Journal articles and submit your own today at: https://www.urncst.com

RESEARCH PROTOCOL OPEN ACCESS

CRISPR-Cpfb1-Mediated Manipulation of EPFL9 in Oryza sativa for Increased Drought Tolerance as a Climate Change Adaptation Strategy: A Research Protocol

Vaneeza Moosa [1]*, Jessie Liu [2], Jenny Liu [2,3], Abidur Rahman [2]

[1] Department of Neuroscience and Department of Physiology, Faculty of Arts & Science and Faculty of Medicine, University of Toronto, Toronto, ON M5S 3G3
[2] Department of Molecular Genetics, Faculty of Medicine, University of Toronto, Toronto, ON M5S 3G3
[3] Department of Immunology, Faculty of Medicine, University of Toronto, Toronto, ON M5S 3G3

*Corresponding Author: vaneeza.moosa@mail.utoronto.ca

Abstract

Introduction: As a result of climate change, increased drought incidence significantly affects the crop yield of rice, *Oryza sativa*. Given that rice serves as a staple food, adaptation strategies to combat climate change-induced drought are critical. Water retention is regulated by stomata size, stomata density, and the opening and closing of the stomata central pore. Previous studies have identified relevant developmental genes in the *Arabidopsis thaliana* model system, encoding for epidermal patterning factor (EPFs) and EPF-like (EPFL) signaling peptides, and their orthologs across various plant species. In barley (*Hordeum vulgare)*, genetic manipulation of *EPF1* has been shown to reduce stomatal density, resulting in improved drought tolerance. In rice, overexpression of *OsEPF1* yields a similar phenotype. The purpose of our study is to develop a proposal for a method to increase drought tolerance of *Oryza sativa* in an effort to battle climate change.

Methods: It has been shown that CRISPR-mediated editing successfully generated knockouts (KOs) of EPFL9—a positive regulator of stomatal development—in *Oryza sativa*. As such, we propose to downregulate *EPFL9* via CRISPR-Cpfb1 gene editing in *Oryza sativa.* Our proposal includes the growth of genetically altered and control *Oryza sativa* under specific conditions, including drought conditions, in order to simulate a natural environment. Following the growth of the plants, we propose conducting tests to determine yield and growth in order to assess drought tolerance.

Discussion: We expect to observe reduced stomatal densities and better drought tolerance in the mutant *Oryza sativa* samples. This should be observed in increased yield and growth from genetically altered samples. Potential implications of our proposal could include improvements in proto-plants developed in the agricultural sector, as well as providing a foundation for future studies to be conducted on drought tolerance.

Conclusion: Our proposal uniquely addresses the impact of climate change on rice by potentially providing an opportunity to scale-up, generating a drought-tolerant rice plant for comparison with previous prototypes, and secondarily, the elucidation of stomatal development. Our proposal may open further opportunities to address and alter plant resistance to climate change.

Keywords: EPFL9; EPF1; drought tolerance; climate change adaptation; Oryza sativa; Oryza genetics; CRISPR-Cpfb1; stomatal development; water retention; gene knockout

Introduction

Climate change poses a significant challenge to global food security. With rising temperatures, growing water scarcity, higher ozone levels, and an increase in extreme weather events, among many other changes, important crops to the world's food systems are at risk of suffering lower yields and decreased nutritional value [1]. In fact, crop yield reduction is estimated to increase to 90% by 2100 for major crops [2,3].

As a staple food, rice (*Oryza sativa*) is of pertinent interest. Indeed, rice is consumed by more than 3.5 billion people, accounting for 20% of an individual's daily caloric intake [4]. Rice is particularly sensitive to drought, especially in arable land where water is limited [5,6]. Rice grows in warm, tropical climates and requires a large amount of water. As a significant percentage of global rice is rainfed, rice is vulnerable to extreme rainfall changes caused by climate change [7].

