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Review

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Genetic resources and precise gene editing for targeted improvement of barley abiotic stress tolerance

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Abstract: Abiotic stresses, predominately drought, heat, salinity, cold, and waterlogging, adversely affect cereal crops. They limit barley production worldwide and cause huge economic losses. In barley, functional genes under various stresses have been identified over the years and genetic improvement to stress tolerance has taken a new turn with the introduction of modern gene-editing platforms. In particular, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) is a robust and versatile tool for precise mutation creation and trait improvement. In this review, we highlight the stress-affected regions and the corresponding economic losses among the main barley producers. We collate about 150 key genes associated with stress tolerance and combine them into a single physical map for potential breeding practices. We also overview the applications of precise base editing, prime editing, and multiplexing technologies for targeted trait modification, and discuss current challenges including high-throughput mutant genotyping and genotype dependency in genetic transformation to promote commercial breeding. The listed genes counteract key stresses such as drought, salinity, and nutrient deficiency, and the potential application of the respective gene-editing technologies will provide insight into barley improvement for climate resilience.

Key words: Clustered regularly interspaced short palindromic repeats (CRISPR); Gene function; Drought; Genetic improvement; Transcription regulation; Breeding

1 Introduction

Agricultural production faces numerous challenges worldwide owing to climate change, insufficient arable land, abiotic and biotic stresses, low carbon input farming, population growth, and ever-increasing food demand which is expected to increase by 35%–56% to feed a population of nearly ten billion by 2050 (van Dijk et al., 2021). Demand for cereals both as food and animal feed is likely to be around three billion tonnes per annum by 2050 (FAO, 2009). Rice, wheat, maize, and barley are the four major cereal crops in terms of worldwide production (Statista, 2022b). Barley is used mainly as animal feed and in brewing, while only a small percentage is used for human consumption (Tricase et al., 2018). It is mainly produced in the European Union, Russia, Australia, Ukraine, Canada, Turkey, the USA, and Argentina (Statista, 2022a). Australia is one of the biggest barley exporters and accounted for USD 2 billion worth of raw barley in 2021 (Trade Map, 2022). However, there is a gap between the demand for food and its supply, which exerts huge pressure on farmers as well as scientists.

Climate change, which affects the growth and development of cereal crops, is yet another challenge in sustainable agriculture (Fatima et al., 2020). The rise of global temperature due to deforestation, burning of fossil fuels, etc. affects the economic yield of crops. It leads to a loss of nutrients and water, resulting in lower nitrogen and water use efficiency in crops (Fatima et al., 2020). Global warming increases flood risks, raises the sea level, and increases desertification, which eventually leads to abiotic stresses (Huang et al., 2016). Abiotic stresses such as drought, flooding,

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waterlogging, frost, high or low temperature, salinity, and excess or deficiency of minerals like aluminium and boron, prevent crops from achieving their full genetic potential (Gürel et al., 2016) and contribute to crop damage, lower yields, and high production costs (Kumar et al., 2021). For example, heat stress accounts for 15% of the loss in wheat yield per annum in Australia (Wardlaw and Wrigley, 1994). Climate prediction models indicate severe effects for Africa, the Arabian Peninsula, and Central South America, where barley is important as human food (Samson et al., 2011). However, agriculture itself contributes to global warming owing to the release of greenhouse gases (Wang et al., 2018). Low carbon input farming is therefore a strategy proposed to reduce energy inputs and the emission of greenhouse gases from agriculture. Achieving this aim while at the same time improving soil carbon content is a new challenge for farmers (Borychowski et al., 2022). Huge annual investments are required to meet the goals of the Paris Agreement (van Veelen, 2021). Therefore, it is sensible to address these issues by developing new cultivars tolerant to stressful environments.

Several barley genes have been exploited to address different stress conditions (Zhou et al., 2016; Hazzouri et al., 2018; Karunarathne et al., 2020; Mwando et al., 2020), including *Hordeum vulgare* aleurone 1 (*HVA1*) for drought, high-affinity K^+ transporter 1;1 (*HKT1;1*) for salinity, and abnormal cytokinin response 1 repressor 1 (*ARE1*) for nitrogen use efficiency (NUE) (Sivamani et al., 2000; Han et al., 2018; Karunarathne et al., 2022). Also, elite stress-tolerant barley genotypes such as 'Golshan' and 'Oxin' for salinity, and 'GrangeR' and 'Bridge' for low-N tolerance have been identified through phenotype screening (Karunarathne et al., 2020; Bahrani et al., 2023). 'Baudin', 'Hamelin', and 'Flagship' were reported to be competitive barley cultivars in the presence of weeds such as ryegrass (Paynter and Hills, 2009). However, these resources are minimal, and more research is required to improve the tolerance of barley and other crops to abiotic stresses. Traditional plant breeding techniques have been widely used to improve crop traits, but are labour-intensive and time-consuming (Zhu et al., 2020). Genetic modification that enables the transfer of genes into elite cultivars has its own drawbacks due to safety and health concerns (Pellegrino et al., 2018). Mutagenesis is more acceptable than transgenesis in breeding, yet in some studies its efficiency is reported to be low (Nonaka et al., 2017). Random mutagenesis also requires large-scale molecular screening to identify a mutation in a given gene (Doll et al., 2019). Therefore, more precise gene-editing technologies such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) gene editing, base editing, and prime editing are imperative to develop new barley lines with superior qualities and better performance under stress conditions (Lin et al., 2021; Karunarathne et al., 2022). The availability of genomic information and more advanced sequencing technologies promotes precise gene editing in crops. For barley, the International Barley Genome Sequencing Consortium (2012) published the first reference genome of 'Morex'. Genome annotation and assembly were subsequently improved (Mascher et al., 2017; Monat et al., 2019).

This comprehensive review aims to provide a gene pool that includes significant genes for tolerance to different abiotic stresses in barley, along with a discussion of the potential applications of modern biotechnologies such as CRISPR/Cas9 gene editing to improve barley tolerance. We focus mainly on the economic impact of abiotic stresses in crop production, how to choose the right target gene, the extension of gene-editing strategies, and how to break genotype dependency in transformation. Knowledge and genetic resources gathered from this review will be useful to generate not only resilient cultivars of barley but also those of other crops, particularly cereals with increased yield and quality.

2 Abiotic stresses threatening crop/barley production

2.1 Drought and extreme high-temperature

Drought is considered the most devastating natural disaster to crop production globally with wide-ranging socio-economic impacts. Drought-related crop losses are reported on all continents, bar Antarctica, and affect all major barley production countries (Kurnaz, 2014; Geng et al., 2016; Stahl et al., 2016; Elliot et al., 2018; Cammarano et al., 2019; Adisa et al., 2020; Kirono et al., 2020; Araneda-Cabrera et al., 2021; Hudzenko et al., 2021; Hunt et al., 2021; Markonis et al., 2021). The European heatwave and drought of 2003 caused a massive 30% reduction in agricultural production (Ciais et al., 2005). Mittler (2006) reported that drought in the USA resulted in USD 20 billion in agricultural economic losses during the period of 1980–2004. When combined with heat extremes, the two stresses were estimated to have caused an astounding USD 120 billion loss. In developing countries, drought not only impacts income and livelihood, but also results in millions of deaths through lack of water and malnutrition, and the displacement of people. As barley is often the only crop grown in some developing countries, particularly in arid and semi-arid regions, the impacts of drought and heat extremes on barley are of great concern (Kebede et al., 2019; Visioni et al., 2019).

