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# Barley Grain Development during Drought Stress: Current Status and Perspectives

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## Abstract

Barley (*Hordeum vulgare* L.) belongs to small grain cereals that cover more than 78% of the daily calorie consumption of humans. With a prediction of 9.7 billion humans in 2050 (FAO stats) and climatic changes, the question of increasing small grain cereal's production has become an agricultural challenge. Drought exerts a strong environmental pressure, causing large yield losses worldwide. Therefore, understanding the mechanisms responsible for grain development from the fertilization to the mature dry grain is essential to understand how drought can affect this developmental program. In this book chapter, we present the physiological, molecular and hormonal regulation of barley grain development. In a second part, we describe the consequences of drought at different stage of barley development, with a special focus on the reproductive phase. Finally, in the last part, we present the different methods used to decipher new genetic information related to drought-tolerance. All this knowledge contributes to understanding the tolerance mechanisms of barley and to developing breeding strategies aiming to bring about new varieties with sustained yield in harsh conditions.

**Keywords:** barley, drought, grain development, QTLs, GWAS

## 1. Introduction

Small grain cereals (rice, maize wheat, barley, rye and oat) are the most important food supply, representing more than 78% of the calories consumed each day by humans (FAO Stat). Cultivated barley (*Hordeum vulgare* L.) is the fourth most important cereal worldwide, serving as a model species for the temperate cereals. Indeed, barley can grow in highly contrasting habitats and tolerate stress conditions such as drought, high and low temperature, and salinity [1, 2]. The use of grains as a source of food begun already during the Middle Stone Age, long before cereal domestication [3]. The early domestication resulted in drastically altered seed size and grain number; later the modern plant breeding in combination with agricultural technics concurred to the nowadays high yields [4]. However, substantial increases in yield have to be reach to ensure food security for the ever-growing worldwide population that is estimated to reach more than 9.7 billions inhabitants in 2050 (FAO statistics). Moreover, in the current context of climatic changes, the sustainability of cereal grain yield has already become a challenge for food security. In the current era, such goals can be reached solely by the use of the molecular breeding that requires a deep understanding of the molecular mechanisms controlling seed and plant development [4].

In agriculture, yield is defined usually by the classical concept of number of inflorescences per cultivated area. Nowadays, grain yield takes also into consideration the grain number per inflorescence and the grain weight, often measured as thousand grain weight (TGW) [5]. Factors affecting the overall plant development (water and nutrients uptake from soil, development of photosynthetic tissues for carbon fixation and storage, carbon and nutrient relocation during grain filling) can have important consequences on grain yield [6]. Whereas the number of florets determines grain number per inflorescence, grain weight is determined by the grain size, and the amount of starch and protein accumulated during grain filling [7]. Grain weight reflects the size of the grain, itself determined by length, width and volume or filling, among other parameters. All these parameters describe the grain architecture. The genetics behind grain architecture is complex, involving maternal and paternal developmental signals, hormonal regulation and integrating the environmental information such as photoperiod, biotic and abiotic stresses [8]. The genetic and molecular bases of this agronomic trait have attracted attention in the last decades. In this regards, several studies based on quantitative trait loci (QTL) mapping or Genome-wide Association Study (GWAS), combined with mutant identification, identified new genes involved in barley grain development and yield-related genes [9–14].

As already mentioned, grain yield is a complex genetic trait, greatly affected by the environment and cultivation conditions. Water deficit or drought is undoubtedly the most important environmental factor affecting the global productivity of crops [15, 16]. However the extent of the damages, the recovery capacity and the impact on the final grain size depend on the developmental stage during which the plant faces the stress [16]. In the last decades, improving crop growth and yield under changing environmental conditions, especially drought, became a major goal of plant breeding programs [17, 18]. Drought tolerance is a complex genetic trait, involving multiple genes [19, 20]. Many studies have investigated the genetic bases of drought tolerance in barley [18–21]. QTL studies are one of the most used approaches to identify genomic regions controlling agronomic performance under water-limiting conditions [22, 23]. Recent advances in barley genome sequencing provide great potential for genetic studies, such as QTL studies and recent Genome Wide Association Studies (GWAS) [24–28]. Very recently, a 50 k iSelect SNP Array, based on exome capture, has been developed from a wide range of European barley germplasm containing 394 cultivated accessions. This large data set is of great interest for further genetic studies in barley [29]. The ability of a plant to adapt to a specific environment relies mainly on the genetic variability that is a long process of adaptation to the environmental pressure. To face fast changing conditions, plants possess epigenetic regulation of gene expression, kind of switch on/off mechanism. Epigenetic relies on structural and chemical modification of the genome without affecting the genetic information. It promotes fast, and most importantly reversible changes in phenotype in response to environment modification [30]. A recent study on the hare barley (*Hordeum murinum subsp. leporinum*) strongly suggested that the response to climate change involves epigenetic regulation of gene expression to maintain homeostasis and ensure functional stability [31].