Given that increased drought incidence critically impacts the crop yield of rice, altering rice plants for drought resistance and/or tolerance serves as a potential adaptation strategy. One method is by improving rice's

ability to retain water through modifying characteristics of its stomata. The stomata consist of two guard cells that surround and control a pore in the leaf, functioning to regulate gas exchange as part of photosynthesis [8]. Importantly, when stomata pores are open, water is lost through transpiration. While transpiration has beneficial effects such as evaporative cooling, it can prove damaging during periods of water stress. As such, manipulating stomata size, stomata density, and the opening and closing of the central pore may serve as avenues of water loss control in drought conditions [8]. That being said, it should be noted that these effects must be moderated to ensure photosynthesis is not significantly impacted [9].

While stomatal development in rice has yet to be fully explicated, previous studies have identified relevant developmental genes in the *Arabidopsis thaliana* model system, encoding for epidermal patterning factor (EPFs) and EPF-like (EPFL) signaling peptides, and their orthologs across various plant species [10,11,12,13]. Specifically, it has been demonstrated in *Hordeum vulgare*, commonly known as barley, that genetic manipulation of *Hordeum vulgare EPF1* (*HvEPF1*) has been shown to reduce stomatal density, successfully improving drought tolerance without impacting crop yield [14]. Seeing that stomatal development relatively parallels the stomatal development of related plant species, manipulating EPF orthologs in rice may yield similar phenotypes [14].

Similar to the study using *HvEPF1*, Caine *et al.* have demonstrated that overexpression of rice epidermal patterning factor *OsEPF1* also results in reduced stomatal density and improved drought tolerance [15]. *EPF1* is a negative regulator of stomatal development such that its overexpression arrests stomatal development. On the other hand, *EPFL9* is a positive regulator of stomatal development [16,17,18]. Yin *et al*,. have established a method involving Cpfb1 to create CRISPR-mediated knockouts (KOs) of *EPFL9* in *Oryza sativa* [16]. Analogous to the CRISPR-Cas9 system, Cpfb1 is a single RNA-guided DNA endonuclease that recognizes a T-rich motif 5' distal to the target gene site, making a staggered double-ended DNA break [16]. Accordingly, we propose to downregulate *EPFL9* via CRISPR-Cpfb1 gene editing in *Oryza sativa*: we expect to observe reduced stomatal densities and better drought tolerance in the mutant *Oryza sativa* samples.

In light of Caine *et al*.'s research with *OsEPF1*, our proposal has several motivations. First, the identification of another modifiable gene candidate, *EPFL9*, for conferring drought tolerance would help facilitate commercial-scale gene editing given that it can be more efficiently accomplished with multiple gene targets. Therefore, our proposal holds long-term potential for future implementation on a larger scale. Second, the creation of more than one genetically manipulated plant, henceforth referred to as a proto-plant, will accommodate for varying environmental conditions wherein drought intensities and incidences vary. For example, an *EPF1*-edited drought-tolerant proto-plant may thrive better in harsh drought conditions whereas an *EPFL9*-edited drought-tolerant proto-plant may thrive better in mild drought conditions. Lastly, our proposal will contribute to the elucidation of stomatal development as our results assess the impact of stomata regulator *EPFL9* on the relationship of the stoma density under drought conditions; this further clarifies the role of *EPFL9* in the context of *Oryza sativa*.

Methods

In this study, a commercially widespread variety of rice, IR64, will be used. The seedlings will be germinated and cultivated under specific conditions as described by Caine *et al* [15]. These conditions include cultivation of seeds in a Sanyo growth cabinet for 7 days with an alternating light and dark photosynthetically active radiation cycle [15]. As seen in Figure 1, there will be three treatment *EPFL9* groups: control plants with the wildtype genotype, heterozygous plants, and homozygous KO mutant plants. Each group will be grown in three sets of conditions: normal, mild drought, and severe drought. Severity of drought condition will be qualified by the frequency of watering: normal conditions will be watered well throughout the course of the experiment whereas mild drought conditions will include one drought for about a week, and severe drought conditions will include two droughts lasting about a week each [15].The plants will then be harvested after 120 days with fertilization every 2 weeks.