The droughts of 2010 and 2012 in the Russian Federation caused extensive losses to wheat and barley harvests, with a combined agricultural loss of RUB 300 billion (about USD 5 billion) (Safonova and Safonov, 2013). The flash drought of 2010 in Western Russia reduced over 70% of wheat harvests and threatened food security domestically and internationally. The ensuing shortages saw domestic wheat price increase, and an export ban was put in place to ensure domestic availability. Major importers of Russian wheat were heavily impacted, with bread prices in Egypt increasing by 300% and cities erupting in rioting and civil unrest (Hunt et al., 2021). Russia is the world's largest producer of barley and the effect of harvest losses caused by the 2010 drought saw that local feed barley prices increase 3.4-fold, and in the drought of 2012, prices rose at least 1.6-fold. In the region of Altai, the 2012 drought caused barley prices to increase by 71.4% (Safonova and Safonov, 2013).

Climate change simulation studies predict drought and heat events to worsen over the century with the most severe changes expected to occur in the second half of the century. The frequency, intensity, and duration of drought events are expected to increase, and terms such as "flash drought" are now used to describe the sudden increase in the intensity of drought episodes observed (Challinor et al., 2014; Otkin et al., 2018; Xie et al., 2018; Ahmadalipour et al., 2019; Cohen et al., 2021; Otkin et al., 2021; Parker et al., 2021). Naturally, there are concerns about how these climatic changes will impact barley production and supply. Notably, the increasing temperature may lead to contradictory impacts on different production areas. For example, crop yields are expected to increase in some regions such as in Canada and northern Spain, where warmer winters would improve yields (Masud et al., 2018; Bento et al., 2021), whilst other regions such as France and southern Spain are likely to experience increasing agricultural losses (Gammans et al., 2017; Bento et al., 2021). To compensate for negative consequences, growers may need to increase expenditure on labour, irrigation, and fertilizers to ensure that barley growing areas do not decrease in size. This will not always be feasible, especially in the context of sustainability and water shortages, and it is expected that there will be a necessary shift in barley supply globally, with consequent changes to import/export markets. The priority will likely remain feed and food supply, with luxury markets such as the brewing industry increasingly impacted and consumers facing increasing costs (Xie et al., 2018; Kebede et al., 2019; Cohen et al., 2021).

2.2 Salinity

Salinity is one of the leading causes of crop losses worldwide, affecting an estimated 32 million ha of dryland agriculture (Wani et al., 2020). Characterized by a high concentration of soluble salts, saline soils impact plant growth through osmotic stress, reduced water availability, and ion excess. The formation of sodic soils or the occurrence of waterlogging can further compound issues for crop producers (Rengasamy et al., 2003; Munns and Tester, 2008). Salinity is a constraint in many of the major barley production countries including Australia, Spain, Turkey, Argentina, the USA, and Canada (Huffman et al., 2000; Houk et al., 2006; Rengasamy, 2006; Acosta et al., 2011; Gorji et al., 2017; Zaman et al., 2018; Taleisnik and Lavado, 2021). Saline soils can be restored, but require substantial investment to leach the soil and large quantities of good quality water, with arid regions suffering larger costs if access to water is limited (Qadir et al., 2014).

In irrigated areas, salt-induced land degradation is estimated to cost USD 27 billion per annum in crop losses (Qadir et al., 2014). About 25%–30% of irrigated land in the USA has crop yields negatively affected by soil salinity (Houk et al., 2006). In Iraq, about 30% of farmland cannot be cultivated due to a combination of soil salinity and lack of water. Yields of wheat, barley, and maize crops are 50%–65% lower than those in non-saline-affected areas, translating to USD 300 million in agricultural losses (Christen and Saliem, 2013). In the southwest region of Western Australia, salinity was calculated to have cost AUD 519–686 million per annum in agricultural losses from 2009 to 2019 (Office of the Auditor General-Western Australia, 2018; Bennett, 2021). In Bangladesh, saline soils are estimated to reduce crop revenue by 20% and have led to diversification into aquaculture as farmers supplement incomes. For those unable to mitigate losses due to salt-induced land degradation, there has been an increase in migration of people away from affected areas (Chen and Mueller, 2018).

Reduced precipitation due to climate change poses increased risks for arid and semi-arid regions as desertification and salinization threaten cropland in the Mediterranean, Africa, parts of Australia, Central America, and parts of the USA (Corwin, 2021). Furthermore, rising sea levels are likely to impact coastal agricultural regions through salt intrusion, with land degradation predicted in Europe (Bosello et al., 2012; Daliakopoulos et al., 2016; Ullah et al., 2021).

2.3 Waterlogging

Waterlogging has been estimated to reduce global crop yields by 10%–20%, impacting North America, Africa, Europe, and Central and South-East Asia. A large proportion of barley producing regions are affected worldwide (Brisson et al., 2002; Setter and Waters, 2003; Dickin and Wright, 2008; Yavas et al., 2012; Ahmed et al., 2013; Rukhovich et al., 2014; Twining, 2014; Sorokin et al., 2016; Borrego-Benjumea et al., 2019, 2020; Ciancio et al., 2021; Tian et al., 2021; Schmitt et al., 2022). Waterlogged soils can occur through floods, heavy precipitation, or irrigation practices, and are compounded by poor drainage, compacted soils, or flat topography (Setter and Waters, 2003; de San Celedonio et al., 2014; Liu et al., 2020c). Excess moisture in the soil limits (hypoxia) or completely depletes (anoxia) oxygen availability, leading to plant oxygen deficiency (Zahra et al., 2021). Global maize, wheat, and even rice yields have decreased by about 33% due to waterlogging (Tian et al., 2021). Barley yields have been reduced by from 35% to as much as 70%, dependent on the timing and duration of waterlogging, and are accompanied by a delay in phenology (Liu et al., 2020a). In Australia, waterlogging causes an estimated crop loss of AUD 180 million per annum, with AUD 100 million and AUD 20 million being attributed to wheat and barley losses, respectively (Manik et al., 2019, 2022). Excess soil moisture has been identified as an agricultural issue in the prairie regions of Canada, where 94% of Canada's barley is grown. Between 1966 and 2017, 37% to 71% of crop losses were reported due to excess moisture (Borrego-Benjumea et al., 2019; de Castro et al., 2022). Wheat yields have decreased by as much as 10% every two years in central China where extreme waterlogging due to climate change is predicted to cause up to 1010 kg/ha wheat yield losses by 2080 (Yan et al., 2022).

Waterlogging has become more frequent and unpredictable due to climate change, with an increased impact on barley cropping regions (Liu et al., 2021). Climate change not only alters the frequency of waterlogging, but also will likely cause a shift in the regions affected. Reduced precipitation and warmer temperatures are likely to improve barley yields in regions prone to waterlogging (Liu et al., 2023). However, modelling predicts more frequent and severe waterlogging stress for countries such as Argentina, Ethiopia, China, the UK, France, and Germany, some of which are the world's largest barley producers (Liu et al., 2023). Furthermore, waterlogged soils release larger amounts of the greenhouse gas nitrous oxide into the atmosphere, contributing to global warming potential, and compounding the issues of climate change (An et al., 2022; Ren BZ et al., 2022).

