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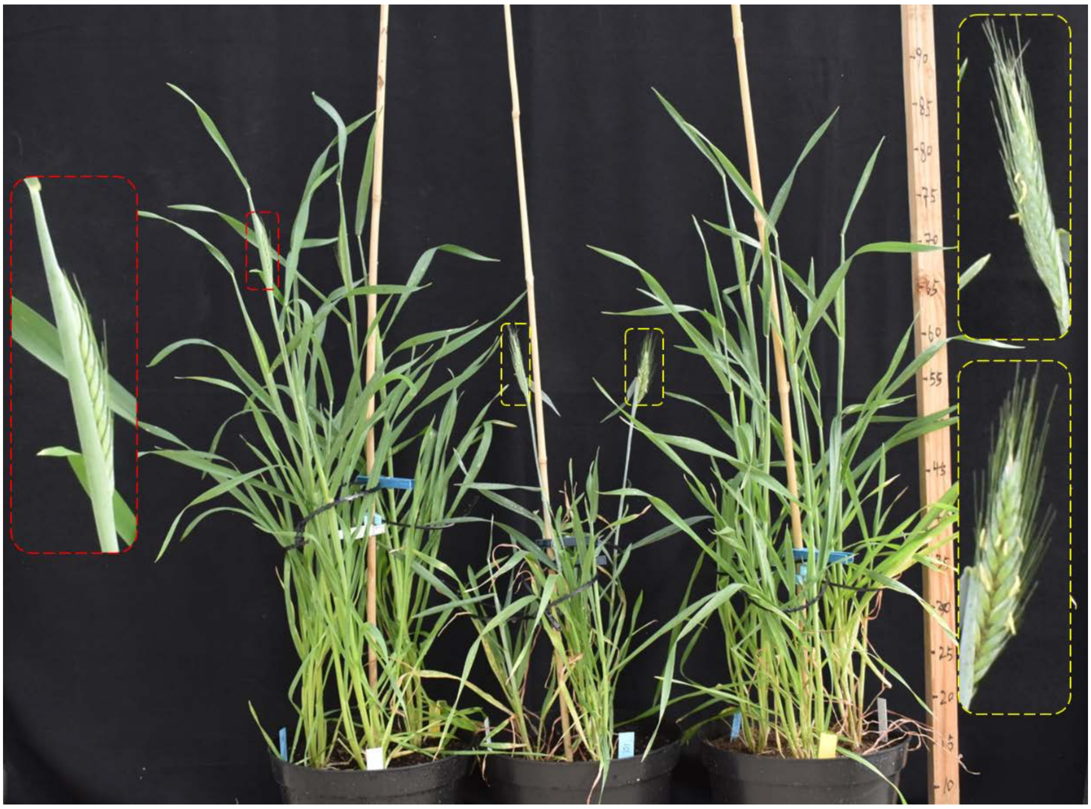
Dissection of Drought Tolerance Mechanism in Wheat Plant

Yuzhou Lan



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Summary

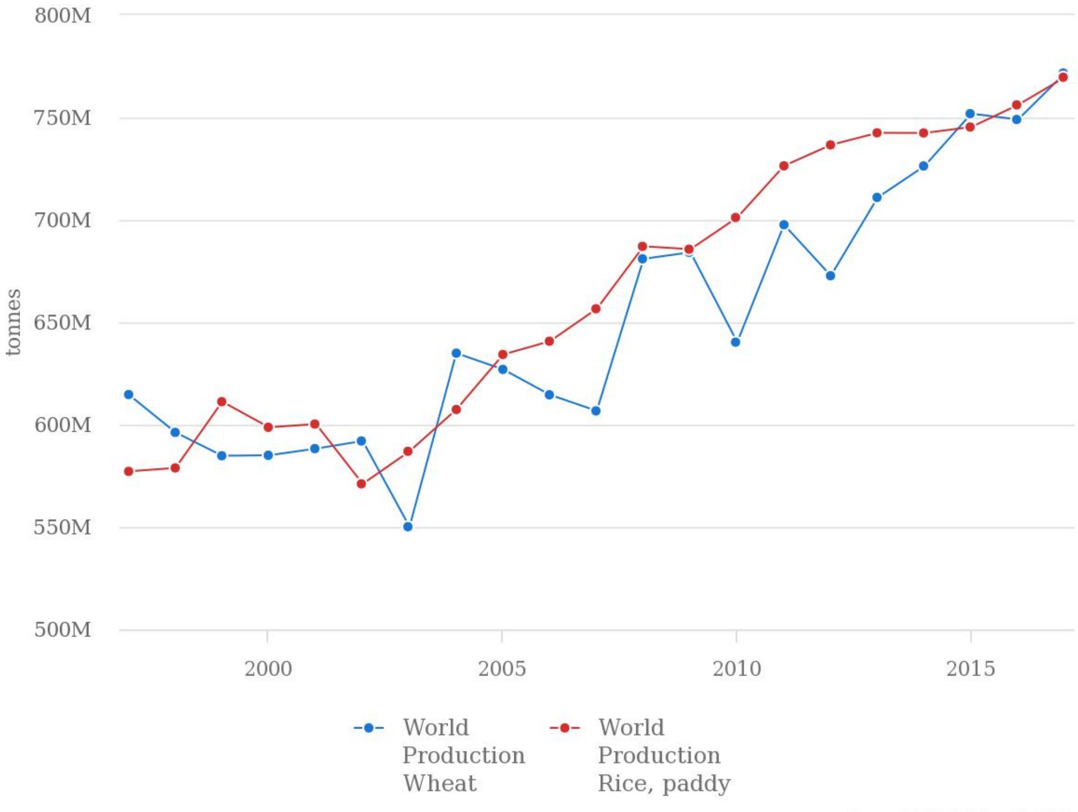
As wheat is one of the three major crops in the world, improving its drought tolerance is crucial for human beings to develop sustainable food in the context of global climate change. This review updates the studies on wheat exposed to drought stress. In this work, the physiological responses of wheat plants under water deficit are discussed from different angles. A comprehensive description of drought-tolerance mechanisms in wheat plants is given. The current state of researches on drought-related traits is reviewed. To further demonstrate the genetic basis of wheat drought tolerance, some knowledge of the powerful genetic research tool, Genome-wide Association study, is elaborated on. In addition, this review also summarizes multiple potential approaches for further studies on drought-related candidate genes. The results obtained utilizing those advanced technologies in this area so far are thoroughly illustrated. Finally, the challenges of investigating wheat genotypes in drought condition and mixtures of natural abiotic stresses are discussed. Traditional difficulties and novel progress in the wheat root system investigation are elaborated.

Contents

1. Introduction

1.1. Importance of Wheat

As one of the main sources of human and livestock calories, wheat is the world's most widely grown crop, providing about 20% of calories and proteins globally to the human diet, while maize contributes 5% of the calories and 4% of the proteins, and rice contributes 19% of the calories and 13% of the proteins [1]. Furthermore, in 2012, approximately 26% of the calories consumed in Europe per day was from wheat [2]. Wheat is currently grown in approximately 220 million ha fields in diverse geographic regions, under a wide range of climate conditions, and over 670 million tonnes grains are produced annually [1]. The statistics of production in the last two decades published by FAO (Food and Agriculture Organization of the United Nations) (http://www.fao.org/faostat/en/#compare) indicate a substantial increase in yield of wheat. A series of technological achievements, including semi-dwarf and high yielding varieties and an appropriate policy by the wheat breeding industry have ensured the access of farmers to new seeds, fertilizer, markets and irrigation infrastructure. [3-6]. Recently, the 749-million-ton and 772-million-ton global production of wheat in 2015 and 2017, respectively, exceeded the production of rice and made wheat the second most-cropped cereal after maize (Figure 1). Several species of wheat are available under the genus *Triticum*. Among them, *Triticum aestivum* (AABBDD, 2n=42), a hexaploid species usually called "common” or "bread" wheat, is one of the main staple crops and the most commonly cultivated wheat species, accounting for 95% of all kinds of wheat [7].



Source: FAOSTAT (Sep 11, 2019)

Fig. 1. Comparison of World Wheat and Rice Production from 1997-2017.

Compared with the other two major crops (rice and maize), wheat has the widest growing area in the world. According to the data published on the FAO website (Figure 2), the area of wheat harvested globally in the past 60 years has been significantly higher than that of rice and maize. Wheat has often been considered as a source of calories instead of an ideal source of protein due to its high content of starch (60-70% of the whole grain and 65-75% of white flour) and the composition of its proteins. However, wheat provides as much protein for human and livestock as the total soybean crop, despite the relatively low protein content of wheat [8].

In 1880, a Bread Reform League was established in London to advocate a return to wholemeal bread, suggesting that officially a minimum standard of 80% flour extraction rate should be adopted. With a clear conflict with general tastes as well as the profits of bread processing companies, this kind of promotion was not supported at that time. However, the League received scientific support in 1911 when Gowland Hopkins stated that 'Standard Bread' may contain 'unrecognized food substances' which were crucial for health: these substances were later defined as vitamins [9]. Thus, in addition to contributing towards solving human starvation and poverty problems, wheat has also been shown to contribute high nutritional value in the human diet, particularly in regions where bread, noodles and other wheat products are a substantial proportion of the diet. After decades of research, wheat, especially in its whole-grain version, has been identified as a source of multiple nutrients, such as minerals, dietary fibre, and bioactive compounds. Whole-grain consumption usually shows a significant connection with reduced levels of the most relevant risk factors for cardio-metabolic disease such as LDL-cholesterol, triglycerides, blood glucose, blood pressure and body mass index [10]. Recently, a meta-analysis confirmed the association between wheat consumption and a reduced risk for disease and cancer [11].