In order to create *EPFL9* mutants, we will be using a CRISPR-Cpfb1 system. Previous research has shown that Cpfb1 can produce stable mutants in plant cell lines [19]. We will transform a known plasmid (pCambia-LbCpf1-EPFL9) into commercial rice strain embryos (IR64) using the method described by Yin *et al* [16]. Following this, a Surveyor Assay (i.e. PCR-testing our sample using *EPFL9* primers) will be performed. Samples found to be positive for the Cpfb1 transgene will undergo Sanger sequencing for their zygosity. Comparing with wildtype IR64 reference data, double peaks on the sequencing chromatogram with alternative sequence: wildtype sequence over 0.8:1 will be considered heterozygous [19]. Following this, the stomata and guardal cell density, water retention, and the overall yield of each group will be examined; this will help determine how *EPFL9* gene dosage and stomatal development is related to rice growth.

Yield and water retention, along with stomatal density, will be determined to assess the results of the experiment. In order to assess yield, plant tissue will be dried at room temperature (~20°C) for one month, after which biomass can be accurately measured. This approach will standardize the yield calculation for each experimental group, enabling analysis of differences between treatment groups and the control group. Stomatal density of the lower surface of the leaf will be regularly recorded. To determine water retention, plants will be grown in an individual pot filled with 1 liter of water, and the pot will be weighed at the end

of each day at 7:00PM EST for one month. A control pot will also be weighed; to provide the overall water loss, the weight of the control group pot will be subtracted from the weight of the experimental group pots. These results will also allow the assessment of the viability of experiments, furthering the validity of the study.

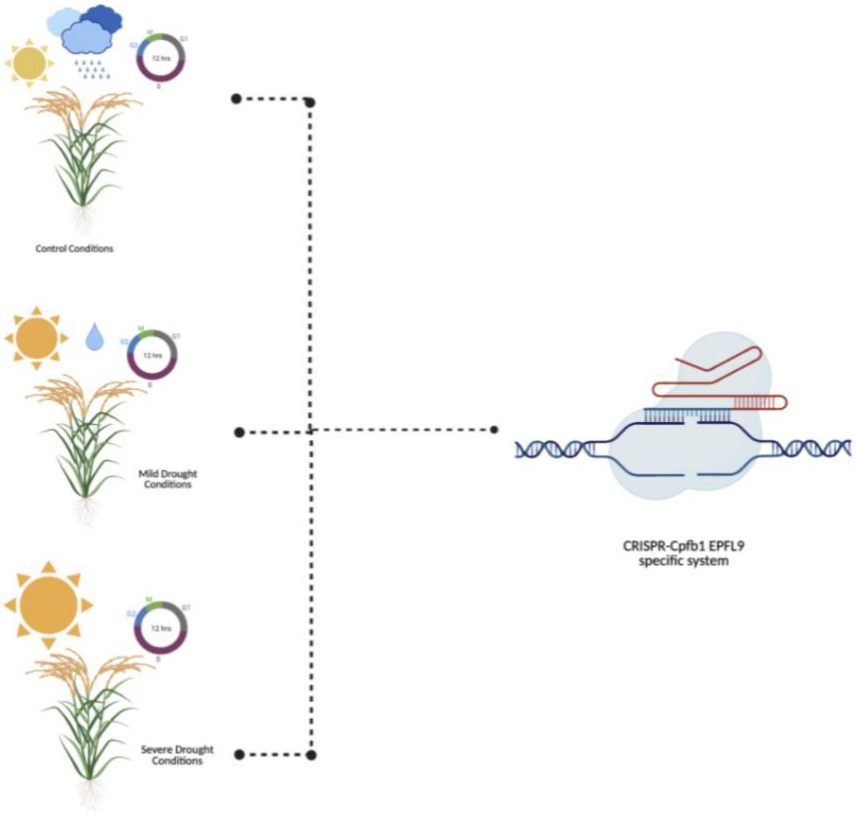

Figure 1. The three conditions under which the crop will be grown, followed by the CRISPR-Cfb1 system (Biorender.com).