2.4 Nitrogen use efficiency

Nitrogen (N) is a major factor limiting crop yield potential and grain quality. The addition of N fertilizers has significantly increased crop production yields and is essential to maintain food security (He et al., 2021; Karunarathne et al., 2022). NUE is the ratio of N uptake by the plant against the total amount of N fertilizer applied. Poor NUE is driven by overfertilization and N loss pathways. It is estimated that only 30%–40% of the current year's applied N fertilizer is taken up by crops, with the remainder remaining in the soil or lost out of the cropping system into the air and water (Yan et al., 2020; He et al., 2021; Gao et al., 2022).

About 57% of the global market share of N fertilizer is consumed by China, India, and the USA (31%, 15%, and 11%, respectively), followed by Brazil, Pakistan, Indonesia, Canada, and France with a combined share of 13% (Heffer and Prud'homme, 2016; Lu and Tian, 2017). About 55% of global N fertilizer is used for cereals crops, with wheat, rice, and maize dominating (Heffer and Prud'homme, 2016). The application of N fertilizers to agricultural land is costly and is an environmental hazard, costing developing countries billions of USD in losses. Production of N fertilizers consumes fossil fuels and contributes to greenhouse gas emission impacting climate change. Furthermore, nitrogen fertilizers negatively impact aquatic ecosystems through nutrient runoff, and contaminate ground and drinking water (Houlton et al., 2019; Langholtz et al., 2021).

Increasing NUE in crops is a sustainable solution that not only maximizes yield potential in the face of increasing global food demands, but also would mitigate the economic and environmental impacts of fertilizer use (Houlton et al., 2019; He et al., 2021; Langholtz et al., 2021; Karunarathne et al., 2022). Modelling of USA cropping systems has estimated that a 10% increase in NUE over a 10-year period would increase crop revenue by USD 350 million per annum. A 20% increase in NUE would increase crop revenue by USD 743 million per annum and reduce the cost of water treatment by about USD 15–136 million per annum (Langholtz et al., 2021).

2.5 Herbicide resistance

Weeds are increasingly becoming a worldwide problem that affects crop productivity. They compete with crop plants for sunlight, nutrients, moisture, and space (Naeem et al., 2022). About 35% of yield is lost in major crops, globally, due to weed infestations (Oerke, 2006). Yield reductions in wheat, rice, and maize are 27%, 37%, and 31%, respectively. Global economic loss owing to the reduction in crop production is USD 32 billion per annum (Kubiak et al., 2022). The total economic loss is estimated to be around USD 11 billion for ten major crops in India (Gharde et al., 2018). Winter wheat yield loss ranges from 2.9% to 34.4% in the USA, which translates to an average loss of USD 2.19 billion between 2007 and 2017 (Flessner et al., 2021). In Russia, the average spring barley yield loss is 13% in plots not treated with herbicides (Mayerová et al., 2018). Barley yield loss is estimated to range from 43% to 78% in Australia (Mahajan et al., 2020). The total cost of weeds, including the expenditure and the income loss, is around AUD 2.5–4.5 billion per annum in Australia (GRDC GrowNotes, 2016; Llewellyn et al., 2016).

Wild oats, annual ryegrass, and flaxleaf fleabane are the common weeds in barley paddocks (Mahajan et al., 2020), while ryegrass, wild radish, brome grass, and wild oats are reported to be among the costliest to control in Australia (Llewellyn et al., 2016). Although weeds can be controlled by mechanical or hand weeding, both methods are restricted owing to the labour-intensive nature of large-scale hand weeding and the loss of soil structure in mechanical weeding (Jabran et al., 2015). Spraying herbicides is currently the most widely used strategy for weed management in Australian grain crops (Mwendwa et al., 2022). However, the evolution of herbicide resistance in weeds and the inability to use certain herbicides due to the increased sensitivity of crops are major concerns in chemical weed control (GRDC GrowNotes, 2016). Some of these weeds have developed resistance to herbicides that inhibit acetyl-coenzyme A carboxylase (ACCase), acetolactate synthase (ALS), and enol pyruvyl shiquimate phosphate synthase (EPSPS), suggesting that herbicides with new modes of action are needed (Galon et al., 2022). Therefore, the development of herbicide resistance in cereals is a cost-effective alternative to avoid crop damage caused by herbicides and to maintain high productivity.

3 Genetic resources for barley improvement

3.1 Candidate genes for enhancing barley abiotic stress tolerance

Plant responses to abiotic stress are complex and multigenic, with changes at the cellular, molecular, and physiological levels. Abiotic stresses activate the abscisic acid (ABA)-independent and -dependent signalling transduction pathways, but also activate signalling pathways typically associated with biotic stresses, such as the jasmonic acid (JA) pathway (Visioni et al., 2019). Plant growth and photosynthesis genes are often downregulated during abiotic stress, impairing plant growth and further exacerbating crop yield losses (Ali and Malik, 2021).

From previous studies, we consolidated a suite of named *Hordeum vulgare* (*H. vulgare*) functional and regulatory genes that participate in plant abiotic stress protection (Fig. 1). Although numerous mapping, microarray, and transcriptome studies were assessed (Talamè et al., 2007; Tommasini et al., 2008; Janiak et al., 2018; Collin et al., 2020; Karunarathne et al., 2020; Nefissi Ouertani et al., 2021; Manik et al., 2022), the summary focused on known barley genes as primary candidates for gene editing and molecular breeding. Genes for secondary traits that allow for stress escape, such as flowering time, were compiled, as well as genes to combat impaired growth due to abiotic stress response. Gene locations and annotations were obtained by blasting the National Center for Biotechnology Information (NCBI), UniProt, or Morex v1 accession information provided in publications against the Morex v3 reference sequence (Colmsee et al., 2015; Afgan et al., 2018; Tello-Ruiz et al., 2022). A compiled gene list with published accession information, gene function, Morex v3 gene ID with annotation, and publication references was supplied in Table S1. More than 150 genes covering all seven chromosomes have been mapped. The list reflects the dominance of drought and salinity studies in the literature, with 44% of identified genes induced by drought stress, followed by 23% induced by salt stress. Several genes were reportedly activated under multiple stresses. These are represented by transcription factors, reactive oxygen species (ROS) scavengers, and protective proteins, such as late embryogenesis abundant (LEA) proteins (Table S1). Regulatory genes, such as transcription factors, were a predominant category. Modifying regulatory genes can alter the expression of multiple downstream stress response genes, and therefore has the potential to produce a more significant and durable phenotypic change than targeting a single functional gene (Umezawa et al., 2006).

3.2 Barley prebreeding resources

A modest range of gene-editing tools have been used to develop abiotic stress tolerance in barley (Tables 1 and S2). Key functional genes, regulatory genes, and genes controlling plant growth and photosynthetic machinery have been modified to elucidate the mechanisms controlling abiotic stress response. The usefulness of this material for breeding tolerant cultivars is largely dependent on the outcomes of these studies.

