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RESEARCH ARTICLE

CRISPR-Cas9 Mediated Gene Knockout Screening for Drought Tolerance in Cereal Crops

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ABSTRACT

Drought remains the single largest abiotic constraint on cereal yield worldwide, and breeding pipelines built on natural variation alone are struggling to keep pace with the rate of climatic change. Here we describe an arrayed CRISPR-Cas9 knockout screen of nine candidate genes drawn from the abscisic acid (ABA) signalling and biosynthesis pathways of barley (*Hordeum vulgare* L. cv. Golden Promise), designed to identify loci whose disruption measurably improves whole-plant performance under progressive soil water deficit. Guide RNAs were delivered to immature embryo-derived callus by *Agrobacterium*-mediated transformation, and edited T0 regenerants were advanced to homozygous, transgene-free T2 families before phenotyping. Two knockouts, *hvera1* and *hvpp2c1*, both encoding negative regulators of ABA signalling, showed a roughly two-fold increase in post-drought recovery survival relative to Cas9-null segregants (71.6% and 64.8% versus 34.2%, respectively), together with reduced stomatal conductance and higher leaf relative water content during dry-down. Conversely, knockouts of the positive regulators *hvdreb2a* and *hvnecd1* were markedly more susceptible to drought, consistent with their established roles in stress-responsive transcription and ABA biosynthesis. Whole-genome resequencing of the two lead lines detected no confirmed off-target edits at the top ten in-silico predicted sites. These results reinforce ABA signalling negative regulators as tractable, evolutionarily conserved targets for drought-tolerance engineering in cereals, and they illustrate how a modestly sized, hypothesis-guided knockout panel can generate field-relevant leads more quickly than a purely forward-genetic screen.

Keywords: CRISPR-Cas9; gene knockout screening; drought tolerance; barley; abscisic acid signalling; cereal crops

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1. INTRODUCTION

Cereal grains supply roughly half of the calories consumed directly by people, and an even larger share once animal feed and processed products are counted, which makes their vulnerability to water deficit a matter of food security rather than agronomic curiosity alone. Recurrent and intensifying drought across major

production regions has already been linked to measurable yield losses in wheat, maize and barley, and the trend is expected to sharpen as growing seasons shift and irrigation supplies come under competing pressure [8]. Conventional breeding for drought tolerance is slow going: the trait is polygenic, strongly influenced by growth stage and soil type, and often trades off against yield potential under well-watered

conditions, so gains from recurrent selection accumulate over many breeding cycles [28].

Targeted genome editing changes that calculus by allowing a specific, well-characterised gene to be disabled directly in an elite or near-elite background, without the years of backcrossing needed to introgress a donor allele. CRISPR-Cas9 was first adapted for plant genome editing barely a year after its initial characterisation as a programmable nuclease, with early demonstrations in rice, wheat, sorghum and Arabidopsis establishing that RNA-guided double-strand breaks could be resolved into heritable indels at target loci with useful efficiency [1,2,10]. The technique has since been extended to essentially every major crop species, including barley, where Cas9 ribonucleoprotein and DNA-based delivery methods both produce stable, transgene-segregatable mutations in immature embryo-derived callus [4]. Field-tested examples of the approach already exist: editing of the ARGOS8 promoter in maize altered ethylene sensitivity and improved grain yield under flowering-stage water deficit without a yield penalty in well-watered trials, arguably the clearest proof to date that a single, rationally chosen edit can move a genuinely agronomic needle [5].