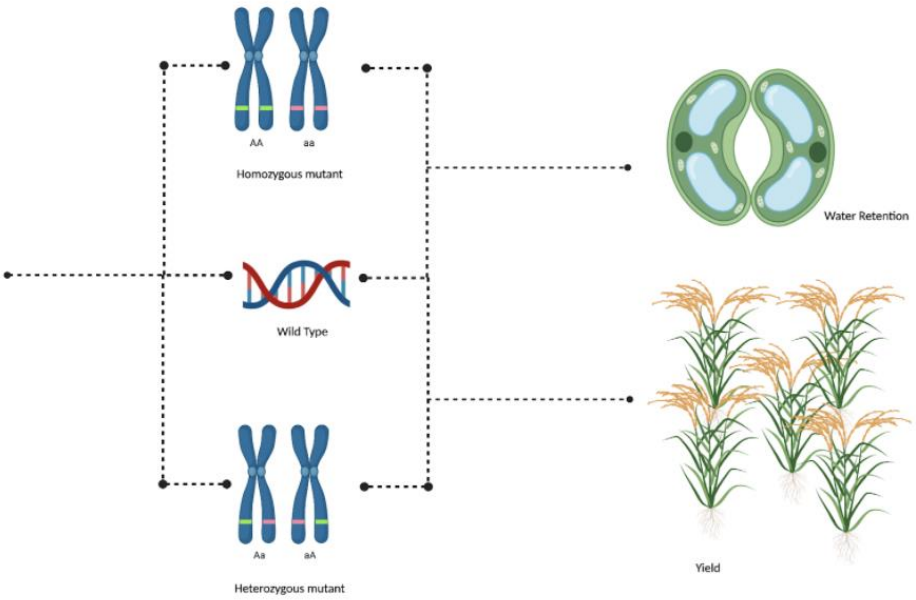

Figure 2. The three experimental groups, followed by measures to determine results (Biorender.com).

Anticipated Results

The study timeline would vary dependent on plant growth and harvesting times. It is estimated that genetic editing will require sufficient time, seedlings will be germinated for 7-8 days, and plants will be ready to be harvested after approximately 120 days [15]. Following this, another 1 month is approximated for biomass determination and water retention calculations. As such, the study timeline is estimated to be 4-6 months, dependent on growth and experimental errors. It is anticipated that there will be reduced stomatal density and better drought tolerance seen in the mutant samples, as compared to controls.

Discussion

We anticipate improved rice growth under drought conditions in the transgenetic rice strain as compared to that of the wildtype rice strain. In terms of plant tissue yield, greater yield is expected in the treatment *EPFL9* groups as compared to the control groups under drought conditions due to expected greater resistance to drought conditions. Within the non-drought condition group, it is expected to see similar yield results regardless of the differing zygosity due to a sufficient amount of water provided uniformly to the group. Furthermore, as a result of improved water conservation, lower stomatal density is expected in the *EPFL9* treatment groups; this finding would be consistent with past literature including that of Caine *et al*. [15] Additionally, as a result of increased drought tolerance and consequent decreases in water loss, the *EPFL9* treatment groups would be expected to exhibit greater water retention as compared to controls.

We aim to address both the impact of climate change on rice as well as consider the scale-up potential of the project via the genetic engineered creation of a drought-tolerant proto-plant. As such, our study serves as an important addition to previous literature focused on translational and scalable modifications for better rice yield. Potential applications of our study include long-term implementation of the proto-plant within the agriculture sector in order to combat worsening climate change. Additionally, our genetic model may serve as a foundational proof-of-principle model for the modification of similar forms of rice and other crops. Potential limitations include accurate control of drought conditions within the laboratory such that they recapitulate natural conditions under typical rice farming conditions.