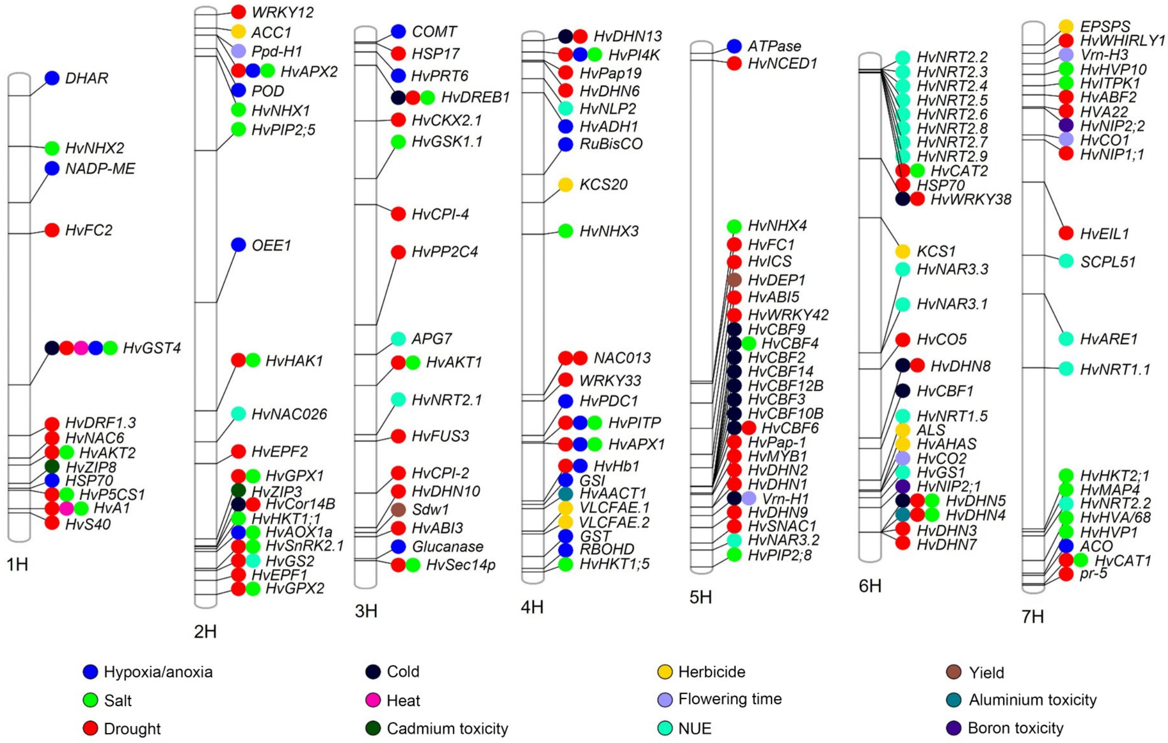

Fig. 1 Potential candidates for gene editing and molecular breeding for improvement of barley abiotic stress tolerance. Identified genes were assigned to physical locations using the Morex v3 reference sequence (comprehensive gene information is available in Table S1). NUE: nitrogen use efficiency. Drawn with PhenoGram software following Wolfe et al. (2013).

Table 1 Genetic resource summary of abiotic stress response genes targeted for gene editing in barley

Gene	Function	Morex ID v3	Mode	Outcome	Engineered lines	Reference
Drought/heat/salt/cold/hypoxia						
HvGST4	ROS scavenger	1HG0051800	BSMV:VIGS	Silencing decreased tolerance to drought, salt, heat, cold, and waterlogging. Increased accumulation of O_2 and H_2O_2 in leaves. No morphological differences observed in unstressed conditions.	BSMV:HvGST4_1, BSMV:HvGST4_2, BSMV:HvGST4_3	Pan et al., 2022
Drought						
HvAKT1	Ion homeostasis	3HG0275790	OE lines, BSMV:VIGS	OE improved drought tolerance through regulation of NO and H_2O_2, resulting in improved K^+ uptake and ion homeostasis. Silencing reduced drought tolerance in wild barley genotype XZ5, with decreased biomass and suppressed K^+ uptake in root cells.	HvAKT1-OX1, HvAKT1-OX2, HvAKT1-OX3, HvAKT1-OX4	Feng et al., 2020b
HvAKT2	Ion homeostasis	1HG0066800	OE lines, BSMV:VIGS	OE improved drought tolerance, increased K^+ absorption and H^+ homeostasis, and reduced H_2O_2. Upregulation of drought-related genes. Silencing reduced drought tolerance in XZ5. Downregulation of drought-related genes and increase of H_2O_2.	HvAKT2-OXs, BSMV:HvAKT2	Feng et al., 2020a
HvCPI-2	Leaf senescence, stay green	3HG0295370	RNAi	Improved drought tolerance compared to Icy4 lines and WT. Upregulation of drought-related genes *Icy4*, *HvPap-1*, *HvPap-12*, and *HvPap-19*. Observed "stay green" phenotype and higher biomass compared to WT in unstressed conditions.	KD Icy2 lines: 1318, 1322, 1390, 1399	Velasco-Arroyo et al., 2018
HvCPI-4	Leaf senescence, stay green	3HG0256160	RNAi	Physiological, biochemical, and molecular changes but no significant improvement to drought tolerance compared to WT. Phenotype under normal conditions similar to WT.	KD Icy4 lines: 1453, 1509, 1558, 1599	Velasco-Arroyo et al., 2018
HvEPF1	Stomata	2HG0206930	OE lines	Enhanced levels of drought tolerance. Reduction in stomatal density and enhanced water use efficiency with no accompanying decreases in biomass or grain yield.	HvEPF1OE-1, HvEPF1OE-2	Hughes et al., 2017

To be continued

Table 1 (continued)

Gene	Function	Morex ID v3	Mode	Outcome	Engineered lines	Reference
HvFC1	Heme biosynthesis, oxidative stress	5HG0471680	OE lines	Improved drought tolerance and reduced oxidative stress. Upregulation of ROS-related genes. Improved photosynthetic capability under stressed and unstressed conditions.	2x35S::FC1	Nagahatenna et al., 2020
HvFC2	Heme biosynthesis, oxidative stress	1HG0033390	OE lines	Improved drought tolerance and reduced oxidative stress. Upregulation of ROS-related genes. Improved photosynthetic capability under stressed and unstressed conditions.	2x35S::FC2	Nagahatenna et al., 2020
HvHAK1	Ion homeostasis	2HG0164700	OE lines, (BSMV:VIGS)	OE improved drought tolerance, increased K^+ absorption and H^+ homeostasis, and reduced H_2O_2. Silencing reduced drought tolerance in XZ5, with downregulation of drought-related genes and increase of H_2O_2.	HvHAK1-OXs, BSMV:HvHAK1	Feng et al., 2020a
HvICS	Salicylic acid biosynthesis	5HG0475370	OE lines, RNAi	Improved drought tolerance in OE lines. Induced of ABA biosynthesis and observed lower levels of ROS. No phenotypic differences compared to WT in unstressed conditions. RNAi lines showed a sensitive phenotype under stress conditions.	Ubi::ICSOE1, Ubi::ICSOE2, Ubi::RNAi1, Ubi::RNAi2	Wang et al., 2021
HvMYB1	MYB transcription factor	5HG0500520	OE lines	Improved drought tolerance with higher RWC in roots and leaves, and reduced water loss and stomatal conductance. Increased proline and dehydrin levels. Reduction of H_2O_2.	OX1, OX2, OX3	Alexander et al., 2019
HvPap-1	Protease	5HG0499760	RNAi	Improved drought tolerance with significantly thicker cuticles than Pap-19 lines or WT, changes in stomata area under stress. Photosynthetic capability more efficient in stressed conditions and similar to WT in unstressed conditions. Less robust than Pap-19 lines and WT in unstressed conditions, slight delay in senescence. Improved protection from *Magnaporthe oryzae* and *Tetranychus urticae* infections.	Pap-1 lines: 1128, 1130, 1175, 1176	Gomez-Sanchez et al., 2019