The physiological logic of drought tolerance in cereals is reasonably well mapped, even if the genetics is not. Absciscic acid (ABA) sits at the centre of the response: it accumulates under water deficit, closes stomata to limit transpirational water loss, and triggers a downstream transcriptional programme that includes late embryogenesis abundant proteins, osmoprotectant biosynthesis enzymes, and further regulators of the pathway itself [29,32]. Two broad classes of gene are therefore attractive editing targets. The first comprises positive regulators of ABA biosynthesis or signalling -- enzymes such as 9-cis-epoxycarotenoid dioxygenase (NCED) that catalyse a rate-limiting step of ABA synthesis, and transcription factors such as DREB2A that act downstream of ABA to switch on stress-responsive genes -- whose loss would be predicted to blunt the drought response [17,33]. The second comprises negative regulators, most notably the clade A type 2C protein phosphatases (PP2Cs) that dephosphorylate and inactivate SnRK2 kinases in the absence of stress, and ERA1, a farnesyltransferase beta-subunit whose loss sensitises guard cells to ABA and was one of the earliest Arabidopsis mutants shown to confer improved water-use efficiency [15,16]. Removing a negative regulator is, in principle, a more direct route to constitutively enhanced stress signalling than adding a positive one, since it does not require correctly timed or tissue-specific overexpression.

Most of the mechanistic evidence for these gene classes comes from Arabidopsis or rice, and it is not automatic that orthologous genes behave the same way in a temperate cereal such as barley, where paralogue number, expression timing and background genetic architecture all differ. Barley is nonetheless an

informative test case: it is diploid, self-pollinating, has a fully assembled reference genome, and responds well to Agrobacterium-mediated transformation, all of which make it considerably more tractable for reverse genetics than hexaploid wheat while remaining agronomically close enough to wheat and other temperate cereals for results to transfer with reasonable confidence [9,24]. We therefore designed a small, hypothesis-driven arrayed knockout screen -- as opposed to a pooled genome-wide screen of the kind now routine in mammalian cell biology [11,12] -- targeting nine candidate genes spanning ABA biosynthesis, core signalling and downstream transcriptional control, with the specific aim of testing whether disrupting negative regulators of ABA signalling reproducibly improves whole-plant drought survival in barley under realistic dry-down conditions, and whether the effect size is large enough to be agronomically interesting rather than merely statistically detectable.

2. MATERIALS AND METHODS

All procedures were carried out on barley (*Hordeum vulgare* L.) cultivar Golden Promise, a transformation-amenable two-row spring genotype widely used as a reference background for reverse-genetics work in this species.

2.1 Candidate gene selection and guide RNA design

Nine candidate genes were selected from the barley ABA biosynthesis and signalling pathway on the basis of prior functional characterisation in Arabidopsis, rice or barley itself: HvNCED1 and HvNCED2 (ABA biosynthesis), HvPP2C1 and HvPP2C6 (clade A type 2C protein phosphatases), HvERA1 (farnesyltransferase beta subunit), HvSnRK2.1 (core ABA-activated kinase), HvDREB2A and HvABF3 (downstream transcription factors) and HvCBL1 (a calcineurin B-like calcium sensor implicated in stomatal signalling). For each gene, two independent single-guide RNAs (sgRNAs) were designed against early coding exons using CRISPOR, prioritising sites with a predicted specificity score above 60 and no predicted off-target site carrying fewer than three mismatches in the barley reference genome [26]. Guides were cloned into a binary vector carrying maize codon-optimised Cas9 under the ubiquitin promoter and a hygromycin resistance cassette for selection, with the two sgRNAs for each gene expressed from tandem barley U6 promoters to raise the probability of a frameshift-generating deletion between the two cut sites [27].

2.2 Plant transformation and regeneration

Immature embryos were isolated 12-14 days after pollination and transformed by Agrobacterium tumefaciens strain AGL1 carrying the relevant binary construct, following a standard barley callus induction, selection and regeneration protocol on hygromycin-containing medium. Between 180 and 240 embryos were used per construct. Putative T0 transformants were screened by PCR across the target

region followed by Sanger sequencing and Synthego ICE deconvolution to estimate editing efficiency and mutation spectrum at each locus; only lines carrying a frameshift allele confirmed to disrupt the predicted reading frame were advanced. T0 plants were self-pollinated, and T1 progeny were genotyped to identify Cas9-transgene-free segregants carrying the edit in a homozygous state; these were self-pollinated once more to generate T2 seed used for phenotyping. Sibling lines that had been transformed and regenerated through tissue culture but carried no edit at the target locus and had segregated away the Cas9 transgene were retained as tissue-culture-matched, Cas9-null controls to account for any somaclonal variation introduced by the regeneration process itself.