It has to be considered that the domestication and breeding strategies, based on inter-crossing elite or high-performance varieties, led to a loss of genetic diversity [32]. This genetic bottleneck could be overcome by the use of wild relative species that constitute a great resource of diversity, useful for new breeding strategies [33].

Understanding the physiology of grain development, as well as the effect of drought in this process are crucial for developing efficient breeding programs aiming to improve or at least sustain barley productivity in water deficit conditions.

In this book chapter, we will focus only on the development of barley grain. For more information related to inflorescence development in barley, one can refer to recent articles [34, 35]. In a first part, we will describe grain development in barley taking into account the hormonal and molecular regulation. In a second part, we will identify how water deficit or drought can affect grain development. Finally, in the last part, we describe methods used to unravel and study drought tolerant-associated genes.

## 2. Grain development in barley

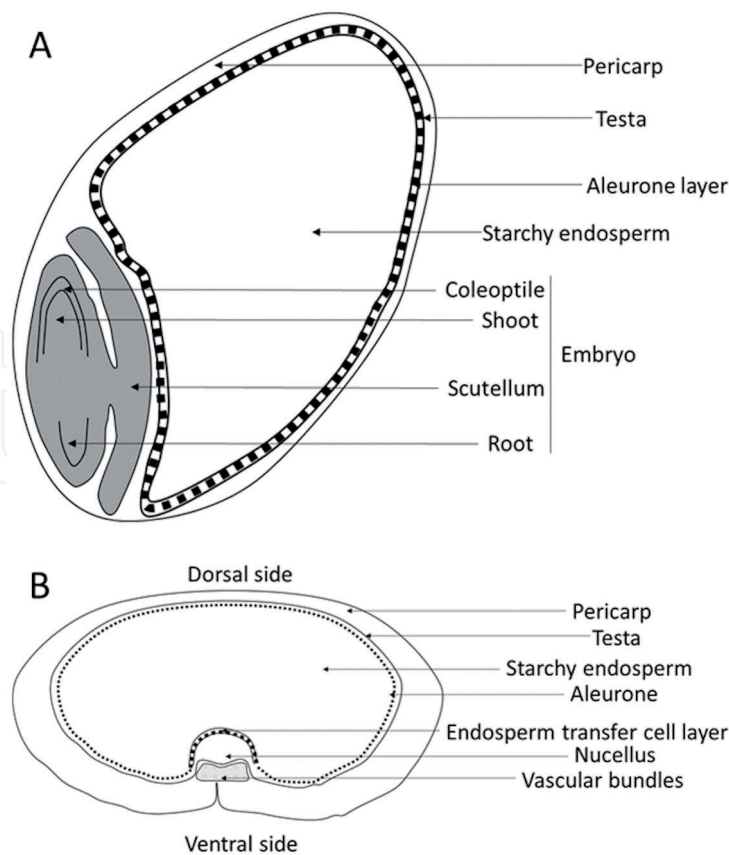
The reproductive phase of development starts with the transition of the vegetative meristem into a reproductive meristem (inflorescence primordia), and ends with the physiological maturity of the grain characterized by dessication of the grain and entrance into dormancy [16]. The entire reproductive phase can be divided into several substages: floral initiation, differentiation of inflorescence and florets, male and female gametogenesis, pollination, fertilization and seed development [16]. From anthesis to maturity, grain development progresses through several phases that are commonly divided into three phases. The phase I, called pre-storage phase, is a phase of active cell division and differentiation that includes double fertilization, syncytium formation and cellularization. At this phase, the potential size is determined by the number of cells formed in the endosperm, as well as the main cell types, such as transfer cells, aleurone, starchy endosperm, embryo surrounding cells. The phase II, called storage or maturation phase, is the period of grain filling; one can observe a fast increase in grain dry weight. Finally, the phase III, or dessication phase, is characterized by water losses [4, 16, 36].