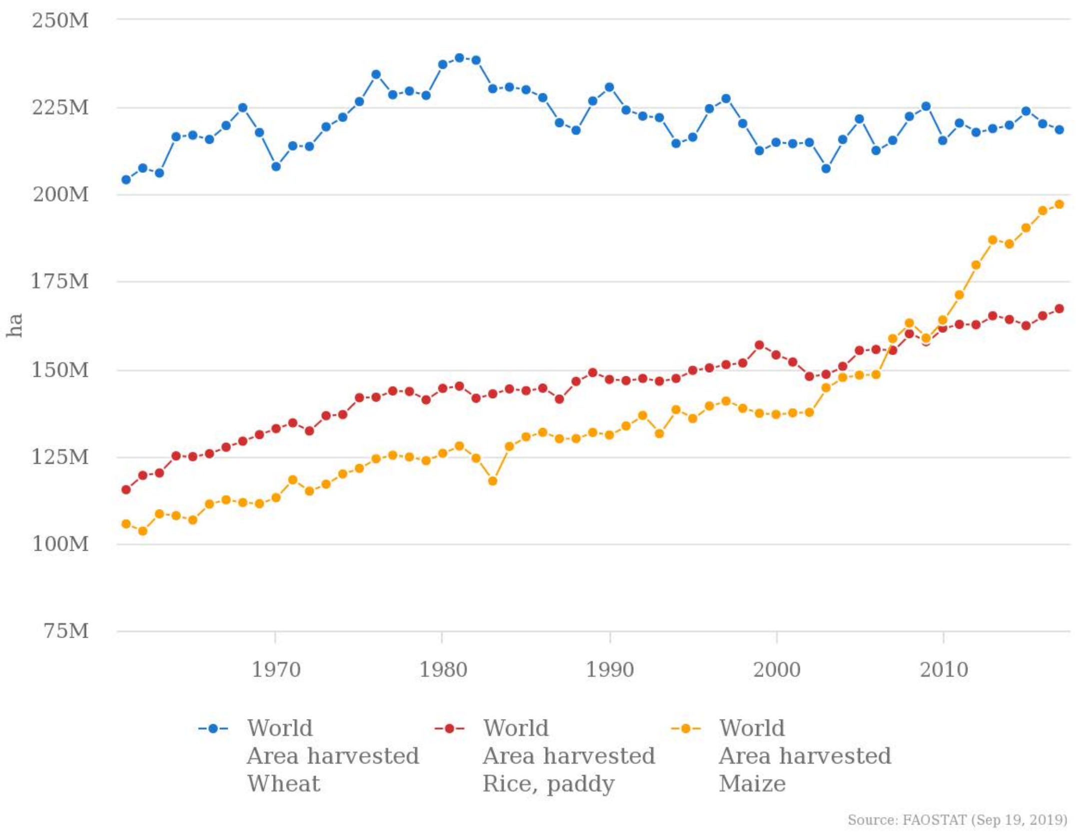


Fig.2. Comparison of the World Harvested Area of Three Main Crops from 1961-2017.

1.2. Relationship between temperature and drought

Some of the staple crops, including wheat, are threatened by different adverse factors negatively affecting their productivity [12]. Moreover, several abiotic stresses such as drought and heat are becoming increasingly frequent, as a result of ongoing progressive global climate changes [13]. Drought is known as an always important environmental constraint that limits crop yield in many regions around the world [14]. According to the statistics from the Intergovernmental Panel on Climate Change [15] (https://www.ipcc.ch/sr15/chapter/chapter-1/), there is at present an upward trend for global temperature change, whether human-induced or natural (sonic and volcanic activity) (Figure. 3). Furthermore, the duration of the drought period is increasing due to climate change, contributing to warming of the air temperature in most cereal growing regions [2]. Drought and high temperature often occur simultaneously and both are known to suppress photosynthesis and productivity of wheat by different mechanisms. Drought stress directly inhibits the expansion of leaves and stomatal conductance and may eventually indirectly affect the photosynthetic process [16]. High temperature is known to directly impact photosynthesis and related processes in wheat in several ways [17].

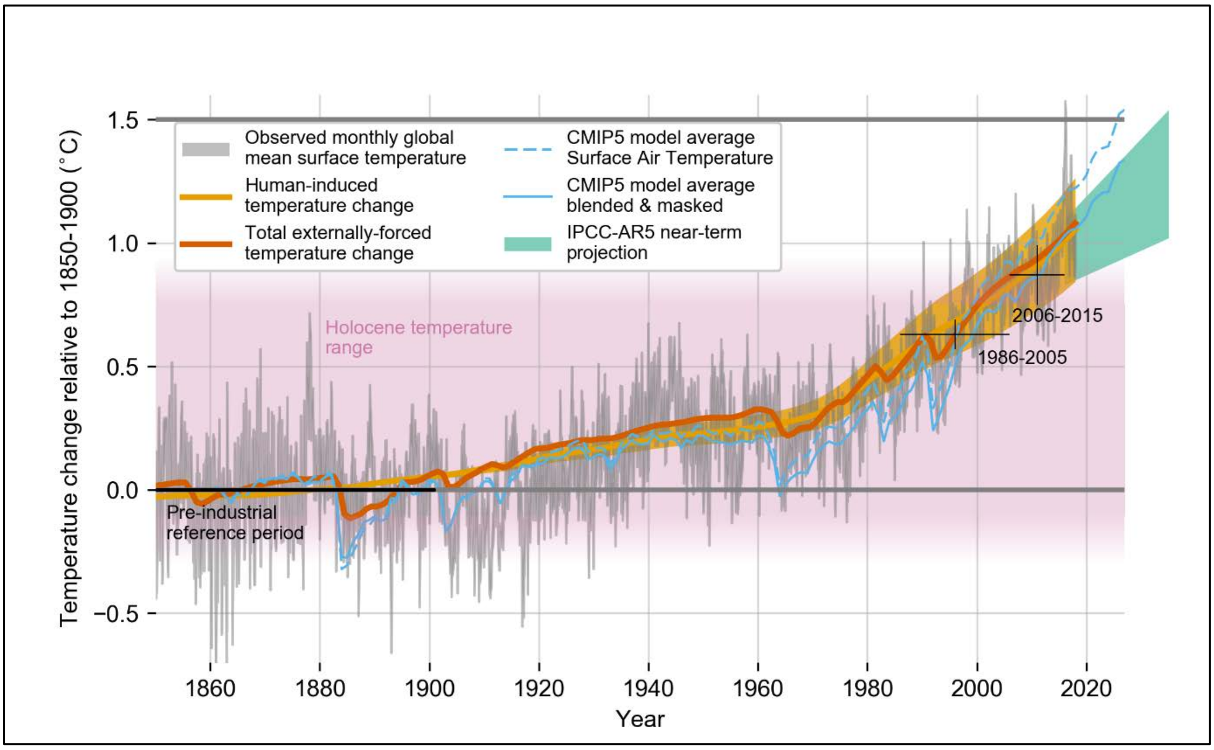


Fig. 3. Evolution of global mean surface temperature (GMST) over the period of instrumental observations

Compared with durum wheat, which accounts for only 5% of all kinds of wheat, bread wheat shows less acclimation to a dry and hot climate. Thus, an improved tolerance to abiotic stresses in bread wheat is urgently needed. Based on recent data on climate change released by IPCC, crops will suffer more severe combinations of abiotic stress in the future than previously estimated, due to anticipated increasing incidences of drought combined with a heatwave [18-20]. Therefore, studies to understand the effect of such stress combinations will help to increase wheat production under adverse conditions globally. It is well-known that drought is mainly caused by limited precipitation and high temperature, but the opposite i.e. that drought is leading to less rainfall and higher environmental temperature has also been proven [21]. Studies on interactions between the two phenomena has been ongoing for decades, and physiological plant parameters were shown more severe under drought at high than at low temperature [22]. The combination of drought with high temperature was found not only to reduce cell division but also to reduce dry matter accumulation and the storage capacity of the grain [23]. One study in sorghum demonstrated that heat and drought stress in the pre-flowering phase dramatically delayed panicle initiation and flowering [24]. In barley drought was found to cause losses in individual grain weight to a higher extent than did high temperature. Consistently, reduction in individual grain weight was found greatest under combined stress [25]. In general, wheat [26], purslane [27], barley [28], arabidopsis [29], tobacco [30] and citrus plants [31] displayed more severe damage unanimously when exposed to the combined stresses of drought and heat than when exposed to only one of the stresses.

1.3. Effects of drought on wheat

Drought is a major limiting factor in crop production in most of the agricultural fields all over the world [32]. Water deficit is restricting the growth of plants by inducing various changes in physiological, biochemical, morphological, and molecular behaviors in plants [33]. In addition, it inhibits the photosynthesis of plants by causing changes in chlorophyll contents, damages to the photosynthetic apparatus, and decreases in activities of enzymes in the Calvin Cycle [34, 35]. Furthermore, the inhibition of photosynthetic activity under drought stress could lead to oxidative stress by generating active oxygen species (AOS) in leaves [36].