To be continued

Table 1 (continued)

Gene	Function	Morex ID v3	Mode	Outcome	Engineered lines	Reference
HvPap-19	Protease	4HG0339740	RNAi	Improved drought tolerance, with changes in stomatal area, greater leaf growth than Pap-1 lines or WT under stress. Unstressed, growth and development similar to WT. Photosynthetic capability similar to WT under unstressed conditions and more efficient under stressed conditions. More susceptible than WT to *M. oryzae* and *T. urticae* infections.	Pap-19 lines: 1770, 1776, 1779, 1782	Gomez-Sanchez et al., 2019
HvSNAC1	NAC transcription factor	5HG0524540	OE lines	Improved drought tolerance. Increased survival rate, reduced water loss, and improved photosynthetic capability under stress conditions. No deleterious effects on growth in well-watered conditions. Yields higher than WT in field trial.	OE#3, OE#11	Al Abdallat et al., 2014
HvWHIRLY1	Leaf senescence	7HG0650090	RNAi	KD lines showed "stay green" phenotype under drought stress. Downregulation or delay of drought stress and senescence genes.	RNAi-W1 lines	Janack et al., 2016
Drought/anoxia/hypoxia						
HvHb1	Phytoglobin, NO scavenger	4HG0394960	OE lines	Uhb OE increased NO scavenging. Lower NO emissions under hypoxia but unchanged under anoxia. Delayed plant development and reduced yield. Reduced resistance to *Blumeria graminis*. Hhb lines had lower yield and reduced iron in grain.	Uhb lines: 7, 8, 9 (maize Ubi-2); 2, 6, 14, 16 (hordein-D)	Hebelstrup et al., 2014
			OE lines, RNAi	Reduction in NO and survival under low oxygen stress. OE plants able to germinate under hypoxia, but stunted growth and reduced germination under normoxia. KD lines have high levels of NO emission.	Uhb8 (Pgb+), RNAi: Pgb−	Cochrane et al., 2017
			OE lines	Increased drought tolerance. Increased protective polyamine levels, with reduced NO and ethylene levels.	Uhb5, Uhb6	Montilla-Bascón et al., 2017
Waterlogging						
HvPRT6	ERF transcription factor	3HG0230560	RNAi	Positive effect on growth and survival during waterlogging, delay in senescence. Decrease in *HvPRT6* expression, increased expression in hypoxia-response genes. Germination efficiency reduced compared to null.	RNAi lines: 23, 25, 55	Mendiondo et al., 2016

To be continued

Table 1 (continued)

Gene	Function	Morex ID v3	Mode	Outcome	Engineered lines	Reference
Cold/hypoxia						
HvPI4K	Kinase, ABA signalling	4HG0337080	OE lines	Enhanced cold stress response and hypoxia tolerance. No significant change in salt tolerance. Developmental abnormalities observed.	PI4K L2, L3, L4, L5, L6, L8	Gierczik et al., 2019
HvPITP	Transfer protein, ABA signalling	4HG0385530	OE lines	Enhanced cold stress response and hypoxia tolerance. No significant change in salt tolerance. Developmental abnormalities observed.	PITP L4, L9, L10, L12, L13, L15	Gierczik et al., 2019
Cold stress						
HvCBF2A	CBF/DREB transcription factor	5HG0497570	OE lines	Improved freezing tolerance under cold stress. Constitutive expression of *CBF2*. Significant upregulation of numerous frost stress-related genes. Negative effect on growth and developmental processes at normal temperatures.	Hv-CBF2A lines 2, 3, 6, 10, 13, 15	Jeknić et al., 2014
Salt						
HvGSK1.1	Kinase, BR signalling	3HG0252610	RNAi	KD lines had greater biomass under normal and stress conditions. Elevated kernel weight under normal conditions. Modified expression of paralogous genes.	Transgenic lines #1, #4, #5, #6, #8, #9, #10	Kloc et al., 2020
HvHKT1;1	Ion homeostasis	2HG0192310	BSMV:VIGS	KD lines had reduced salt tolerance and higher Na^+ accumulation in roots and leaves, and reduced K^+ in shoots. Significant reduction in dry biomass compared to control.	BSMV:HvHKT1;1-1 (H1), BSMV:HvHKT1;1-2 (H2)	Han et al., 2018
HvHKT1;5	Ion homeostasis	4HG0415690	RNAi	KD lines had improved salt tolerance with decrease in Na^+ translocation and increase in K^+/Na^+ under salt stress compared to WT.	RNAi-14, RNAi-23, RNAi-27	Huang et al., 2020
HvHKT2;1	Ion homeostasis	7HG0727320	OE lines	Increased tolerance to salt stress and increase in NA^+ accumulation in leaves. Higher growth rate than WT under salt stress.	HvHKT2;1-OX1, HvHKT2;1-OX2	Mian et al., 2011
HvHVP10	Ion homeostasis	7HG0657860	CRISPR/Cas9, RNAi	KD and KO decreased salt tolerance. Inhibited growth, and higher shoot Na^+ concentrations.	CRISPR: CR-1, CR-2; HVP10-RNAi: L3, L5, L7	Fu et al., 2022
HvITPK1	ABA signalling	7HG0661290	CRISPR/Cas9	Improved plant growth under high salt stress, but impaired growth under normal and low salt stress. Delay in germination. Distinct phenotypic differences between insertion and deletion mutants.	*itpk1-2*, *itpk1-33*	Vlčko and Ohnoutková, 2020

To be continued

Table 1 (continued)

Gene	Function	Morex ID v3	Mode	Outcome	Engineered lines	Reference
HvMPK4	Kinase, SA signalling	7HG0728160	OE lines, RNAi	OE lines had greater salt tolerance. Constitutively enhanced JA and ethylene levels. Less salt accumulated in leaves and enhanced level of proline. Biomass affected by stress but less so than WT. No differences observed between OE lines and WT after *Magnaporthe grisea* infection.	HvMPK4-OX (overexpression), HvMPK4-AS (antisense)	Abass and Morris, 2013
NUE						
HvARE1	Nitrogen assimilation	7HG0693130	CRISPR/Cas9	Higher accumulation of N in shoots. Delayed leaf senescence and increased yield. Yield remained high under optimal growth conditions.	1*are1*-E-7-6, 2*are1*-K-4-7	Karunarathne et al., 2022
HvNLP2	Nitrate assimilation	4HG0343670	RNAi	Reduction in NUE, biomass, and seed yield. Downregulation of related N assimilation genes. No observed changes in nitrogen uptake.	*hvnlp2-2*, *hvnlp2-3*	Gao et al., 2022
Aluminium toxicity						
HvAACT1	Citrate transporter	4HG0396210	OE lines	Increased Al^{3+} tolerance and enhanced Al^{3+}-activated citrate efflux from roots. Transgenic plants not as successful as tolerant cultivar 'Dayton'.	Hv:T2_17A, Hv:T2_33A, Hv:T2_51, Hv:T2_52B	Zhou et al., 2013
HvAACT1	Citrate transporter	4HG0396210	Promoter modification	The 1-kb insertion in the promoter enhanced and altered expression pattern conferring Al-tolerant phenotype.	B1:1-1,2-3,4-2,5-3; B2:1-1,1-4,2-1; B3:1-2,1-3,3-2,7-3	Fujii et al., 2012
Cadmium toxicity						
HvZIP3	ZIP transporter	2HG0191210	RNAi	Significantly higher accumulation of toxic Cd plus reduction of essential minerals Zn and Mn in grains.	W-ZIP3-2	Sun et al., 2015
HvZIP8	ZIP transporter	1HG0071750	RNAi	Significantly higher accumulation of Cd. Reduction of Zn and Mn in grains.	W-ZIP8-1, W-ZIP8-6, Z-ZIP8-6, Z-ZIP8-7, Z-ZIP8-8	Sun et al., 2015