In barley, the grain is referred to as a caryopsis, type of fruit in which seed and fruit coats are fused. Surrounded by husks, the caryopsis consists of a diploid embryo and a large triploid endosperm, surrounded by tissues of maternal origin (pericarp and testa) (**Figure 1**). The endosperm, tissue of nutritional value, is composed of several types of cells: aleurone (AL), starchy endosperm and endosperm transfer cells (ETC). Besides starch, the cells of the endosperm accumulate hordeins, the major source of proteins in barley grain [37]. The aleurone layer is source of lipids and vitamins; it also contains soluble proteins, including enzymes required for the remobilization of carbohydrates during the germination [6]. The endosperm develops with the synchronous division of nuclei without completion of cytokinesis. This phase of development is called the syncytial or coenocytic stage [38, 39]. The embryo is made of two main parts: the embryo axes and the scutellum, a nursing tissue. During grain development, scutellum drives the transport of nutrients to the developing embryo while later, during germination, it will contribute to the redistribution of sugars from the endosperm to the germinating embryo [6, 37, 40].

Few days after pollination (DAP), cellularization and differentiation occur, enclosing nuclei in cell walls and leading to the cell fate specification of the endosperm (**Figure 1**). The overall size of the grain partially depends on the number of nuclei formed during the syncytial phase; this number can exceed 2000 in *Triticum* and *Hordeum* [36, 41].

The main steps of barley grain development are summarized here. However, detailed information can be found in [6, 12, 36]. During the first 6 days after pollination (DAP), in barley, the endosperm cellularizes. Programmed cell death (PCD) occurs in the nucellus, except in the region in vicinity with the vascular bundle, leading to the differentiation of the nucellar projection (NP). In the same time, the region of the syncytium close to the NP initiates cellularization and differentiates into the endosperm transfer cells (ETC). During the transition phase (6 to 8 DAP),





**Figure 1.** Longitudinal (A) and transversal (B) schematic representation of sections of barley grain showing the different tissues (adapted from [6]).

transcriptional and physiological reprogramming occurs, involving the expression of genes encoding proteins with function in energy production and storage product synthesis [39, 42]. This phase is marked by endoreduplication, a modified mitotic cycle during which nuclei undergo one or more additional rounds of DNA replication; endoreduplication is common in plant and is often associated with higher cell volume [36, 43]. The final composition of the mature grain is then determined during the storage phase that lasts from 9 to 23 DAP. Both NP and ETC control the fluxes of assimilates into the endosperm [38].

The extremely complex mechanisms defining the grain structures composing the seed are tightly controlled by hormones and involved permanent exchange of signals from and to the maternal tissues, but also between embryo and endosperm. Whereas the role of abscisic acid (ABA) and gibberellins (GAs) is well documented in their role in controlling dormancy and germination [44–46], information related to the hormonal control of grain development are more scarce. Most of the advanced insights come from studies conducted on rice, that is considered as a plant model for cereals. The overall point of view is that cytokinin (CK) are most probably synthesized in endosperm where they act as a negative regulator of grain width but a positive regulator of grain length. Auxin, brassinosteroid and GAs (synthesized in the embryo) all promote grain length. The accumulation of CK, short after fertilization, corresponds with the formation of syncytium [47]. A recent study, based on transcriptomic studies, paved the first steps towards understanding the role of hormones during grain development in barley [48]. The NP, a mitotically active tissue, is characterized by events of differentiation/elongation/cell death that form a top-down gradient, persisting throughout grain development. GAs might contribute to establishing and maintaining this gradient [48]. PCD is an essential process throughout grain development, participating in the formation of the NP at

the early phase of development, of the starchy endosperm, of the vascular tissue, and of the scutellum. PCD of the pericarp cells is also important during grain's enlargement [49]. These processes are regulated by ethylene, jasmonate, ABA, auxin and GAs [49]. During grain filling, ABA, auxin and CK regulate the source photoassimilates during remobilization. Notably they can alter the synchronization between source activity and sink strength [50].