1.3.1. Grain Yield

The development of crop varieties with high and satisfactory yield potentials is the key to increasing productivities in dry regions where yield reduction by drought is a major concern [37]. Furthermore, grain yield is also a crucial agronomic trait serving as a criterion for assessing drought tolerance in wheat. The most widely used criteria for selecting high yield performance include mean yield, mean productivity and relative yield performance in drought and favorable conditions [38]. However, it is difficult to make progress for grain yield under drought conditions as it is a complex trait that is characterized by large genotype × environment interactions under drought stress [39]. Even though, breeders and plant physiologists will never stop exploring opportunities to develop drought tolerant cultivars with high yield due to the importance of this trait. The drought susceptibility index (DSI) derived from the yield difference between drought and favorable environments was introduced to characterize the stability of grain yield for each genotype [40], and has been widely applied in several studies on yield stability under water deficit conditions [41, 42]. Other traits, such as water-soluble carbohydrates and flag leaf glaucousness, have been proposed as key traits reflecting grain yield under drought conditions [43, 44].

Large cultivar differences in grain yield under drought have been verified [45]. There is a general agreement that modern high yielding wheat cultivars are more adapted to optimal environments, while old cultivars and landraces display a more reliable yield under unfavorable conditions such as drought [46]. This view is supported by another research, in which 30 wheat cultivars and 21 landraces were employed. The result indicated that grain yield was more drought sensitive than plant height and number of spikelets per spike in cultivars, while no significant diversity was observed among these traits in landraces. This might suggest that landraces adapt better to drought conditions [47]. A two-year field trial with rainout shelter on wheat proved that the grain yield under drought was correlated with canopy temperatures [40]. Further efforts were put into constructing an SSR/EST-STS marker map in wheat, and a major quantitative traits locus (QTL) on chromosome 4AL was identified to have a significant influence on wheat grain yield under drought [48]. Another two QTLs located on chromosome 3B were found correlated to canopy temperature and grain yield through a series of experiments over three field seasons by using a wheat population containing 368 individuals [49].

1.3.2. Grain Quality

In general, wheat grain quality can be divided into three groups (i) botanical (species and varieties), (ii) physical and (iii) chemical characteristics [50]. However, scientific research has mainly been focusing on physical and chemical characteristics. Physical quality characteristics of the wheat grain include grain weight, hardness, grain size and shape, vitreousness and color [51, 52]. Chemical characteristics of wheat grain include moisture content, protein (gluten)-, amylase- and fiber contents [53]. In fact, the grain quality is also connected to the grain yield. For instance, yield losses are often caused by a decline of starch content, as over 65% of cereal grains are composed of starch [54]. The starch accumulation and cell division were also found to be influenced by post-anthesis drought [55]. The diversity in grain quality also depends on genotypes, the soil, nutrient supplies, weather conditions and their interactions. In respect of environmental conditions, the adverse weather conditions like insufficient precipitation and high temperature during the growing season has a substantial impact on the plant metabolic systems, and therefore on wheat grain quality [56].

The bread-making quality of the wheat kernels is determined by grain protein concentration and -composition, which is greatly influenced by drought and heat stress [57]. An investigation on winter wheat revealed that a water deficit during grain filling showed a drastic influence on protein components [58]. Another study on five winter wheat cultivars indicated that drought stress could significantly affect starch granule and relative protein content [59]. Variation of grain quality in winter wheat cultivars were found associated with changes in protein composition under drought stress during grain filling [60]. It was reported that wheat protein content could be significantly increased under drought stress during grain filling [61], and this conclusion has also been verified by other studies [62, 63]. Increased protein content may be attributed to higher rates of grain nitrogen accumulation and/or to lower rates of carbohydrate accumulation under stress conditions [64]. Furthermore, the transcriptional progress of storage proteins was inhibited due to the repressed expression of wheat storage protein genes, i.e. for expression and production of gliadins and glutenins under a short-term drought stress [65]. A comparative proteomic analysis of grains in two spring wheat varieties exhibited that two previously identified drought-tolerant proteins, triosephosphate isomerase and oxygen-evolving complex, showed diverse expression patterns between Chinese Spring and Ningchun 4. The differentially expressed proteins might be the reason for the stronger drought tolerance of Ningchun 4 compared to Chinese Spring [66]. Additionally, drastically enhanced flour yellowness with drought stress was observed in a two-year trial in durum wheat cultivars [67]. The values of micronutrients such as Fe and Zn in wheat grains were found to increase in severe drought conditions [68].

2. Physiological response of wheat to drought stress

After thousands of years of evolution, plants developed several strategies to survive in water-deficit conditions. Compared with well-watered plants, those under drought stress show differences in physiological conditions such as transpiration, photosynthesis, shoot and root growth, and reproductive development [69]. In wheat, several transcription factors, such as bHLH, bZIP, ERF, HD-ZIP, NAC, and WRKY were found to be differentially expressed in a drought-tolerant wheat genotype [70]. Furthermore, a proteomic study on durum wheat revealed that the activity level of enzymes representing the tricarboxylic acid cycle could also be considered as an important response to drought [71].

2.1. Root system

Unlike the shoot system, roots are a hidden part underground of a plant, responsible for growth and supplying the above-ground part with water and nutrients [72]. As the root system is where the plant has direct contact with soil, studying the root system in water-limited soil can help to understand the plant response to drought better. Based on soil moisture, root traits vary from species to species [73]. In wheat, genotypic variation has been proven in several root characteristics, which include the depth of rooting, root elongation rate, root distribution at depth, xylem vessel diameter, and root to shoot dry matter ratio [74-80]. Severe water deficit was found to cause oxidative stress in plant root tissues, so the reactive oxygen species restriction and well-coordinated induction of antioxidant defense in roots were reported as the key to enhanced drought tolerance in wheat [81]. Even though scientists have long found that the root system was highly associated with drought tolerance in wheat [82], our knowledge on root interaction with the soil environment is still limited. Fortunately, the advances in imaging and sensor technologies in recent decades are making thorough root research more possible and effective [83]. Compared with standard wheat variety, the drought-tolerant wheat displayed a more uniform rooting pattern and greater root length at depth [76]. This is supported by the result of another study, which suggested that deep rooting is one of the alternative strategies to drought stress in wheat [84]. Also, studies on winter wheat demonstrated that roots of drought treated plants reached 20 cm deeper in subsoil than the well-watered ones [85]. Greater wheat yield with a deeper root system was assessed using a validated crop simulation model [86]. Furthermore, the number of nodal roots of wheat was found to be reduced significantly under drought while the seminal roots were less affected [87]. A recent study with six commercial and breeding forms of spring wheat indicated that drought-tolerant genotypes displayed a lower shoot-to-root ratio. Additionally, elevated dry matter, length and diameter of nodal and lateral roots was found in tolerant genotypes [88].

2.2. Shoot system

Shoots of plants consist of stems and leaves, with lateral buds and flower buds germinating on the stems [89]. It is well-known that water-soluble carbohydrates (WSCs) can accumulate in the stem and leaf sheath of cereals [90, 91]. There are several components of WSCs while fructan is the major form accumulated in wheat stems at the late stage of the accumulation phase, and those components can account for more than forty percent of total stem dry weight [92, 93]. WSCs were proven to mobilize from the stem to grain during the later phase of grain filling [94]. Furthermore, a study on wheat revealed that the carbohydrates reserve in stems could be largely relocated to grains under drought conditions, fifty to eighty percent higher than the amount in well-watered group [95]. The stem WSCs in wheat were reported to potentially contribute to more than fifty percent of total grain yield under drought conditions [96].

Stomatal conductance is another important drought-related physiological trait. In wheat, stomatal conductance was found to be correlated with canopy temperature in a variety of climatic conditions [97, 98]. A noticeable decrease in stomatal conductance was observed in wheat when exposed to water-deficit condition [99]. By comparing different wheat varieties under drought stress, lower stomatal conductance and transpiration rate were observed in drought-tolerant varieties at both vegetative and reproductive stages [100]. Stomatal conductance was reported to be mediated by abscisic acid (ABA) as a response of wheat to drought conditions [101]. Furthermore, the stomatal closure is also regulated by ABA in wheat under drought stress [102]. A study with six spring durum wheat cultivars suggested that the inhibited photosynthesis can be attributed to the drought-induced reduction of stomatal conductance [103]. This result is supported by another study which confirmed that the limitation of photosynthesis in water deficit wheat is mainly caused by stomatal closure [104]. Those early studies suggested that the stomatal closure was triggered to reduce the water loss under drought but could also lead to a decrease of leave gas exchange and then inhibit photosynthesis. However, a later study on durum wheat questioned it and pointed out the flaw of early research. By measuring the internal CO2 concentration in wheat leaves under drought, they found a similar CO2 level to well-watered conditions. Thus, it means that the decreased stomatal conductance was not the main cause for the inhibited photosynthesis of leaves under drought [105].

2.3. Floral organs

In wheat, anther development can be severely affected by drought during the pollen mother cell meiosis. Microspore mother cells were observed to complete the meiosis, but further microspore development was stopped at drought conditions. This behavior can be due to the dislocation of the microspores from their normal position in water deficit wheat [106, 107]. The grain development starts with the fertilization of egg and polar nuclei. Fertilized egg and polar nuclei form the zygote and triploid endosperm respectively [108]. Grain filling can be regarded as the result of starch biosynthesis and accumulation in wheat. The grain filling is strongly determined by current photosynthesis and reserve assimilates in vegetative tissues [90, 109]. Under drought conditions, the grain-filling rate decreases due to declined photosynthesis and accelerated leaf senescence [110]. The dramatically affected grain number largely accounts for the reduction of wheat yields under drought [111]. Furthermore, the lack of fertilization of the egg is the major cause of the reduction of grain number [112].