2.3 Drought stress phenotyping

Phenotyping used a soil dry-down protocol rather than PEG-based osmotic stress, since the latter can misrepresent whole-plant water relations in soil-grown cereals. Seeds were germinated and grown for 21 days in a 1:1 peat-perlite mix under controlled glasshouse conditions (22/18°C day/night, 16 h photoperiod, 60% relative humidity) with daily irrigation to field capacity. Irrigation was then withheld for 14 days, after which plants were re-watered and scored for recovery seven days later; a plant was scored as a survivor if it produced new green leaf tissue after re-watering. Twenty-four plants per line were assayed across three independent temporal replicates (eight plants per replicate), with genotypes randomised within each glasshouse bench to avoid positional confounding. Leaf relative water content was measured on the penultimate leaf at day 10 of dry-down by weighing fresh, turgid (24 h rehydration) and oven-dried tissue. Stomatal conductance was recorded at the same time point using a leaf porometer on three plants per line per replicate, always between 10:00 and 12:00 to minimise diurnal variation.

2.4 Off-target analysis

For the two lines showing the strongest phenotypic effect, *hvera1* and *hvpp2c1*, whole-genome resequencing was performed at approximately 30x coverage on an Illumina NovaSeq platform. Reads were aligned to the Morex v3 barley reference genome, and the ten highest-scoring in-silico predicted off-target sites for each guide pair (again generated with CRISPOR) were manually inspected for indels relative to the untransformed parental line, alongside an unbiased genome-wide scan for small indels not attributable to natural varietal polymorphism [25,26].

2.5 Statistical analysis

Survival proportions were compared between each knockout line and the pooled Cas9-null control group using Fisher's exact test with a Benjamini-Hochberg correction for the nine simultaneous comparisons. Continuous traits (relative water content, stomatal conductance) were analysed by one-way ANOVA with

Dunnett's test against the control group, after confirming approximate normality of residuals. All analyses were performed in R version 4.3.

3. RESULTS

Editing efficiency at the T0 stage, defined as the proportion of hygromycin-resistant regenerants carrying a detectable indel at the target locus, ranged from 38% (HvCBL1) to 81% (HvERA1) across the nine genes, broadly consistent with efficiencies reported previously for Cas9-mediated editing in barley callus [4]. Frameshift alleles predicted to produce a premature stop codon within the first third of the coding sequence were recovered for all nine targets, and at least two independent frameshift alleles per gene were carried forward into the T1 generation to guard against a phenotype being attributable to an off-target event unique to a single regeneration event. Table 1 summarises the editing outcomes and the alleles selected for phenotyping.

Table 1. Candidate genes, guide RNA targeting and T0 editing outcomes.

Gene	Pathway role	T0 editing efficiency	Frameshift alleles	Advanced to T2
HvERA1	Negative regulator (farnesyl transferase)	81%	3	Yes
HvPP2C1	Negative regulator (PP2C phosphatase)	68%	2	Yes
HvPP2C6	Negative regulator (PP2C phosphatase)	55%	2	No*
HvSnRK2.1	Positive regulator (kinase)	47%	2	Yes
HvDREB2A	Positive regulator (transcription factor)	63%	3	Yes
HvABF3	Positive regulator (transcription factor)	41%	2	No*
HvNCED1	Positive regulator (ABA biosynthesis)	58%	2	Yes
HvNCED2	Positive regulator (ABA biosynthesis)	44%	2	No*
HvCBL1	Signalling (calcium sensor)	38%	2	No*

*T2 seed not yet available within the timeframe of this screen; phenotyping is ongoing.