In a recent study, Sharma et al. [51] carried out a GWA study using nested association mapping populations combining the genetic information of 25 ancestor genotypes of *H. vulgare* subsp. *spontaneum* into the cultivated barley elite cultivar Barke. Authors identified a hotspot located on chromosome 7 showing a highly significant association with almost all traits. The ancestral allele increases several grain parameters, especially grain length. The region contains two genes: THOUSAND GRAIN WEIGHT 6 (TGW6), an IAA-glucose hydrolase, and MAP KINASE 6 (MAPK6), a mitogen-activated protein kinase. Interestingly, both genes have been demonstrated to influence grain size, weight and biomass in rice [52]. In the rice cultivar Nipponbare, *TGW6* expression peaked two DAP before decreasing rapidly in older seeds. In the Indian landrace rice Kasalath, *TGW6* contains 7 SNPs, including a – 1 bp deletion causing a frameshift that prevents the production of the mature, active protein. The Nipponbare near-isogenic lines containing the Kasalath haplotype accumulated markedly less IAA than wild type at 3 DAP, and had larger mature grains [52]. The authors concluded that the functional *TGW6* affects the duration of the coenocytic stage by controlling IAA supply, limiting cell number and the subsequent grain size. It is noteworthy that the *tgw6* loss-of-function resulted in increased carbohydrate storage capacity before heading [53].

### 3. How drought affect the grain development and grain yield

Drought stress can drastically affect plant growth and development at any time of the crop life cycle. However, the extent of damages, the recovery capacity, as well as the impact on the yield depend on the stage of development at which the crop undergoes the stress [17]. Early drought stress at the seed germination stage reduces seed germination ratio. Drying soil surface after seedling emergence can cause seedling's failure [54, 55]. During the early vegetative phase, shoot elongation, leaf area, and tillering can be limited by drought stress [56]. Drought affects the most yield when it occurs at the onset of meiosis, i.e. during gametogenesis, and at the early grain initiation [16].

The most sensitive stage of barley growth to drought stress is the spike emergence and the initial stage of grain development [16, 50]. At the beginning of the reproductive stage, drought stress can affect the differentiation of floral meristem, and subsequently the spikelet's number. Exposure to the drought stress during the gametogenesis leads to pollen sterility; during flower induction and inflorescence development, it leads to a delay or complete inhibition in/of flowering. Later, drought results in the reduction in the grain size and weight by limiting the number of endosperm cells, consequently reducing the potential size of the grain. Finally, at later phase of development, drought affects the rate and duration of starch accumulation in the endosperm [16, 17].

Seed filling is the terminal stage of cereal grain development. Several biochemical processes associated with carbohydrate, protein and lipid synthesis in seeds and import of constituents are involved [6]. During the storage phase, endosperm cell division and accumulation of seed reserves are largely influenced by the moisture status of the cells. Water deficit elevates endogenous ABA concentration, reduces starch accumulation and results in ovary abortion leading to poor grain yield [57].

Flag leaf and ear are the main photosynthetically active organs that provide assimilates during grain filling at the end of the plant's life cycle [58]. Drought stress during this period negatively affects the net photosynthetic rate of the flag leaf. However, despite the high vapor pressure deficit condition, there is no significant effect on the grain-filling [59]. Perhaps, the remobilization of vegetative reserves maintains the grain growth rate under drought stress [60]. Whereas drought stress during the grain-filling stage enhances assimilate remobilization, it fastens senescence, reducing the grain-filling duration [61].

If one needs to summarize, drought stress is characterized by a low soil moisture that negatively affect nutrient uptake and assimilation at the root level. The consequence is a reduced photosynthetic ability, an altered sugar translocation, a pre-mature leaf senescence, an altered source/sink equilibrium. Finally, this is translated into alteration of the reproductive developmental stage and a shorter period of grain filling. All together, this participates to reduce the number of grains whose size and quality are highly deteriorated [50].

#### **4. QTL, GWAS and other studies to identify new genetic resources of tolerance to drought**

Breeding programs are the most effective method to improve the yield stability under drought stress condition [62]. The genetic and molecular bases of grain yield, quality and sustainability under drought have been studied in mapping quantitative trait loci (QTL) or Genome-wide Association (GWA) studies, combined with mutant identification. The number of studies focusing on the discovery of genes controlling yield in cereals and understanding their functions has increased in the last years [10]. However, it has to be considered that the domestication and breeding strategies, based on inter-crossing elite or high-performance varieties, led to a loss of genetic diversity [32]. This bottleneck can be overcome by the use of wild relative species and landraces that constitute a great resource of diversity, useful for new breeding strategies [32, 33, 63]. In this regards, *Hordeum spontaneum*, the wild barley ancestor, shows larger adaptation abilities to the unfavorable environmental conditions, including drought, compared to the cultivated barley, and an unexploited genetic variability [62]. Therefore, screening drought-tolerant germplasms from wild barley to integrate elite traits to the cultivated barley is one of the breeding approaches to improve drought tolerance in barley [19].