When subjected to drought stress, the fertility in different winter wheat genotypes was all inhibited significantly [113]. The result of a recent study demonstrated that the seed-setting of wheat could be reduced by up to 51 percent when exposed to short-term drought stress at meiosis stage meanwhile, 60 drought-responsive protein spots were identified via proteome analysis [114]. Furthermore, both male and female reproductive parts are responsible for the seed-setting reduction in wheat when drought treated during meiosis, but the male reproductive part contributes more to yield reduction [115]. By analyzing the possible links between pollen fertility and drought tolerance under different levels of stress at young microspore stage, the grain number in wheat exhibited a sharper decline under more severe water deficit [116]. Another study suggested that the reduction of potential grain number in wheat under water deficit conditions might be due to the premature abortion of florets [117]. Several differentially expressed genes were detected in wheat both during early reproductive periods and for the flowering period via a transcriptomics analysis [118]. An increase of ABA was observed in ovaries and anthers under drought stress [119]. The drought-induced accumulation of ABA in spike was also proven to be associated with the loss of male fertility in wheat [120]. An investigation on five wheat varieties indicated that the number of sterile florets per head can be increased in water deficit wheat [121].

3. Drought tolerance

Drought tolerance is defined as the ability of a plant to survive, grow, and reproduce with limited water supply or under periodic conditions of water deficit [122]. For plants, drought tolerance is a complicated trait that can be influenced by multiple characteristics [123]. For crop plants, given the common use of crops, special attention should be paid to the ability to produce a harvestable yield under drought conditions. As drought tolerance is a quantitative trait, with complex phenotype and genetic control [124], dissecting the characteristics of such a trait from multiple aspects is a theoretical prerequisite for developing tolerant genotypes through conventional breeding.

3.1. Drought-tolerance mechanisms in wheat

In terms of mechanism, scientists normally consider drought tolerance from two aspects, drought avoidance and dehydration tolerance [125]. Drought avoidance is mainly reflected in changes in root depth, reasonable use of available water, and changes in lifestyle to use rainfall. Additionally, early maturity, small plant size, and reduced leaf area were reported to be connected with drought tolerance [126]. Dehydration tolerance is reflected in the capability of plants to withstand partially dehydration and grow again when rewatered [127]. Some compatible solutes such as K^+, glycinebetaine, proline, and glucose were identified to actively contribute to osmotic adjustment in wheat under drought stress [128]. A study with four different wheat cultivars revealed that the level of inorganic solutes was decreased while the level of organic solutes was increased under drought treatment [129].

Furthermore, increased levels of NO (nitric oxide) was found in drought-tolerant wheat varieties and may be related to the drought-induced inhibition in root growth [130]. Salicylic acid (SA) was identified as another signaling molecule to enhance drought tolerance through increased accumulation of solutes [129]. The increase of cysteine protease, functioning upon limiting protein loss in dehydration, was detected in leaves of a drought-tolerant wheat cultivar [131]. Results of another study confirmed the crucial role of a cysteine protease protein by its overexpression in *Arabidopsis* [132].

To better understand the drought-tolerance mechanism in wheat, the mechanism of drought damage to plants needs to be elaborated. Inside wheat plants, drought stress can lead to a reactive oxygen species (ROS) generation in chloroplast and mitochondria, which then causes further oxidative damage to cells [133]. Therefore, enhanced drought tolerance can be partially attributed to an enhanced tolerance to oxidative stress in wheat seedlings due to both ROS restriction and well-coordinated activation of antioxidant defense [81]. In addition, a different study with two drought-tolerant and two drought-sensitive wheat genotypes also suggested that the drought tolerance in wheat can be determined by the number of antioxidant enzymes [134]. The activity of some antioxidant enzymes such as glutathione reductase (GR) and ascorbate peroxidase (AscP) in leaves of wheat cultivars was observed to be increased on the 3rd and 5th days of drought [135]. Furthermore, the acclimated wheat plants displayed lower accumulations of superoxide radicals and less membrane damage in leaves and roots than non-acclimated plants when subjected to severe drought stress [136]. Additionally, ABA was reported to be correlated with a more efficient mechanism to scavenge ROS under drought in a drought-tolerant wheat cultivar. The activity of an antioxidant enzyme, superoxide dismutase (SOD), was observed to be significantly enhanced by drought-induced endogenous ABA in wheat. Meanwhile, the decline of the activity was also observed on rewatering the wheat [137].

3.2. Drought-tolerance genes in wheat

As mentioned before, drought tolerance is a complex trait regulated by multiple genes [124]. Through decades of efforts, several drought-related genes in wheat were identified. One of the well-characterized drought-related groups of genes is *Lea* genes, which encode the Late Embryogenesis Abundant (LEA) proteins. LEA proteins have been classified into six groups according to their sequence similarities [138]. The ABA-responsive barley gene *HVA1* regulates the synthesis of a kind of protein which is in group 3 LEA. After introducing *HVA1* into spring wheat, scientists observed higher water use efficiency values in two homozygous and one heterozygous transgenic line expressing the *HVA1* gene [139]. In wheat seedlings, higher levels of group 3 LEA proteins were induced in shoot and scutellar tissue after being dehydrated to 90% [140]. Furthermore, using *Agrobacterium*-mediated transformation, scientists introduced the wheat LEA gene, *TaLEA3*, into *Leymus chinensis.* The transgenic lines displayed increased relative water content, leaf water potential, and relative average growth rate compared with the non-transgenic lines [141]. In a study with three winter wheat varieties, *TaLEA2* and *TaLEA3* expression was induced in drought-stressed plants. Additionally, the delayed expression of *TaLEA2* and *TaLEA3* was observed in the least drought-tolerant variety [142].

Some transcription factors (TF) were identified to be involved in drought tolerance in plants [143]. Those TFs binding to the dehydration responsive element (DRE) were named DRE-binding (DREB) proteins [144]. In a study with wild emmer wheat (Triticum turgidum ssp. *dicoccoides*), the gene for an emmer DREB protein, TdicDRF1, was specifically upregulated in drought-tolerant wheat lines in drought conditions. [145].

Another large TFs family is the NAM/ATAF/CUC (NAC) family, which was first described according to the names of three genes, namely *NAM* (no apical meristem), *ATAF* (*Arabidopsis* transcription activation factor), *CUC* (cup-shaped cotyledon) [146, 147]. In a study, scientists introduced the rice *NAC1* gene (*SNAC1*) into an elite Chinese wheat variety. A significantly enhanced drought tolerance was observed in transgenic plants expressing *SNAC1* [148]. The overexpression of *TaNAC1* in roots of wheat was proven to be an effective approach to enhance tolerance to drought due to elevated biomass and root length [149]. Besides, when the wheat NAC gene, *TaNAC2a*, was integrated into tobacco, scientists found higher fresh weight and dry weight in transgenic tobacco plants overexpressing *TaNAC2a* than non-transgenic plants under drought stress [150]. Results of a different study suggested that the expression of *TaNAC8* in wheat can be significantly up-regulated by drought stress simulated with polyethylene glycol (PEG) treatment [151]. Moreover, the strong expression of another NAC gene, *TaNAC69-1*, was detected in a durum wheat variety by reverse transcription quantitative PCR under drought [152].

Vacuolar H^+-translocating pyrophosphatase (V-PPase) is a crucial enzyme related to plant abiotic stress. The V-PPase gene members in wheat include *TaVP1*, *TaVP2*, and *TaVP3*. Among them, the *TaVP2* gene was found to be more correlated to drought tolerance due to its earlier and more remarkable down-regulation in a drought-tolerant spring wheat cultivar under dehydration stress [153]. Furthermore, enhanced tolerance to drought was observed in transgenic *Arabidopsis* lines with overexpression of *TaVP1* gene from wheat [154].

The term zinc finger refers to protein domains that have a finger-like structure held together by one or more zinc ion(s). Among the entire zinc finger protein family, a really interesting new gene (RING) is known to be involved in protein-protein interaction [155]. In wheat, four (*TaRZF70*, *TaRZF8*, *TaRZF38* and *TaRZF59*) of seven RING zinc finger genes exhibited drastic changes in transcript level under water deficit [156].

3.3. Breeding of drought-tolerance wheat varieties

Scientists have made great efforts to improve wheat drought tolerance. Since 1979, investigations on plant's response to drought have intensified and most of the results emphasized the complexity of the plant's dehydration tolerance mechanism [157]. Researchers soon realized that development of drought-tolerant wheat varieties required the integration of plant breeding and plant physiology [158]. Several greenhouse and field screening indices were evaluated for the ability to differentiate drought tolerance in winter wheat cultivars [159]. However, as mentioned in last subsection, a vast number of genes are involved in drought tolerance in wheat. Different genes respond differentially to drought stress. Expressions of those genes can be induced or repressed by drought. Therefore, the complex nature of this trait, drought tolerance, makes breeding work complicated. On the other hand, conventional breeding is labour-intensive and time-consuming. Breeders need to make great efforts to separate undesirable traits from desirable traits generated by random mixing of genomes. This process usually takes several growing seasons [160]. The lack of efficient selection procedures and low genetic variance for yield components under drought conditions might also result in limited break-through with conventional breeding approaches. Nowadays, the advent of new techniques, such as genome-wide tools, proteomics, and thermal or fluorescence imaging, makes it possible to bridge the genotype-phenotype gap in traditional breeding. Plant genetic engineering and molecular-marker technology are the two major biotechnological approaches allowing further development of drought-tolerant germplasm [161].