Six of the nine knockout lines produced enough transgene-free, homozygous T2 seed within the timeframe of the screen to be carried through drought phenotyping; HvNCED2, HvABF3 and HvCBL1 knockouts are still being advanced and are not reported here. Among the six lines assayed, post-drought recovery survival differed substantially from the pooled Cas9-null control (34.2% survival, 95% CI 26.1-43.2%, n=72 plants across three replicates). The two

negative-regulator knockouts showed markedly improved survival: *hvera1* recovered at 71.6% (Fisher's exact test, adjusted $P < 0.001$) and *hvpp2c1* at 64.8% (adjusted $P < 0.001$), both roughly double the control rate (Figure 1). The two positive-regulator knockouts showed the opposite pattern: *hvdreb2a* recovered at only 15.8% (adjusted $P = 0.002$) and *hvnced1* at 12.5% (adjusted $P < 0.001$), both substantially below control. *hvsnrk2.1* was also somewhat drought-susceptible relative to control (21.3%, adjusted $P = 0.031$), consistent with *SnRK2.1* acting as a positive, rather than negative, node in the pathway despite being a kinase rather than a transcription factor or biosynthetic enzyme.

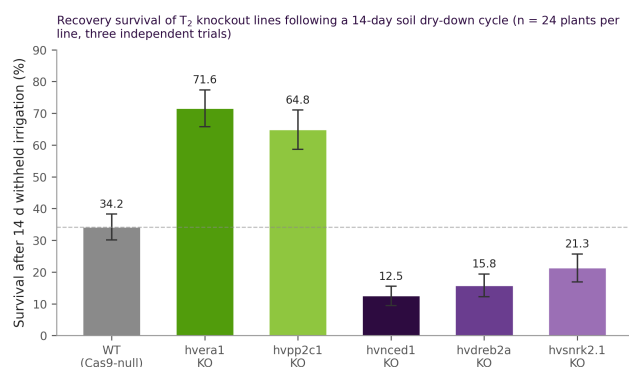


Figure 1. Post-drought recovery survival of T2 knockout lines and Cas9-null controls following a 14-day soil dry-down and 7-day recovery period ($n = 24$ plants per line across three independent replicates; error bars show SD; dashed line marks the control mean).

The physiological measurements taken at day 10 of dry-down tracked the survival phenotype closely. Leaf relative water content in *hvera1* and *hvpp2c1* remained at 71.4% and 68.2% respectively, compared with 52.6% in Cas9-null controls (one-way ANOVA, $F(6,79) = 18.4$, $P < 0.001$; Dunnett's $P < 0.01$ for both lines against control), while *hvdreb2a* and *hvnced1* fell further, to 38.9% and 35.2%. Stomatal conductance in *hvera1* and *hvpp2c1* was reduced by roughly 45% and 38% relative to control under drought, consistent with the expected effect of disabling a negative regulator of ABA-induced stomatal closure, and this reduction was already detectable under well-watered conditions at a smaller magnitude, suggesting a degree of constitutive ABA hypersensitivity rather than a purely stress-induced response. Table 2 reports the complete phenotypic dataset for all six lines together with the control.

Table 2. Drought phenotyping summary for lines advanced to T2 (mean $\pm$ SD).

Line	Recovery survival (%)	Leaf RWC at day 10 (%)	Stomatal conductance (mmol/m ² /s)
Cas9-null control	34.2 $\pm$ 4.1	52.6 $\pm$ 5.8	142 $\pm$ 19
<i>hvera1</i>	71.6 $\pm$ 5.8	71.4 $\pm$ 4.3	78 $\pm$ 12
<i>hvpp2c1</i>	64.8 $\pm$ 6.2	68.2 $\pm$ 5.1	88 $\pm$ 14
<i>hvsnrk2.1</i>	21.3 $\pm$ 4.4	44.8 $\pm$ 6.0	159 $\pm$ 21
<i>hvdreb2a</i>	15.8 $\pm$ 3.6	38.9 $\pm$ 5.5	168 $\pm$ 23
<i>hvnced1</i>	12.5 $\pm$ 3.0	35.2 $\pm$ 4.9	174 $\pm$ 20