As already mentioned, drought tolerance in plants is a complex quantitative trait which is controlled by several genes with small effect or by QTLs [56]. Functional genomics and QTL mapping are the most useful approaches to identify the key genes and networks mediating the yield response under drought stress [64]. A large coverage of the plant genome by markers is essential to identify most relevant QTL associated with a trait of interest. Among others, single nucleotide polymorphism (SNP) is the most widely used type of markers in genomic studies. Recent advanced technologies based on high throughput next-generation sequencing (NGS) allow cheap and quick deep sequencing of genome of model and non-model crops, largely increasing the available genetic information. NGS encompasses different sequencing technologies and genotyping methods including restriction site-associated sequencing (RADseq) [65], diversity array technology sequencing (DArTseq) [66], and exome capture [67]. The recent barley genome sequencing [25] makes possible to identify the accurate positions and locations of the markers on the chromosome, as therefore to perform an effective QTL search in barley germplasm. Genome-wide association studies (GWAS) are a powerful tool to dissect the genetics of complex traits such as drought stress [23, 27, 68]. Genes identified in these studies



can be used directly in molecular breeding in countries where the GMO regulation allows it, or indirectly in marker-assisted selection (MAS). In MAS, the selection is performed as soon as during early developmental stage of plants, reducing the time and cost of breeding researches [69–71].

Several factors have to be taken into consideration while considering reliable QTLs and markers in GWAS. First, allele frequency differences due to population stratification (systematic ancestry differences) is one of those factors. Population stratification can cause spurious associations in QTL mapping studies [70]. Therefore, the population structure (i.e. geographical origins and breeding history) needs to be analyzed prior to QTL/marker mapping, using statistical methods such as the principal component approach (PCA) [70]. Second, one might consider the environmental effects on the QTL expression. Specific environmental conditions such as abiotic stresses can increase the expression of specific QTLs named adaptive QTL. The presence and quantity of adaptive QTLs vary between different environments and experiments. The interaction between QTLs and environment ( $Q \times E$ ) can therefore modify the effect of a specific QTL according to the environmental conditions that can be the intensity of drought stress or the combination with different stresses such as heat or salinity [72]. An alternative to QTL studies, based on inheritability of markers across offspring, is to use natural populations and map traits by an association analysis, named linkage disequilibrium (LD) mapping. LD or gametic disequilibrium is the “nonrandom association of alleles at different loci”. In simpler words, it reflects the correlation between polymorphisms. LD is caused by the mutation and recombination in a large, randomly mated population with independent loci segregations. In small populations with less individuals, the rare allelic combinations might be lost because of genetic drift [73].

To date, some QTLs involved in drought stress response have been identified in barley. Jabbari et al. [68] reported eight markers over the 3H, 5H and 6H chromosomes, significantly associated with grain number per spike using association mapping based on LD under the irrigated and water deficit conditions in barley. Honsdorf et al. [74] found an unknown wild barley QTL allele on chromosome 4H that improved thousand-grain weight under terminal drought stress. Similarly, a QTL on chromosome 4H related to increased biomass under both drought and control conditions was identified by GWAS, in a study involving offspring of a cross between wild barley accessions and an elite barley cultivar [27]. These results show that wild barley *Hordeum spontaneum* is a useful source of drought tolerance alleles in barley breeding programs. The use of recombinant inbred lines (RILs) resulting from a cross between Syrian and European cultivars identified that the earliness allele from the Syrian parent conferred higher yield performance under drought conditions [75]. Drought response-specific QTLs were identified within the confidence intervals of candidate genes encoding antioxidants, carboxylic acid biosynthesis enzymes, heat shock proteins, small auxin up-regulated RNAs, nitric oxide synthase, ATP sulfurylases, and flowering time regulation proteins [75]. Adjustment of flowering time and in particular early flowering represents an escape strategy of plants to complete the sensitive reproductive stage before unfavorable environmental conditions. In barley, most of the seed dry weight is composed of carbohydrates which are produced and transferred to the seeds from the photosynthetic organs of the spike such as lemma, palea and awn [76]. Spike is more resilient to drought stress compare to the flag leaf and awn is the major photosynthetic organ under terminal drought stress and plays a crucial role in grain filling [77]. Several QTLs for grain plumpness and yield in doubled haploid populations of barley with significant  $Q \times E$  interaction have been identified [78].