Numerous drought-related molecular markers are currently available for marker-assisted breeding (MAB) technique in different crops including wheat [162]. By using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers, scientists detected a QTL responsible for flag leaf senescence on chromosome 2D in drought-stressed winter wheat [163]. In another study, several random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers linked to the flag leaf senescence gene in wheat were detected under drought stress [164]. Furthermore, yield-related QTLs in wheat were identified using molecular markers like restriction fragment length polymorphism (RFLP), AFLP, and SSR [165]. These markers derived from modern techniques can be further used as a potential selection tool in wheat breeding programs. On the other hand, transgenesis has emerged as an additional approach to tackle single-gene breeding or transgenic breeding of crops. Genetically modified (GM) drought-tolerant wheat has already been reported to be of potential benefit [166]. Although some drought-tolerant transgenic plants are still under pot experiments or field evaluation, great progress on modern breeding can still be achieved by combining molecular-marker technology and genetic engineering technology [161].

Apart from the shortage of technology in the past, limited achievements in drought tolerance improvement can also be related to isolated scientists putting breeding efforts in various crops. Thus, more collaboration between breeders and other experts with a common goal can be helpful to the development of drought-tolerant and high yielding wheat varieties [167]. After decades of experience, breeders started to realize that most of the time drought does not occur independently from heat stress [168]. Therefore, combining heat and drought stress is crucial in future breeding for drought-tolerant wheat varieties.

4. Traits associated with drought stress

4.1. Flag leaf size

Traits of flag leaf in wheat can partly indicate the drought tolerance of the whole plant. It is generally accepted that the longer time genotypes can maintain the photosynthesis in flag leaf the higher yield they will produce. The photosynthetic rate of a drought-tolerant wheat cultivar was found to be less affected by drought than that of a sensitive cultivar [169]. The products of flag leaf photosynthesis in wheat have been reported to contribute 30-50% of the composition for grain filling [163]. This is easy to understand from the short distance to spike and the fact that flag leaves sustain green for longer than other leaves. More recently, the grain yield of wheat treated with the removal of flag leaf was observed to drop considerably [170].

Flag leaf area has long been exploited as a selection criterion contributing to drought tolerance in different crops [171, 172]. In sorghum, flag leaf area was proven to be a reliable morpho-physiological parameter for drought tolerance [173]. In rice, a study with 30 genotypes revealed that flag leaf length and width were positively correlated to rice yield under drought [174]. An early study on seven spring wheat genotypes suggested that large flag leaf area might be correlated with a higher yield [175]. Similarly, another study reported the significant association between flag leaf area and grain yield in wheat variety Chinese Spring [176]. Flag leaf sheaths were found to be positively correlated to plant height and grain yield, which means that wheat varieties with longer flag leaf sheaths also displayed yield advantage [177]. Later on, another study with 29 wheat genotypes reported the strong and positive correlation between grain yield and flag leaf area in rainfed condition [178]. Results of a modern investigation on wheat exhibited positive and significant correlations between grain yield and flag leaf area under either drought or normal conditions [179]. Moreover, the results of correlation studies showed that grain yield per plant had a significantly positive correlation with flag leaf area in wheat under drought conditions [180]. A comparison of drought stress responses was made between tolerant and sensitive wheat varieties. A significant decline in glucose, fructose and sucrose content of flag leaves of the sensitive variety was observed in drought condition [181].

4.2. Heading date and Flowering time

Normally, wheat plants enter the boot stage in five days after the flag leaf emerges, and right after the boot stage is the heading stage [182]. Heading date of a plant can be regarded as the date when the plant shows its flower. The anthers sticking out from the spikelet mark the beginning of flowering stage (anthesis). The heading date in wheat has been proven to be regulated by some genes which were also associated with grain weight [183].

A two-year field experiment with four wheat varieties suggested that the days to heading were significantly reduced under drought stress. Based on those results, researchers illustrated that wheat genotypes with earlier heading and flowering dates may have a better chance to partially escape from drought and complete their life to reproduce the next generation before getting dehydrated [184]. In a field trial with a recombinant inbred line (RIL) population, researchers detected a QTL for days to heading on chromosome 7D and this QTL was further validated in a drought trial [185]. By conducting Pearson's correlation analysis, scientists also found that the days to heading were significantly correlated with days to maturity, plant height, spike length, and spikelet per spike under drought stress [186].

For flowering date, a similar theory with heading date was proposed that early flowering can be regarded as a favorable trait to better adapt the drought by escaping and minimizing the exposure to the stress [187]. As one of drought tolerance indices, in 2002, the flowering date was reported to be strongly negatively correlated with yield in wheat under drought stress [188]. On the contrary, another study in 2014 revealed that the number of days to flowering was significantly positively correlated with grain yield per plant in wheat in a dry environment [189]. Therefore, the strong correlation between flowering time and grain yield in wheat has been generally accepted, however, more carefully-designed studies are needed to figure out whether the correlation is positive or negative.

4.3. Canopy temperature

With the development of remote-sensing technique, canopy temperature has been introduced as an effective index into wheat yield estimates [190]. Remotely sensed canopy temperature was also reported to be a powerful indicator in screening for drought-tolerant wheat genotypes due to its correlation with leaf water potentials under moisture stress. Researchers found that lower canopy temperatures were accompanied by higher leaf water potentials [191]. It is not difficult to learn that a leaf temperature depressed below air temperature is because of the water evaporation from leaves. That is why canopy temperature can also be considered as an indirect measure of the transpiration in wheat [192]. Wheat plants exposed to drought stress were found to display higher canopy temperatures compared to those in optimum condition [193]. Results of a 2-year field trial revealed a positive correlation between drought-susceptibility index and canopy temperatures, suggesting that drought-susceptible wheat genotypes had warmer canopies at midday [40]. Furthermore, another study on spring wheat observed strong correlations between canopy temperature and yield in drought conditions [194]. The canopy temperature depression value was also found to be valuable to efficiently predict grain yield and water stress in wheat [195].

Apparently, the canopy temperature measurement is a cheap, fast, and non-destructive process. Since traits of roots in the soil are difficult to measure, scientists studied the possibility of using another above-ground trait as an indicator. The canopy temperature was found to be linked to the ability of roots to extract water from the soil, which was also proven to be associated with yield under drought in wheat [84]. In addition, another study confirmed the conclusion that cooler canopy temperature was associated with optimized root distribution for better water extraction in stressed wheat [196]. Moreover, the biomass of wheat was also reported to be correlated with canopy temperature depression in dry land [197]. In terms of genetics, two QTLs associated with both yield and canopy temperature depression were detected on chromosome 3BL and 5DL. Interestingly, the QTL located on 3BL was also pleiotropic for biomass [198]. In another study, a QTL located on chromosome 3B was detected and proven to have a large effect on canopy temperature and grain yield. [49].

4.4. Number of tillers, Number of spikes and Number of grains

Tillers can be described as additional stems that develop from the main shoot of the plant. Number of tillers in wheat is an easily measurable parameter. Tillering patterns determines the number of heads per acre which is an important yield component in spring wheat [199]. In a field experiment conducted under rainfed conditions with ten drought-tolerant wheat breeding lines, several traits were observed to be significantly and positively correlated with grain yield. Among those traits, tillers per plant displayed the highest positive effect on grain yield [200]. However, single culm was suggested as a favorable trait of wheat ideotype. The concept proposed earlier was that fewer tillers would be helpful to reduce the competition between the developing spikes so that the plant can invest more in the growth of each spike [201]. In a modern study, comparing conventional cultivars with freely tillering cultivars, scientists recorded a 6% yield increase in wheat lines containing a tiller inhibition (*tin*) gene in drought conditions [202]. They also confirmed that the reduced number of tillers would be an advantageous trait for wheat to cope with terminal drought.

In terms of spikes, a spike of wheat, also called the ear, consists of a central stem with tightly packed rows of flowers growing on it. It is also where the edible grains grow. The direct effect of number of spikes/m2 on grain yield of wheat under drought stress was found to be significantly positive [203]. Furthermore, results of a study revealed that number of spikes/m^2 was an effective parameter affecting grain yield. It was suggested that selecting wheat materials with high number of spikes/m^2 could be beneficial to develop varieties with high yield under drought conditions [204]. In another experiment with sixteen advanced wheat lines, a genotypic correlation between spikes/m2 and grain yield under a late drought regime was reported.

The number of grains per spike is one of the most important yield components in wheat. Early on, a drought-induced reduction in number of grains per spike was observed in two spring wheat genotypes [205]. Subsequently, a field experiment conducted under different moisture levels demonstrated that severe drought stress could result in a significant reduction in the number of grains per spike in wheat [206]. Results of another study with fourteen durum spring wheat varieties showed a positive association between the number of grains per spike and grain yield under drought stress [207]. Furthermore, a study with 37 durum wheat varieties applying regression analysis (stepwise method) also indicated that the number of grains per spike had a positive effect on yield in drought conditions [208].