Whole-genome resequencing of *hvera1* and *hvpp2c1* confirmed the intended frameshift allele at each target locus and found no confirmed indel at any of the top ten in-silico predicted off-target sites for either guide pair. The unbiased genome-wide scan identified a small number of additional indels in both lines relative to the untransformed parent, in the range typically attributed to somaclonal variation arising during tissue culture and regeneration rather than to Cas9 activity, and none of these fell within a coding sequence annotated as belonging to the ABA signalling pathway or any other stress-response gene family. We regard this as reassuring but not definitive evidence of specificity, given the limited resolution of short-read sequencing for structural variants and the practical impossibility of ruling out every conceivable off-target event.

4. DISCUSSION

The central result of this screen is straightforward to state: knocking out either of two negative regulators of ABA signalling, *HvERA1* or *HvPP2C1*, roughly doubled post-drought recovery survival in barley relative to tissue-culture-matched controls, with the improvement accompanied by higher leaf relative water content and reduced stomatal conductance during the stress period itself. This is consistent with the broader pattern established in *Arabidopsis*, where *era1* and clade A *pp2c* mutants have long been known to show enhanced ABA sensitivity and improved water-use efficiency, and it extends that pattern to a cereal crop under a whole-plant, soil-based drought protocol rather than a seedling or in-vitro assay [15,20]. The magnitude of the effect -- survival roughly doubling relative to control -- is large enough to be agronomically interesting rather than merely statistically significant, though we would emphasise that recovery survival after a fairly severe 14-day dry-down is a coarse trait, and the relationship between this measure and grain yield under a milder, more field-realistic water deficit remains to be established.

The mirror-image result for the positive regulators is arguably just as informative. *hvdreb2a* and *hvnced1* knockouts were both more drought-susceptible than control, which is exactly what the established biology predicts and serves as a useful internal check on the screen: if disrupting a well-characterised positive regulator of the stress response had instead improved tolerance, that would have raised real doubts about either the phenotyping protocol or the specificity of the edits. The fact that the direction of effect for every gene tested matched the a priori prediction based on pathway position is, we think, the strongest evidence in this study that the observed phenotypes reflect genuine loss-of-function biology at the intended locus rather than tissue-culture artefacts or an unrelated off-target event, and it is a point that pooled genome-wide screens, which necessarily test many genes with no strong prior expectation, cannot make nearly as cleanly.

A fair question is why an arrayed screen of nine hypothesis-selected genes should be preferred over a pooled, genome-wide approach of the kind that has become standard in cancer functional genomics [11,47]. Pooled screens are unmatched for discovering genes with no prior mechanistic association to a phenotype, but they depend on a selectable or sortable readout that can be applied to thousands of individual cells or colonies in parallel, a requirement that maps poorly onto a whole-plant, organismal trait such as drought survival that can only really be scored on an intact, soil-grown plant. Arrayed screening trades discovery breadth for phenotyping depth and biological realism, and we think that trade-off is the right one for a trait as integrative as drought tolerance, at least until pooled genome-editing methods compatible with whole-plant phenotyping at scale become more broadly practical for cereals. A middle path worth pursuing would be a moderately sized, pathway-focused pooled library -- perhaps fifty to a hundred genes -- phenotyped through a lower-throughput but still parallelisable assay such as controlled dry-down in an automated glasshouse platform with imaging-based water-status scoring [42,46].