ABA: abscisic acid; Al: aluminium; BR: brassinosteroid; BSMV: barley stripe mosaic virus; CBF: C-repeat binding factor; Cd: cadmium; CRISPR/Cas9: clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9; DREB: dehydration responsive element binding; ERF: ethylene responsive factor; JA: jasmonic acid; KD: knockdown; KO: knockout; Mn: manganese; MYB: myeloblastosis; N: nitrogen; NAC: NAM (no apical meristem), ATAF1/2 (*Arabidopsis thaliana* activating factor), and CUC2 (cup-shaped cotyledon 2); NO: nitric oxide; NUE: nitrogen use efficiency; OE: overexpression; RNAi: RNA interference; ROS: reactive oxygen species; RWC: relative water content; SA: salicylic acid; VIGS: virus-induced gene silencing; WT: wild type; ZIP: Zn- and Fe-regulated transporter-like protein (expanded experiment data are available in Table S2).

Numerous overexpression lines have been developed with significant phenotypic improvement reported across a range of abiotic stresses (Table 1). However, constitutive overexpression yielded variable results on plant development and growth, depending on the gene targeted. In some cases, plant growth remained healthy under normal and stressed conditions and even exhibited enhanced growth and plant biomass, compared to the wild type. In other instances, abnormal growth and reproductive development were observed, particularly under normal and unstressed conditions. In transgenic barley, the use of stress-inducible or modified promoters has been shown to address abnormal growth associated with constitutive overexpression and could plausibly be applied here (Kovalchuk et al., 2013).

Gene silencing is an elegant tool for elucidating the functional role of a gene of interest, but outcomes are not necessarily predictable. For instance, knockdown of *HvHKT1;1* ultimately led to reduced salt tolerance, whereas knockdown of negative regulator *HvHKT1;5* enhanced salt tolerance (Table 1). Whilst both outcomes are academically valuable in establishing the role of these genes in salt stress response, only one of the exhibited phenotypes is desirable for breeding purposes. Likewise, virus-induced gene silencing (VIGS) rapidly provides valuable insights into gene function, but its transient nature is of limited benefit to plant breeders.

The CRISPR/Cas9 system is a more precise technique than overexpression, yet only Vlčko and Ohnoutková (2020), Fu et al. (2022), and Karunarathne et al. (2022) had used this technology for barley germplasm development (Table 1). In two of these cases, a series of mutants with variable phenotypes were successfully generated. Other editing tools, such as transcription activator-like effector nucleases (TALENs) or prime editing, were unrepresented for the abiotic stresses, and only Fujii et al. (2012) had examined promoter modification.

It was shown that selecting early flowering cultivars with high yield potential mitigated some of the impacts of heat and drought stresses occurring during the maturation stage (He et al., 2022). Liu et al. (2020b) highlighted the importance of identifying optimal sowing and flowering periods to alleviate the negative consequences of water, heat, frost, and potentially waterlogging for Australian barley. Targeting genes that maintain plant growth and photosynthetic capabilities whilst under abiotic stress is therefore a valuable approach to improve tolerance in barley germplasm (Atkinson and Urwin, 2012; Ali and Malik, 2021). Constitutive overexpression of *HvFC1* and *HvFC2* not only improved drought tolerance, but also improved the photosynthetic rate under normal and drought-stressed conditions (Table 1). This suggests that modification of such genes has the potential to mitigate some of the yield losses associated with abiotic stress responses.

A single candidate gene approach is often undertaken in germplasm development, but there is some skepticism that this is enough to confer acceptable tolerance in a field setting over an entire season. Zhou et al. (2013) showed that although overexpression of *HvAACT1* enhanced plant tolerance to aluminium (Al^{3+}), engineered lines were not as tolerant as the existing cultivar ‘Dayton’, leading the authors to propose multiple mechanisms conferring Al^{3+} tolerance in barley (Table 1). Furthermore, of the material presented in Table 1, only al Abdallat et al. (2014) had examined plant response in a field environment, with many studies focusing only on the seedling stage of growth (Table S2). Significant differences that are observed for short durations under controlled laboratory or glasshouse conditions may yield only minor or negligible improvement once in the field (Hirayama and Shinozaki, 2010; Atkinson and Urwin, 2012). Plant stress response is interactive and non-additive, with some genes acting synergistically and others antagonistically. Once an initial abiotic stress response is activated, plants may alter how they respond to secondary stress, resulting in a phenotype that is unexpected from observing a single stress in isolation (Mittler, 2006; Atkinson and Urwin, 2012). While it is essential to test specific variables in isolation for preliminary studies, such as in functional genomics, it is also essential to assess responses to multiple stresses simultaneously (both abiotic and biotic), observed over multiple growth stages, and in the field.

4 Precise gene editing in barley—advances, strategies, and challenges

4.1 Current progress using CRISPR/Cas9 system

As highlighted under barley prebreeding resources, the CRISPR/Cas system is a precise technique in gene-editing. It is an integral part of the bacterial and archaeal immune systems, which has now been adapted by scientists in various fields including agriculture. The working mechanism of CRISPR is well explained in many recent reviews (Najera et al., 2019; McCarty et al., 2020; Wada et al., 2020; Nejat et al., 2022). The introduction of CRISPR as a gene-editing platform has greatly improved the efficiency of breeding (Nonaka et al., 2017) and the functional characterization of genes (Zhang et al., 2021; Karunarathne et al., 2022). It is a revolutionary tool for basic research and biotechnology. Gene knockout is the widely used application of CRISPR/Cas9, and single or multiple gene knockouts have been successfully observed in maize, barley, wheat, and rice (Li et al., 2013; Doll et al., 2019; Kim et al., 2019; Lawrenson and Harwood, 2019; Křenek et al., 2021; Zhang et al., 2021; Karunarathne et al., 2022). Advancement of the technique to multiplex gene editing allows the simultaneous editing of several targets in a single genome (Xing et al., 2014). Engineered CRISPR systems have become more efficient and flexible, with base and prime editing tools among the most recent advances of CRISPR. Base editing targets single nucleotide mutations with the use of deactivated Cas9 (dCas9) or nickase Cas9 (nCas9). Prime editing allows for all types of desired base substitutions, small insertions up to 44 bp and deletions up to 88 bp at selected target sites with the use of CRSIPR/Cas9 nickase-reverse transcriptase fusions (Anzalone et al., 2019; Lin et al., 2020). CRISPR/Cas9 is the most widely used system, but new systems such as Cas12, Cas13a, and Cas13b are in the pipeline (Smargon et al., 2017; Zaidi et al., 2017; Aman et al., 2018). Products of gene editing are classified as site-directed nuclease-1 (SDN-1), SDN-2, and SDN-3 types. SDN-1 products rely on a non-homologous end joining (NHEJ) pathway to introduce a few base insertions or deletions. SDN-2 and SDN-3 rely on homology-directed repair (HDR) in which a template DNA sequence is used (Grohmann et al., 2019).