4.5. Roots

Root traits include root length, root elongation rate, root distribution at depth, root to shoot dry matter ratio, emergence angle, tip angle, root biomass and root count [80, 209-211]. The root system determines the water use efficiency (WUE) of a plant. It was reported that the soil moisture capture ability in root is a major factor influencing crop yield in water-limited conditions [212]. The roots distribution in soil has been proven to be influential in grain yield in wheat under drought conditions. Scientists found that a wheat variety with less grain yield reduction under drought stress tended to develop more deep roots (below 30 cm) than the same wheat variety in well-watered conditions [213]. A reasonable explanation is that the upper layer of the soil always loses its moisture first when drought occurs so developing longer roots is advantageous for plants to reach the water in subsoil. It is also widely accepted that the root system is closely related to the grain yield in wheat [84, 213]. By conducting a simulation analysis with a modeling technique in Australian dry environments, it was estimated that the absorption of each additional millimeter of water by the root system during grain filling stage could produce an additional 55 kg/ha of grain in wheat [76].

A big variation was found in the growth angle of seminal roots among different wheat varieties [214]. Similarly, a significant difference was also found in the root length among different varieties [215]. It was also observed that wheat varieties with a deep root system showed a narrower angle of seminal roots, while varieties with a shallower root system tended to develop their seminal roots with a wide-angle [214]. Likewise, another study described a significant genetic variation in the growth angle of seminal roots among different wheat varieties. Besides, they also reported the lack of correlation between the growth angle and number of seminal roots [216]. In another study, scientists observed a positive correlation between grain yield and root biomass under terminal drought. Comparing to other genotypes, they further observed a less grain yield reduction in a wheat genotype which developed more roots under drought stress. These results suggested that wheat genotypes with a longer root system tend to have a more stable grain yield under drought stress [213].

5. Genome-wide Association Study (GWAS)

5.1. What is GWAS

5.1.1. Genetic makers

With the development of new technologies, plant breeding has come into a new era due to emergence of the revolutionary molecular breeding. Molecular breeding comprises two major areas, the transgenic crops and the molecular marker technology. In Molecular marker technology, SNPs are the third generation molecular markers. The term SNP is the abbreviation for single-nucleotide polymorphism, which describes a substitution of a single nucleotide at a certain position in the genome. With the development of modern DNA microarray technology, simultaneously analyzing thousands of SNP markers can be conducted in an efficient and cost-effective way [217]. Significant efforts towards large-scale SNP discovery have begun in the end of the twentieth century [218]. However, most of the early studies were conducted in the human genome. Fortunately, plant molecular biologists began to notice the existence of SNPs and saw the potential of extensive use of SNP maps in plants soon [219]. In rice, an SNP marker was found to be linked to the fragrance gene (*fgr*) [220]. Furthermore, by using a modified allele-specific PCR method, scientists developed abundant SNP markers in *Piz* and *Piz-t* genes, which were proven to be correlated to blast resistance in rice [221].

Compared to other plant species, wheat has a very large genome size [222], which is seven times the genome size of maize and 40 times the genome size of rice (Figure 4) [222-227]. Therefore, the use of molecular markers developed less smoothly than in rice. Initially, scientists introduced the first generation molecular markers, RFLP (Restriction fragment length polymorphisms) technology, to wheat breeding. However, RFLP has not been widely used for segregation analysis in wheat breeding for its low polymorphism in wheat breeding materials [228]. Then the second generation molecular markers arose and the most typical one is the microsatellite or SSR markers. The second generation molecular markers have then been dominant in wheat genetic studies for more than ten years [229-231]. At the advent of the third generation molecular markers, SNP markers started to take over the SSR makers' place. Already at the early stage, breeders realized the potential of SNP markers to be a valuable tool for genomics studies. In durum wheat, researchers identified a total of 2659 SNP markers using the complexity reduction of polymorphic sequences (CRoPS®) technology. Totally, 275 of those SNPs were found highly robust and uniformly distributed across the wheat genome [232]. While studying 172 elite European winter wheat lines, scientists found a difference between results of population structure and genetic relatedness obtained with SNP and SSR markers. They suggested that it would be beneficial to increase the number of SNP markers so that a higher coverage of the chromosomes could be obtained [233]. When studying a wheat stem rust resistance gene *SrCad*, scientists identified 27 SNP markers located closely to *SrCad*. By evaluating the diagnostic potential of these SNPs, they found four SNP markers completely predictive of the presence or absence of *SrCad* [234]. In addition, this research group also identified six highly predictive SNP markers linked to the wheat leaf rust resistance gene Lr16 [235]. In another study, scientists identified SNP markers which can be used to distinguish two low-molecular-weight glutenin subunits (LMW-GS) genes Glu-D3 and Glu-B3 in bread wheat [236].

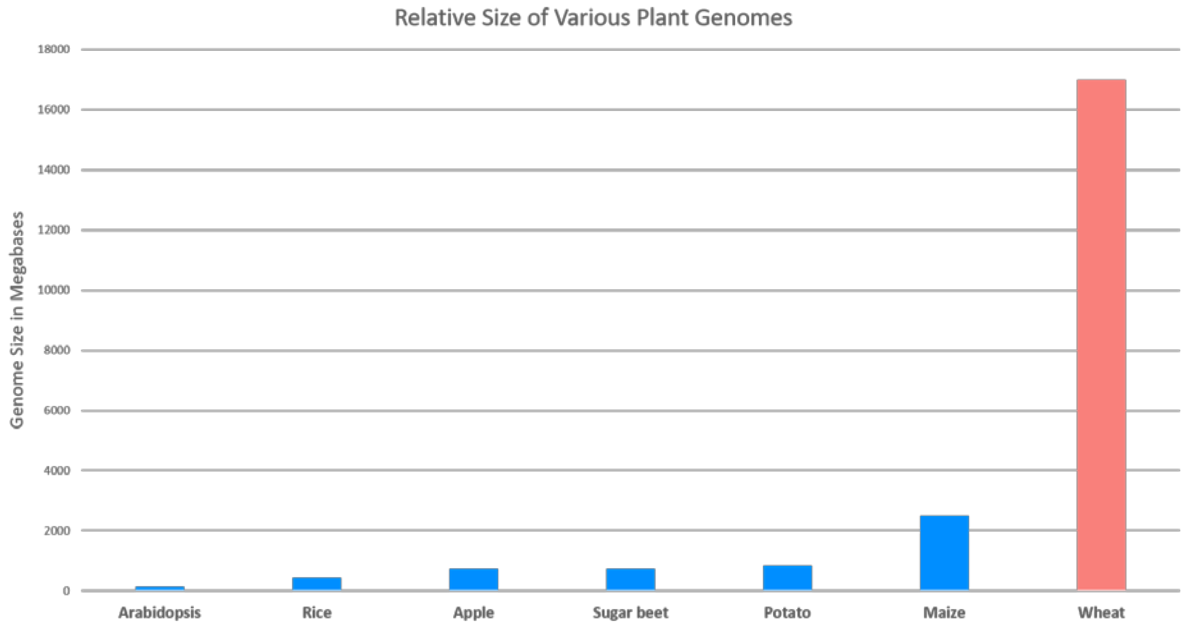


Fig. 4. Comparison of genome sizes of different plant species

5.1.2. Linkage disequilibrium (LD) decay

Linkage disequilibrium refers to a nonrandom association of alleles at different loci. It represents a tendency of alleles to be transmitted together more or less often than expected by chance alone [237]. Theoretically, alleles located on separate chromosomes get sorted into gametes independently of one another due to independent chromosome segregation. When it comes to two alleles located on the same chromosome, because of crossing over in meiosis, the frequency of alleles inherited from one parent in a given population should have the same value as the alleles at each locus were combined at random, which is also called linkage equilibrium. However, alleles on the same chromosome do not always separate randomly when they are close enough to each other. Therefore, close proximity is the cause of linkage disequilibrium.

The essence of GWAS (Genome-wide association study) is a marker-based study of associations between a genome-wide set of genetic variants in a population and a trait. Once markers significantly associated with variation of the trait are detected, the physical locations of the loci on chromosomes can be identified. To determine the screening range of candidate genes, the coefficient of LD and LD decay distance need to be introduced. The coefficient of LD describes the correlation between a specific pair of alleles [237]. The closer two alleles the higher value of the coefficient. When the value reaches 1, it represents that the pair of alleles are fully associated. By examining the coefficient of LD of bread wheat and durum wheat populations, researchers successfully compared two population structures and clustered wheat materials into different subpopulations [238]. The LD decay distance refers to the physical distance between any two alleles when their coefficient decays to a certain value. In other words, with the increase of the physical distance between a pair of alleles, the corresponding coefficient of LD decays. Once the coefficient drops below a certain threshold value, there is a corresponding threshold distance, which differs in different species. In wheat, results of a study based on 172 European winter genotypes suggested that LD decayed within approximately 5-10 cM [233]. In another study on 250 bread wheat accessions, LD decay distances of below 5 cM and 5-10 cM were identified in landraces and modern varieties respectively [239]. The conclusion can be made that any two loci with a distance greater than this threshold distance are not associated. Therefore, by calculating the physical distance between detected markers and candidate genes, scientists can determine whether a candidate gene is associated with the marker.

5.1.3. Phenotypic data

The term phenotype used in genetics refers to the observable characteristics of an organism. These characteristics include the organism's appearance, development and behavior. In association study, traits measured across the whole population are considered as the phenotypic data. Good phenotypic diversity brings the data considerable phenotypic variation, which is the key of the ideal phenotypic data in GWAS.

In wheat, an association study used seminal axis root length, seminal root length, root diameter, branched root length, and dry root matter as the phenotypic data. With the detected markers associated with the phenotypic data, scientists identified multiple proteins with different functions for those traits [240]. In a series of wheat field trials carried out for GWAS, researchers included the heading date, plant height and other three yield component related traits as the phenotypic data. Ninety-seven chromosome regions were identified to be associated with those traits [241].