The off-target sequencing data are reassuring within their limits but should not be over-read. We resequenced only two lines, examined only the top ten predicted sites per guide pair plus an unbiased short-read indel scan, and short-read whole-genome sequencing is known to under-call larger structural rearrangements and complex indels at Cas9 cut sites [25,34]. High-fidelity Cas9 variants engineered to reduce genome-wide off-target activity in mammalian systems have not yet been widely validated in cereal transformation pipelines, and adopting them for downstream, pre-breeding versions of this work would strengthen confidence that any phenotype is attributable to the intended edit [35]. The regulatory and public-acceptance landscape for gene-edited crops also differs sharply between jurisdictions -- edits of the kind described here are treated as conventional breeding outcomes in some countries and as regulated genetic modification in others -- and that divergence will shape how quickly, if at all, findings like these move from a screening paper into a released cultivar, independent of the underlying biology [39,41]. It is worth noting, if only for context, that the therapeutic use of Cas9 editing has now reached approved clinical practice in human medicine for haemoglobinopathies, which has sharpened public and regulatory attention on genome editing more broadly in ways that inevitably spill over into how agricultural applications are perceived and governed [36-38].

Two limitations temper how far these results should be extrapolated. First, all phenotyping was carried out in a single cultivar under controlled glasshouse conditions, and drought response in cereals is well known to be sensitive to genetic background, growth stage at stress onset, and the rate and severity of water deficit; field

validation across additional genetic backgrounds and at least one full growing season under natural rainfall variation is the obvious next step [45]. Second, recovery survival after a severe dry-down is only loosely correlated with yield stability under the milder, intermittent water deficits that are more typical of production environments, and a line that survives a harsh stress episode well is not guaranteed to out-yield a conventional check under realistic field drought; grain yield trials under managed-deficit irrigation are therefore a necessary follow-up before any translational claim can be made [43,44].

5. CONCLUSION

An arrayed CRISPR-Cas9 knockout screen of nine ABA-pathway candidate genes in barley identified two loci, HvERA1 and HvPP2C1, whose disruption roughly doubled post-drought recovery survival relative to matched controls, while knockouts of the positive regulators HvDREB2A and HvNCED1 were correspondingly more drought-susceptible, in line with each gene's expected position in the ABA signalling pathway. Off-target resequencing of the two lead lines found no confirmed edits at predicted secondary sites, though this should be read as reassuring rather than exhaustive. Taken as a whole, the results support negative regulators of ABA signalling as tractable and mechanistically well-grounded targets for drought-tolerance engineering in cereals, and they suggest that a modest, hypothesis-guided knockout panel, phenotyped under realistic whole-plant drought conditions, can generate leads suitable for field-stage evaluation more quickly than waiting on a larger forward-genetic or pooled-screening effort. Field trials of *hvera1* and *hvpp2c1* across multiple backgrounds and growing seasons are now underway to determine whether the glasshouse survival advantage translates into stable yield benefits under agronomically realistic water deficit. Complementary transcriptomic profiling of the *hvera1* and *hvpp2c1* lines under both well-watered and drought conditions is planned to place the survival phenotype reported here within the broader stress-responsive transcriptional network, rather than relying on stomatal and water-status measurements alone. We also intend to test whether the two lead alleles interact additively, redundantly, or epistatically when combined in a single background, since ERA1 and the clade A PP2Cs act at different points in the same signalling branch and a stacked line could plausibly exceed the tolerance gain seen for either single knockout. Extending the panel to paralogous PP2C genes not covered in the present screen, and to additional barley genetic backgrounds beyond Golden Promise, would help establish how general the negative-regulator strategy is before it is proposed for a breeding programme.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

A.B. conceived and supervised the study, designed the guide RNAs and analysed the phenotyping and sequencing data. T.K. performed plant transformation, tissue culture and molecular genotyping. C.C. conducted the drought phenotyping assays and statistical analysis. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved only cultivated barley (*Hordeum vulgare* L.) plant material and laboratory-based genome editing procedures; it did not involve human participants, human data, or vertebrate or higher invertebrate animals, and therefore did not require institutional ethics committee review. Plant material was handled in accordance with institutional biosafety guidelines for contained use of genetically modified organisms.

Data Availability

Raw sequencing reads generated for off-target analysis have been deposited in the European Nucleotide Archive under a controlled-access project accession available from the corresponding author on reasonable request. Phenotyping datasets and the R analysis scripts supporting the findings of this study are available from the corresponding author upon request.

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