Compared to other gene-editing methods such as TALEN or zinc finger nuclease (ZFN), CRISPR/Cas9 has advantages in simple target design, multiplexed mutation, versatility, and efficiency (Leong et al., 2018). There have been numerous proof-of-concept experiments conducted using the CRISPR/Cas9 gene-editing platform (Lawrenson et al., 2015), but few developments have reached commercialization (Nonaka et al., 2017). A γ-aminobutyric acid (GABA)-enriched tomato from Japan was the first CRISPR-edited food to enter the market. Introduction of a stop codon to the *Solanum lycopersicum* L. glutamate decarboxylase *SlGAD2* and *SlGAD3* genes through CRISPR/Cas9 technology increased the GABA content in the tomatoes by 7-fold to 15-fold (Nonaka et al., 2017). In barley, CRISPR/Cas9 gene editing has been successfully used to create insertion/deletion (InDel) mutations in eukaryotic translation initiation factor 4E (*elF4E*), *H. vulgare* mitogen-activated protein kinase 6 (*HvMPK6*), *H. vulgare* nudum (*HvNud*), *H. vulgare* purple acid phosphatase phytase (*HvPAPhy*), *H. vulgare* protein targeting to starch 1 (*HvPTST1*), *H. vulgare* granule-bound starch synthase 1 (*HvGBSS1*), *H. vulgare* homogentisate phytyltransferase (*HvHPT*), and *H. vulgare* homogentisate geranylgeranyl transferase (*HvHGGT*) genes to improve desirable traits (Holme et al., 2017; Zhong et al., 2019; Zeng et al., 2020; Křenek et al., 2021; Kershanskaya et al., 2022; Zang et al., 2022). This marks the potential application of the technology in developing abiotic stress tolerance and accelerating barley breeding.

4.2 CRISPR gene-editing strategies to develop abiotic stress tolerance in barley

Gene-editing strategies that need to be used to develop stress tolerance in barley differ based on their positive or negative effects or transcriptional regulation of the potential genes (Tables 1 and S2). For instance, downregulation of the *ARE1* gene has been identified to improve NUE in cereals, which makes CRISPR/Cas9 gene knockout the best strategy to create loss of function mutant barley lines (Karunarathne et al., 2022). Choosing the wrong gene-editing strategy could produce plants with impaired growth or an unfavourable phenotype under stress conditions. The knockout mutant lines generated for vacuolar *H. vulgare* H^+-pyrophosphatase (*HvHVP10*) in barley had decreased salt tolerance (Fu et al., 2022). This might have been because *HvHVP10* is known to be upregulated in response to salt stress in barley roots. The study was doubtlessly invaluable to elucidate the gene's function, but inducing overexpression using CRISPR would have been a better approach in terms of commercial breeding. Most of the genes in Table 1 have been overexpressed to achieve drought and frost tolerance, but not through CRISPR/Cas9. Where gene knockouts in the coding region cause unfavourable pleiotropic effects, modification of the promoters to induce gene activation is desirable in developing stress tolerance in crops (Ren C et al., 2022). CRISPR/Cas9 can be reprogrammed for transcriptional regulation by fusing transcriptional activator domains to a dCas9 in barley. In rice, simultaneous multigene activation is possible using the CRISPR activation (CRISPRa) system (Lowder et al., 2018). Therefore, this may be a promising application in other monocots too, including barley, to study the transcriptional network underlying abiotic stresses.

Some agronomic traits are controlled by a few quantitative trait loci (QTL), and in such cases editing one gene is less likely to give a favourable phenotype. Multiplex gene editing can solve this problem by facilitating the simultaneous editing of two or more genes, or multiple loci of the same gene (Xing et al., 2014). Especially when improving abiotic stress tolerance in crops, we can develop a mutant line with tolerance to multiple stresses through multiplex gene editing, which otherwise demands prolonged conventional crossing. An editing efficiency of 21% was observed with heritable mutations in multiplex editing of two barley cytokinin oxidase/dehydrogenase (*CKX*) genes, *HvCKX1* and *HvCKX3*, using a polycistronic transfer RNA (tRNA)-guide RNA (gRNA) construct (Gasparis et al., 2018). The Western Crop Genetics Alliance, Murdoch University (Australia) has successfully used multiple independent cassettes as a proof of concept to target *WRKY* transcription factors to develop a high-throughput mutant library in barley (Nejat, 2022). A number of mutants were obtained with improved agronomic and plant architecture traits such as seed number, spike length, tiller number, and root structure, and are currently being screened under different stress conditions (Nejat, 2022).

Alteration of one nucleotide in a gene is reported to have the potential to improve important crop traits in agriculture (Tian et al., 2018). These single nucleotide polymorphisms (SNPs) can now be achieved through base or prime editing (Lin et al., 2021; Xiong et al., 2022). Base-editing studies conducted in *Arabidopsis* and rice provide confidence in using this technique in barley to improve useful traits, for instance, inducing early or late flowering to escape stress windows such as those for frost or drought (Li et al., 2020; Xiong et al., 2022). More importantly, herbicide-resistant barley lines can be developed through base editing (Shimatani et al., 2017; Tian et al., 2018). It can also be repurposed as a gene loss of function tool by creating a stop codon or a gene knockdown by creating an upstream open reading frame (uORF) (Billon et al., 2017; Kuscu et al., 2017; Xiong et al., 2022). Prime editing is the latest technique that can induce precise mutations (Lin et al., 2021). Plant prime-editing systems have been used in rice and wheat, but not in barley (Lin et al., 2020; Xu et al., 2020). They provide another platform for generating herbicide resistance in barley, especially as they can introduce pre-determined single base substitutions precisely. For instance, the rice gene *Oryza sativa* acetolactate synthase (*OsALS*) was targeted to develop resistance to the herbicide bispyribac sodium (Butt et al., 2020). We can maximise the use of prime editing by combining it with multiplex gene editing to generate improved barley lines with resistance to multiple herbicides with different modes of action.

4.3 Confidence in targeting genes

We have collated a long list of genes related to different abiotic stresses in barley (Fig. 1, Table 1). However, choosing the most significant genes for downstream experiments is challenging. Researchers have sometimes used gene expression studies with reverse transcription-polymerase chain reaction (RT-PCR) or differential gene expression to determine potential genes for any given trait (Feng et al., 2020a; Karunarathne et al., 2022). VIGS is an effective tool widely used to study gene function in plants. It allows a quick study of several genes without the need for transformation (Han et al., 2018; Feng et al., 2020b). This is an ideal tool to narrow down our gene list and eventually pick only the most significant genes. VIGS has been used to functionally analyse the *Triticum aestivum* enhanced response to abscisic acid 1 (*TaEra1*) and basic transcription factor 3 (*TaBTF3*) genes in wheat, and inwardly rectifying potassium (K^+) channels *HvAKT1* and *HvAKT2*, *HVA1*, and *Hv* dehydrin 6 (*HvDhn6*) genes in barley for drought tolerance (Liang et al., 2012; Kang et al., 2013; Manmathan et al., 2013; Feng et al., 2020a). Barley stripe mosaic virus-induced gene silencing has been conducted in barley by Han et al. (2018), and our lab has an established platform to use VIGS to back up gene selection (unpublished data). Since VIGS is transient, after confirming the gene, CRISPR/Cas9 gene editing should be used to generate stable successful mutants. Furthermore, the availability of barley pan-genome sequences facilitates the identification of structural variations and conserved sequences within the genes of interest and pinpoints the most plausible gene for editing (Jayakodi et al., 2020).