5.1.4. Genotypic data

Genotyping is a process of determining genetic differences among lines by examining the individual's DNA sequence and comparing it to another individual's sequence. As mentioned above, based on the polymorphic SNP markers physically anchored to the genome assembly, the GWAS can identify chromosome regions affecting crop adaptation in combination with phenotypic data.

In wheat, the complexity of its genome used to be the greatest obstacle in developing stress adaptation improved varieties. The international wheat genome sequencing consortium (IWGSC) was established in 2005 with the aim of sequencing the genome of wheat. It took 13 years for scientists to finally map the massive wheat genome [242]. In 2018, the release of the annotated reference sequence of wheat marked a new chapter in wheat genotyping.

In general, genotyping consists of two phases, a polymorphism discovery phase and a genotyping phase. Next-generation sequencing (NGS) has been widely applied in sequencing the genomes of multiple genotypes of the same species to identify sequence polymorphisms such as SNPs [243, 244]. In the genotyping phase, large number of samples can be genotyped using a more cost-effective method such as high-density SNP-genotyping arrays [245]. However, the whole-genome sequencing in species with a large and complex genome was still costly in the last decade. In order to improve the efficiency and cost-effectiveness of the process, sequencing of restriction site-associated genomic DNA (RAD) and genotyping-by-sequencing (GBS) were introduced to the genotyping workflow [246, 247]. The GBS has been widely used to derive the genotypic data of durum and bread wheat [248-250].

5.2. The role of GWAS for drought stress research

As drought tolerance is controlled by multiple interacting genes [251, 252], it is not easy to study the drought tolerance of a crop directly but the crop's response to drought stress can be reflected in variations of target traits under drought conditions. Thus, investigating the traits under drought stress in combination with studying the crop's genome can be a promising entry point to identify candidate genes associated with its drought response. Furthermore, the drought tolerance of a crop can be evaluated based on the way it responds to drought stress.

By studying a barley population under drought stress, scientists detected 2 stable QTLs for internode length and flag leaf length with association study [253]. Furthermore, two candidate genes correlated to spikelet number and grain number per spike under drought stress were identified using 121 barley accessions and a 9K SNPs chip [254]. Using the survival rate as an indicator of drought tolerance in GWAS analysis, researchers identified the gene *ZmVPP1* contributing to drought tolerance in maize seedlings [255]. In another GWAS analysis on 209 maize accessions, 62 loci for seminal root length were revealed for two water treatments [256]. By investigating 7 traits including final grain yield, total number of ears per plot, kernel number per row, plant height, anthesis-silking interval, days to anthesis, and days to silking under water-stressed and well-watered regimes, researchers identified 115 candidate genes correlated to the variations of these traits under drought in maize [257]. With the development of new phenotyping approaches, scientists introduced a non-destructive phenotyping facility to GWAS. Fifty-one image-based digital traits (i-traits) for 507 rice accessions were derived. The gene *OsPP15* was identified to be correlated to drought tolerance in rice [251]. Moreover, based on the knowledge that the grain yield of rice can be used as a selection criterion under reproductive-stage drought [258], a GWAS analysis for yield performance of rice under drought stress resulted in 50 genes identified and 30 of them were annotated [259]. Another group focused on the root traits of rice. By performing a GWAS analysis on 21 root traits under normal and drought stress, they detected 143 significant associations and identified 11 reported root-related genes co-located with the association loci [260]. In sorghum, researchers utilized an aerial drone to measure the biomass, plant height, and leaf area of 648 lines under drought treatments. Multiple candidate genes were identified in GWAS analysis [261]. In pea, researchers investigated the onset of flowering and grain yield under terminal drought. A big number of markers associated with high yield and early flowering were detected in GWAS analysis [262]. In industrial crop sesame, by performing GWAS to 490 accessions, scientists detected 130 significant SNPs for drought stress. A total of 13 potential candidate genes coding transcription factors, antioxidative enzymes, osmoprotectants and involved in hormonal biosynthesis, signal transduction or ion sequestration were identified for drought tolerance [263]. In another industrial crop cotton, scientists introduced an automatic phenotyping platform (APP) for measuring 119 i-traits as the phenotypic data under drought stress. Results of GWAS on 56 morphological and 63 texture digital traits identified four drought-related genes as the candidate genes [264]. Another group of scientists utilized hydroponics to study 7 traits of 306 upland cotton accessions. Drought treatment was performed using Polyethylene glycol 6000 (PEG 6000). By combining results of GWAS, RNA-seq and qRT-PCR verification, 4 candidate genes were identified to be potentially important for drought tolerance in cotton [265].

5.3. GWAS applied in wheat research

With the advances in new sequencing technology in recent years, high-quality genome data can be provided for breeders [266, 267]. Different high throughput SNP arrays including 35K [268], 90K [269], 660K [270], and 820K [271] have been developed for wheat research. These SNP arrays have been successfully used to map multiple traits with diverse wheat populations while the most widely adopted is the 90K SNP array. It has been used for GWAS in European winter and spring wheat [272], CIMMYT spring wheat [273], United States elite wheat breeding genotypes [274], and Chinese bread wheat cultivars [275].

For studying the genetic bases of important traits under water-limited conditions, the powerful tool GWAS has been widely adopted in wheat research recently. Several QTLs for drought-related traits were successfully identified on different chromosomes through GWAS [276]. In a 2-year field trial, researchers observed a 60% mean yield reduction of a durum panel under drought stress and further identified a large number of significant marker-trait associations on chromosome 2A and 2B by performing GWAS [277]. Besides, researchers identified 14 drought-related candidate genes with 493 durum wheat accessions. Based on their GWAS results in combination with previous reports, they confirmed the presence of a drought tolerance related QTL in a region on chromosome 4B [278]. In a study with 210 elite European bread wheat varieties under different water stresses, scientists detected a total of 590 QTLs (126, 238, and 226 on A, B, and D genomes respectively) through GWAS analysis [279]. In another study, scientists investigated 5 traits of elite bread wheat lines in irrigated and drought stress environments. Significant associations on 4A, 2D, 3B, and 3A were identified for yield, spike length, 1000-kernel weight, and kernel abortion respectively. Likewise, associations for the number of grains per spike were identified on chromosome 6B and 3A [280]. Furthermore, scientists phenotyped a bread wheat panel for days to heading, days to maturity, shoot biomass, root biomass, root to shoot ratio and grain yield. GWAS analysis identified 37 marker-trait associations in drought conditions [281]. A GWAS analysis on 645 Chinese wheat landraces revealed 29 QTLs for eight traits under drought conditions. On the basis of these markers, 21 candidate genes were identified for drought response and four of them were validated by qRT-PCR [282]. To study the drought tolerance of wheat, researchers also adopted drought tolerance indices which were calculated based on grain yield, 1000-kernel weight, and the number of kernels per spike. GWAS analyses identified a total of 175 SNPs associated with at least one drought tolerance index. [283]. Most associations from this study were further located on chromosome 4A, which is consistent with the hypothesis from several previous QTL analyses that chromosome 4A plays a crucial role in drought tolerance [284-286]. In a study on synthetic hexaploid wheat, scientists identified a total of 194 marker-trait associations, and 90 of these were novel (45, 11, and 34 on the A, B, and D genomes respectively). Forty-five of these associations were further found in genes annotated as having a potential role in drought stress [287]. At the micro-level, cell membrane stability has been reported to be related to drought tolerance in wheat [288]. By performing GWAS to the cell membrane stability data of 2111 spring wheat accessions treated with PEG, a total of 20 significant SNPs were detected. In addition, the linked candidate genes were found to be involved in transporting solutes through the cell membrane or generally involved in abiotic stress tolerance mechanisms [289].

6. Further studies on candidate genes associated with drought stress

After identifying potential candidate genes by association study, some further studies are needed to validate the roles of the candidate genes from different aspects. Nowadays there are multiple methods of verifying the function of candidate genes.

6.1. RNA interference

To study the physiological role of the gene product, researchers can exploit RNA interference (RNAi) technique in further study. Once the double-stranded RNA synthesized with a sequence complementary to a gene of interest is introduced into a cell or organism, it could be recognized as exogenous genetic material and activate the RNAi pathway. Under this mechanism, the expression of the target gene could be drastically decreased so that scientists can observe the phenotypic difference between normal samples and gene-knockdown samples [290]. The first use of gene silencing via RNAi technology in plants was reported in a study on flower identity genes in arabidopsis. They successfully inhibited the mRNA level of a gene *AGAMOUS* and further proved the ability of RNAi to selectively reduce gene function in *A. thaliana* [291]. In wheat, scientists investigated the function of gene *TaGW2* using RNAi. Significant decreases in grain size and number of endosperm cells were observed in transgenic lines compared with the controls. Thus, the grain-size-regulating function of the gene *TaGW2* was verified and they further speculated that the grain size was regulated by *TaGW2* controlling the number of endosperm cells [292]. In a study on wheat under drought stress, scientists investigated the function of a gene *TaBZR2*, which encodes transcription factors belonging to the brassinazole-resistant (BZR) family. A significantly elevated drought sensitivity was observed in transgenic lines, whose *TaBZR2* was downregulated by RNA interference. Interestingly, they also constructed overexpressor (OX) lines and observed lower levels of ROS in drought-treated TaBZR2-OX lines than the wild type. Through RNA sequencing analysis of those OX lines, they found the drought stress induced the expression of *TaGST1*, which is thought to encode a reactive oxygen species-scavenging enzyme. Moreover, the results of their conducted electrophoretic mobility shift assay and luciferase reporter analysis indicated that *TaBZR2* bound to the promoter of *TaGST1* [293]. In another study, to verify the function of a dehydrin gene *WZY2*, scientists used PEG to create osmotic stress to wheat plants. Combining RNA interference and real-time PCR, they found that plants with an inhibited expression of *WZY2* showed a lower relative water content, oxidative-related enzyme activities, and higher malondialdehyde (MDA) content than wild types [294].