Another widely used method to identify drought-associated genes is to analyze gene expression profiles at the transcriptional level of the drought-resistant and



sensitive line exposed to drought stress [79–81]. Barley transcriptomic data reported differentially expressed genes in drought-tolerant and sensitive genotypes, in relation to terminal drought stress. Several genes with known or predicted function were found to be constitutively expressed in the drought-tolerant barley genotypes, representing a potential mechanism of adaptation to the stress. To date, several drought tolerance-associated genes have been reported in plant species [82, 83]. However, their overexpression in transgenic plants did not significantly improve drought tolerance [84] indicating that drought tolerance is a complex mechanism that might involve different regulation at the genetic level.

The regulation of gene expression is an important process, in part controlled by microRNAs (miRNAs). MiRNAs are single-stranded noncoding RNAs composed of 20–24 nucleotides that play an important role as gene regulators in a wide range of organisms by cleavage of target messenger RNAs (mRNA), translational repression and DNA methylation [85]. Drought-responsive miRNAs have been reported in many plants to participate in the regulation of drought-responsive genes [86]. Moreover, the expression of miRNAs itself is altered in response to drought stress. Four drought stress-induced miRNAs (hvu-miR156a, hvu-miR166, hvu-miR171 and hvu-miR408) were reported in barley leaves differentially expressed under drought conditions [87]. Lv et al. [88] reported three miRNAs (miR-n026a, miR-n029 and miR-n035) up-regulated under drought and salinity stresses in barley leaves. Hackenberg et al. [89] identified a miRNA, hvu-miR5049b up-regulated, under the drought conditions. Additionally, authors indicated that hvu-miR168-5p was up-regulated under drought stress only in leaves while its expression level remained unchanged in barley roots suggesting that some of the drought-regulated miRNAs can be expressed differently in barley tissues. Ferdous et al. [90] determined that Hv-miR827 enhances drought tolerance in barley. Several miRNAs were identified with different abundance in two different drought-sensitive and tolerant barley cultivars as drought-responsive miRNAs [91]. Recently, 2 conserved and 10 novel miRNAs were identified as drought-tolerant miRNAs in two different drought-tolerant and sensitive wild barley genotypes [92]. These miRNAs can regulate many different genes involved in numerous biological and metabolic processes in plants such as growth, development, hormone signaling, consequently affecting the stress response in plants.

Besides miRNAs, epigenetic factors such as DNA methylation and histone modifications in response to environmental conditions lead to changes in chromatin structure. Open and closed chromatin states cause gene activation and gene silencing, respectively, and regulate a wide range of developmental processes in plants in response to changing environment [93]. Chromatin dynamic and DNA-methylation have been reported as tolerance mechanisms to drought stress in crops [93, 94].

Beyond that what has been discussed above, the role of transcription factors (TFs) in the regulatory networks underlying plant responses to abiotic stresses is crucial [95]. Recently, Collin et al. [96] showed that the barley mutant carrying *ABA INSENSITIVE 5 (ABI5)* genes (HvABI5) is drought tolerant compared to its parents. ABI5 is a basic leucine zipper (bZIP) transcription factor which acts in the ABA network. ABA is the crucial regulator of plant responses to abiotic stresses. ABA-dependent signaling alters the activity of stress-responsive genes and thus regulate physiological processes, such as photosynthesis, stomatal closure and osmoprotectant biosynthesis in response to drought stress [97, 98]. MYB genes encode another class of TFs known for their involvement in the regulation of drought stress responses [99]. Harb et al. [79] reported that NAC transcription factors are specifically induced in drought-tolerant barley compared to sensitive genotype. The improvement roles of NAC genes in response to the drought stress have been reported previously [100].

Alternative splicing (AS) was also found to differ between genotypes as a key mechanism controlling the expression of the drought-responsive gene in barley [101]. In the gene expression process, during the transcription of DNA to RNA, first precursor mRNA (pre-mRNAs) are produced, containing the introns which interrupt the protein-coding regions. Splicing is an essential step to remove the introns through the pre-mRNAs [102]. In AS, a single pre-mRNA can produce more than one mRNA through the use of alternative splice sites. Alternative mRNAs encoding different isoforms of proteins increases the diversity of an organism's transcriptome and proteome [103]. AS can regulate the gene expression at the transcript levels by producing unstable mRNA isoforms, which can be degraded by nonsense-mediated decay (NMD) [104].

## 5. Concluding remarks

Plant responses to drought stress, including different tolerance mechanisms and genetic controls, are complex. Further studies are required to determine the molecular basis of yield-related traits in barley before their integration into breeding programs focused on tolerance to drought stress and sustainable yield under adverse conditions.

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