4.4 Breaking genotype dependency for commercial breeding

Gene editing in barley is performed mainly by delivering the CRISPR/Cas9 gene-editing complex through particle bombardment or *Agrobacterium*-mediated transformation (Han et al., 2021). Stable transformation of barley from immature embryos, microspore-derived embryos, and callus from young embryos was first reported by Wan and Lemaux (1994). Regardless of the delivery method, the process involves tissue culture, which is tedious, time-consuming, and genotype-dependent. The spring barley cultivar 'Golden Promise' has been used for decades with a successful transformation and regeneration rate. A reference genome assembly for this genotype is now available (Schreiber et al., 2020). Other cultivars such as 'Compass', 'Spartacus', and the breeding line WI4330 have been used in previous research, but with low efficiency (Ismagul et al., 2014; Han et al., 2021; Karunarathne et al., 2022). 'RGT Planet' has now been found to have a high transformation and regeneration efficiency almost similar to that of 'Golden Promise' (Nejat, 2022). A barley pan-genome is now available that provides more sequence information for gene editing, but the transformation capacity of pan-genome accessions is yet to be tested (Jayakodi et al., 2020). Overexpression of developmental regulators such as the maize BABY BOOM (*BBM*) and WUSCHEL 2 (*WUS2*) genes has increased the transformation efficiency in sorghum, rice, and sugarcane (Lowe et al., 2016; Mookkan et al., 2017). The latest breakthrough is the overexpression of the *T. aestivum* WUSCHEL-related homeobox 5 (*TaWOX5*) gene to overcome genotype dependency in wheat, barley, and maize genetic transformation (Wang et al., 2022). The use of virus-based vector, namely sonchus yellow net rhabdovirus (SYNV), to deliver the entire CRISPR/Cas9 cassette is an alternative way to bypass the tissue culture process. This does not require the isolation of plant cells or tissues and can be delivered directly into intact plants. It is reported to achieve both single and multiplex mutagenesis with high efficiency in tobacco (Ma et al., 2020). De novo induction of gene-edited meristems also avoids the tissue culture step and can be induced in aseptically grown seedlings and soil-grown plants (Maher et al., 2020). Injection of *Agrobacterium tumefaciens* carrying the development regulators *WUS2* and SHOOT MERISTEMLESS (*STM*) into tobacco seedlings led to the development of gene-edited shoots with heritable mutations (Maher et al., 2020). The use of nanoparticles, such as mesoporous silica nanoparticles, liposomes, and layered double hydroxides, as vectors for the delivery of the CRISPR/Cas9 system is a significant development in gene editing (Chen and Mueller, 2018; Alghuthaymi et al., 2021). It can avoid genotype dependency in plants but requires further insight in terms of application.

4.5 High-throughput mutation screening

Identification of the genotype of mutants is the most important task in gene editing. Several molecular techniques from agarose gel electrophoresis to sequencing can be used for this task (Zhang et al., 2021; Karunarathne et al., 2022). PCR-restriction enzyme digestion has proved to be useful in genotyping mutants. Enzymes that can digest the wild-type DNA, but not the mutant DNA, can be used to pinpoint mutations as heterozygous or homozygous on agarose gels (Han et al., 2021; Karunarathne et al., 2022). Enzyme recognition sites are usually chosen close to the protospacer adjacent motif (PAM), and thus any mutations away from PAM/recognition site can give false negative results, limiting the application of this method. Single-strand conformation polymorphism (SSCP) on the other hand can be used to detect SNPs due to its simplicity and low cost. Altered conformation due to a single base change in the DNA can cause different band patterns on non-denaturing gels which separate mutant and wild-type DNA (Zhu, 2005). High-resolution melting (HRM) is useful in a large-scale screening of mutants. It generates sequence-related melting profiles and reveals the genotype at the level of a single nucleotide (Chatzidimopoulos et al., 2019). Third generation digital PCR (dPCR) enables rare mutation detection and trace DNA detection but is not suitable for large amplicons and cannot detect multiple targets in one sample (Mao et al., 2019). Regardless, all the above techniques must be followed by sequencing, which is the gold standard for genotyping mutants (Xu et al., 2020; Zhang et al., 2021). Sanger sequencing is widely used and useful for low-volume high-quality DNA sequencing (Nonaka et al., 2017; Doll et al., 2019). The more advanced next generation sequencing (NGS) is useful for sequencing large mutant populations at lower cost (Peterson et al., 2016; Yang et al., 2022). Both techniques enable the identification of the exact order of a gene sequence, and the alignment of this DNA or protein sequence helps us to detect the respective amino acid change in mutants. TA-cloning can be used to clone multiple DNA fragments for sequencing, which can detect heterozygous or chimeric mutations (Ma et al., 2020). Transgene-free homozygous mutants are desirable and in barley are feasible in the T1 generation (Zeng et al., 2020). Tracking of InDels by Decomposition (TIDE) is an effective web tool (https://tide.nki.nl) that can be used to analyse sequencing data, which estimates the spectra and frequencies of small insertions and deletions generated from gene editing (Brinkman et al., 2014).

5 Conclusions and perspectives

Cereal crops including barley suffer significant yield and economic losses in the face of increased abiotic stresses due to climate change. Thus, improving abiotic stress tolerance in barley is imperative to ensure food security. CRISPR/Cas9 gene-editing provides an ideal platform with high efficiency and versatility, which has already been applied in both monocots and dicots to improve numerous agronomic traits, and some products have already been commercialised. CRISPR/Cas9-based multiplexed gene-editing is useful to develop new barley lines with tolerance to more than one stress condition by targeting multiple genes simultaneously. A combination of gene-editing with speed breeding techniques can accelerate crop breeding to develop new cultivars with desirable traits. Further applications of CRISPR/Cas9 not discussed in this review include haploid induction and generating male sterile lines to support hybrid vigour. Barley wild species have huge genetic diversity and CRISPR/Cas9-aided domestication for climate resilience and other desirable traits takes only a short time compared to traditional domestication. Even though CRISPR/Cas9 gene-editing has advanced over the years, more insight and further development of the toolkit are essential in certain crops. New and efficient methods for delivery of the gene-editing complex such as the use of nanoparticles must be tested within crops to accelerate the transformation process.

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Author contributions

Sakura KARUNARATHNE and Esther WALKER performed the literature search and data analysis, and drafted the manuscript. Chengdao LI, Darshan SHARMA, and Yong HAN critically revised the work. Yong HAN conceptualized the work. All authors have read and approved the final manuscript.

Compliance with ethics guidelines

Sakura KARUNARATHNE, Esther WALKER, Darshan SHARMA, Chengdao LI, and Yong HAN declare that they have no conflict of interest.

This review does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary materials

Tables S1 and S2