Conclusions

Overall, the aim of this study is to develop a method to improve the drought tolerance of rice by increasing its water retention via reduced stomatal density. *EPFL9* holds promise as a positive regulator of stomatal development. Future research is required to determine the safety and nutritional value of ingesting an *EPFL9* knockout plant in humans, as it may cause off-target effects in other biochemical pathways, leading to potential biotoxicity. Nevertheless, our proposed adaptation strategy may open further avenues in methods to induce plant resistance to climate change.

List of Abbreviations Used

CRISPR: clustered regularly interspaced short palindromic repeats
EPF: epidermal patterning factor
EPFL: epidermal patterning factor-like
KO: knockout

Conflicts of Interest

The author(s) declare that they have no conflict of interests

Ethics Approval and/or Participant Consent

This study does not require Ethics Approval and/or Participant Consent as it is wholly conducted in plants/plant genes.

Authors' Contributions

VM: made contributions to the design of the study, collected and analysed data, drafted the manuscript, and gave final approval of the version to be published.
JL: made contributions to the design of the study, collected and analysed data, drafted the manuscript, and gave final approval of the version to be published.
JL: made contributions to the design of the study, collected and analysed data, drafted the manuscript, and gave final approval of the version to be published.
AR: made contributions to the design of the study, collected and analysed data, drafted the manuscript, and gave final approval of the version to be published.

Funding

This study was not funded.

References

[1] Myers SS, Smith MR, Guth S, Golden CD, Vaitla B, Mueller ND, Dangour AD, Huybers P. Climate change and global food systems: potential impacts on food security and undernutrition. Annual review of public health. 2017 Mar 20;38(1):259-77. https://doi.org/10.1146/annurev-publhealth-031816-044356
[2] Li Y, Ye W, Wang M, Yan X. Climate change and drought: a risk assessment of crop-yield impacts. Climate research. 2009 May 26;39(1):31-46. https://doi.org/10.3354/cr00797

[3] Porter JR, Xie LY, Challinor AJ, Cochrane K, Howden SM, Iqbal MM, Lobell DB, Travasso MI, Chhetri N, Garrett K et al. 2014. Food security and food production systems. In: Field CB, Barros VR, Dokken DJ, Mach KJ, Mastrandrea MD, Bilir TB, Chatterjee M, Ebi KL, Estrada YO, Genova RC, et al., eds. Climate change 2014: impacts, adaptation, and vulnerability, part A: global and sectoral aspects. Working Group II contribution to the fifth assessment report of the Intergovernmental Panel on Climate Change. Cambridge, UK: Cambridge University Press, 485–533.

[4] United Nations Environment Programme. Rice may be cheap, but production comes at a cost.UNEP. 2021 Available from: https://www.unep.org/news-and-stories/story/rice-may-be-cheap-production-comes-cost

[5] Bernier J, Atlin GN, Serraj R, Kumar A, Spaner D. Breeding upland rice for drought resistance. Journal of the Science of Food and Agriculture. 2008 Apr 30;88(6):927-39. https://doi.org/10.1002/jsfa.3153

[6] Korres NE, Norsworthy JK, Burgos NR, Oosterhuis DM. Temperature and drought impacts on rice production: An agronomic perspective regarding short- and long-term adaptation measures. Water resources and rural development. 2017 Jun 1;9:12-27. https://doi.org/10.1016/j.wrr.2016.10.001

[7] Farooq M, Wahid A, Lee DJ, Ito O, Siddique KH. Advances in drought resistance of rice. Critical Reviews in Plant Sciences. 2009 Jul 15;28(4):199-217. https://doi.org/10.1080/07352680902952173

[8] Stevens J, Faralli M, Wall S, Stamford JD, Lawson T. Stomatal responses to climate change. InPhotosynthesis, Respiration, and Climate Change 2021 (pp. 17-47). Springer, Cham. https://doi.org/10.1007/978-3-030-64926-5_2

[9] Bertolino LT, Caine RS, Gray JE. Impact of stomatal density and morphology on water-use efficiency in a changing world. Frontiers in Plant Science. 2019 Mar 6;10:225. https://doi.org/10.3389/fpls.2019.00225

[10] Liu T, Ohashi-Ito K, Bergmann DC. Orthologs of Arabidopsis thaliana stomatal bHLH genes and regulation of stomatal development in grasses. https://doi.org/10.1242/dev.032938

[11] Raissig MT, Abrash E, Bettadapur A, Vogel JP, Bergmann DC. Grasses use an alternatively wired bHLH transcription factor network to establish stomatal identity. Proceedings of the National Academy of Sciences. 2016 Jul 19;113(29):8326-31. https://doi.org/10.1073/pnas.1606728113

[12] Chater CC, Caine RS, Tomek M. Origin and function of stomata in the moss Physcomitrella patens. Nature Plants, 2, 16179. https://doi.org/10.1038/nplants.2016.179

[13] Caine RS, Chater CC, Kamisugi Y, Cuming AC, Beerling DJ, Gray JE, Fleming AJ. An ancestral stomatal patterning module revealed in the non-vascular land plant Physcomitrella patens. Development. 2016 Sep 15;143(18):3306-14. https://doi.org/10.1242/dev.135038

[14] Hughes J, Hepworth C, Dutton C, Dunn JA, Hunt L, Stephens J, Waugh R, Cameron DD, Gray JE. Reducing stomatal density in barley improves drought tolerance without impacting on yield. Plant physiology. 2017 Jun;174(2):776-87. https://doi.org/10.1104/pp.16.01844

[15] Caine RS, Yin X, Sloan J, Harrison EL, Mohammed U, Fulton T, Biswal AK, Dionora J, Chater CC, Coe RA, Bandyopadhyay A. Rice with reduced stomatal density conserves water and has improved drought tolerance under future climate conditions. New Phytologist. 2019 Jan;221(1):371-84. https://doi.org/10.1111/nph.15344

[16] Yin X, Biswal AK, Dionora J, Perdigon KM, Balahadia CP, Mazumdar S, Chater C, Lin HC, Coe RA, Kretzschmar T, Gray JE. CRISPR-Cas9 and CRISPR-Cpf1 mediated targeting of a stomatal developmental gene EPFL9 in rice. Plant cell reports. 2017 May;36(5):745-57. https://doi.org/10.1007/s00299-017-2118-z

[17] Lee JS, Hnilova M, Maes M, Lin YC, Putarjunan A, Han SK, Avila J, Torii KU. Competitive binding of antagonistic peptides fine-tunes stomatal patterning. Nature. 2015 Jun;522(7557):439-43. https://doi.org/10.1038/nature14561

[18] Zoulias N, Harrison EL, Casson SA, Gray JE. Molecular control of stomatal development. Biochemical Journal. 2018 Jan 31;475(2):441-54. https://doi.org/10.1042/bcj20170413

[19] Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, Van Der Oost J, Regev A, Koonin EV. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell. 2015 Oct 22;163(3):759-71. https://doi.org/10.1016/j.cell.2015.09.038

Article Information
Managing Editor: Jeremy Y. Ng
Peer Reviewers: Siobhan O'Brien, Vanessa Susevski
Article Dates: Received Mar 19 22; Accepted Jul 13 22; Published Aug 22 22

Citation
Please cite this article as follows:
Moosa V, Liu J, Liu J, Rahman A. CRISPR-Cpfb1-mediated manipulation of EPFL9 in *Oryza sativa* for increased drought tolerance as a climate change adaptation strategy: A research protocol. URNCST Journal. 2022 Aug 22: 6(8).
https://urncst.com/index.php/urncst/article/view/360
DOI Link: https://doi.org/10.26685/urncst.360