6.2. Virus-induced genes silencing

Virus-induced genes silencing (VIGS) is another powerful genetic tool for plant genes function study. With this technique, scientists can trigger a targeted gene transcript suppression by infecting the plant with a viral delivery vector carrying a short sequence of the target gene [295]. It is based on a natural post-transcriptional gene silencing (PTGS) defense mechanism that is developed to protect plants from virus infection [296]. The VIGS was first used to describe a phenomenon that tobacco plants can recover from virus infection and further develop a resistance to the virus [297]. The major advantage of VIGS is that it is very rapid and cost-effective and has a moderately high throughput compared with RNAi. Scientists quickly realized the potential of VIGS as an efficient tool for the study of gene function when they found that it allowed the targeted downregulation of a particular gene [298]. Early on, many vectors for use in VIGS were based on the model RNA virus, tobacco mosaic virus (TMV) [299]. Thus, this technique was usually performed in a virus-susceptible tobacco species *N. benthamiana* in early studies. To overcome the limitation of host range and meristem exclusion, scientists have made substantial efforts for developing new vectors. One of the famous VIGS vectors developed later is based on the tobacco rattle virus (TRV), which can efficiently spread throughout the entire plant. The only drawback of TRV was its mild symptoms of infection. This flaw was resolved soon with the advent of improved TRV vectors named pYL156 and pYL279 [300, 301] that were successfully used for silencing endogenous genes in tomato and other species [302, 303]. In wheat, researchers have reported the use of barley stripe mosaic virus (BSMV) as the vector to perform VIGS could efficiently silence a leaf rust resistance gene [304]. The BSMV-VIGS system was also used to study wheat stripe rust resistance. By silencing two heat shock protein genes *TaHsp90.2* and *TaHsp90.3,* scientists observed increased numerous rust sori on the leaves [305]. Another disease studied with BSMV-VIGS is wheat powdery mildew. The experiment was based on the knowledge that Calcium-dependent protein kinases (CPKs) are playing important role in plant complex immune and stress signaling networks. After downregulating a corresponding gene, *TaCPK2-A*, by performing VIGS, scientists observed loss of resistance to powdery mildew in resistant wheat lines [306]. In addition, VIGS has also been widely employed in functional validation of potential drought-responsive genes in wheat. In a study, scientists successfully verified the functions of two genes under drought stress. They performed VIGS to wheat plants and significant reductions in transcripts of *Era1* (enhanced response to abscisic acid) and *Sal1* (inositol polyphosphate 1-phosphatase) were confirmed by the results of qRT-PCR. Those VIGS-treated wheat plants exhibited increased relative water content, improved water use efficiency, inhibited gas exchange, and improved vigor compared to plants in the control group under drought stress [307]. Similarly, another study adopting VIGS also provided concrete evidence to support the vital drought-related roles of two candidate genes in wheat. After knocking down *TaDrSR1* and *TaDrSR2* by performing VIGS, scientists observed significant increases in relative electrolyte leakage rate, malonaldehyde content, and reductions in relative water content, and proline content in VIGS-treated plants under drought stress [308]. The function of a gene encoding a wheat ERF transcription factor, TaERF3, was thoroughly examined by gene overexpression and silencing approaches. In terms of gene silence, scientists chose VIGS to silence *TaERF3*. *TaRTF3*-silencing wheat lines were generated and subjected to salt and drought stresses. Those *TaRTF3*-silencing lines showed a lower survival rate than did the control plants [309]. Researchers also investigated the function of a gene, *TaH2B-7D,* belonging to a wheat histone H2B family. They utilized VIGS to generate *TaH2B-7D* knockdown plants that displayed lower relative water content and proline content compared with control plants under drought stress. Moreover, those VIGS-treated plants showed observable sagging, wilting and dwarf phenotypes in drought conditions [310].

6.3. Gene overexpression

Another powerful genetic tool to study the function of target genes is overexpression. The popularity of overexpression as a screening tool started in the molecular genetics era shortly after the arising of yeast transformation techniques [311, 312]. Overexpression screens were confined to perform in yeast because of stable maintenance and easy recovery of 2µ vector-based plasmids. With the advent of some conceptually analogous overexpression screens using sib selection of expressed cDNA library clones in *Xenopus* [313], or transposon-based overexpression collections in *Drosophila* [314] and *Arabidopsis* [315]. Overexpression has grown into a viable research option in multiple organisms. In wheat, overexpression has been successfully exploited to study the functions of several genes, including a sharp eyespot related gene, *TiERF1,* from *Thinopyrum intermedium* [316], a high molecular weight (HMW) gene [317], and some fusarium head blight (FHB) related genes [318]. Additionally, by cloning the aquaporin gene *TaAQP7* from wheat and transferring it to tobacco, scientists observed increases in drought tolerance in transgenic tobacco lines with *TaAQP7* overexpressed [319]. Similarly, scientists also transferred another wheat aquaporin gene *TaAQP8* to tobacco. The roots of transgenic lines overexpressing *TaAQP8* displayed increased root length, higher K^+/Na^+ ratio, Ca^{2+} content, enhanced SOD, CAT, and POD activities under salt stress [320]. A wheat gene *TaNAC2a*'s role was also verified in tobacco. Transgenic tobacco plants overexpressing *TaNAC2a* displayed higher fresh weight and dry weight under drought stress [150]. In another study, researchers transferred two genes *TNHX1* and *TVP1* encoding wheat vacuolar transporters to arabidopsis plants. Transgenic plants overexpressing those two genes exhibited better salt and drought tolerance than wild types due to their increased vacuolar solute accumulation and water retention [154]. In order to study the function of a wheat abiotic-stress-related gene, *TaSnRK2.8,* scientists cloned and transferred it to arabidopsis. Enhanced drought tolerance in transgenic lines overexpressing *TaSnRK2.8* was evidenced by longer primary roots, higher relative water content, better cell membrane stability, lower osmotic potential, elevated chlorophyll content and enhanced PSII activity [321]. In addition to verify the function of wheat genes in tobacco or arabidopsis, researchers can also create gene overexpression in wheat lines. Scientists successfully made TaNAC69-1 expression constructs by inserting the coding region of TaNAC69-1 between a barley promoter and rice rbcS 3' in pSP72 vector. The transgenic wheat plants overexpressing TaNAC69 showed more shoot biomass, root biomass, and increased root length under drought stress [322]. *TaRNAC1* is another member of the NAC transcription factors family. Researchers developed *TaRNAC1*-overexpressing wheat lines using a predominantly root-expressed promoter. They found that transgenic lines could produce more aboveground biomass and grains under PEG treatment than wild type plants [149]. Another research group generated *TaWRKY2*-overexpressing transgenic wheat lines. By comparing with wild type plants, they observed a higher survival rate and lower water loss rate of detached leaves in transgenic lines after drought treatments at three leaves stage. Moreover, after 20-25 days of drought treatment, increased grain yield was identified in transgenic lines in comparison with wild type plants at maturity [323]. In the WRKY transcription factors family, another gene from Arabidopsis (*AtWRKY30*) was also reported to be associated with heat and drought tolerance. To verify its function, scientists generated the overexpression of *AtWRKY30* in wheat plants. In transgenic wheat lines, they found multiple enhanced physiological parameters including biomass, gas-exchange attributes, chlorophyll content, relative water content, prolines content, soluble proteins content, soluble sugars content, and antioxidant enzymes activities. Noticeably, the expression level of a number of antioxidant enzyme-encoding genes and stress-responsive genes including *ERF5a*, *DREB1*, *DREB3*, *WRKY19*, *TIP2*, and *AQP7*, were detected to be induced in *AtWRKY30*-overexpressing wheat lines [324].

7. Challenges, Hypothesis and Perspectives

Looking at the efforts of scientists in plant drought tolerance study over these decades, our understanding of the drought-responsive mechanism has progressed a lot. With the development of numerous novel technologies, researchers are allowed to combine their results of morphological changes of wheat plants with new studies on the genetic character of the entire panel. However, there are still some obstacles standing between scientific research and realistic improvement in wheat drought tolerance.

Drought is a term we used to describe a regional environment with prolonged shortages in the water supply. Severe drought can further influence the local ecosystem and agriculture. It is mainly caused by below-normal precipitation. The lack of adequate rainfall can significantly affect crop production and further lead to a potential food crisis [325]. However, drought condition is not as simple as it seems. There are always connections between drought and other abiotic stresses. For instance, heat can also cause a reduction in soil moisture because high temperature accelerates the evaporation of water. Therefore, it is important for researchers to study drought stress in conjunction with heat stress [23]. In addition, salt stress in the soil can lead to similar osmotic pressure to drought stress. It is also important to take salt stress into account when investigating drought tolerance [326]. But in respect of scientific research, it is not recommended to introduce too many factors to a single experiment as it makes it difficult for researchers to conclude the effect of each factor on the result.

Another challenge lies in the complexity of plants' response to drought stress. As mentioned in section 3.1, when exposed to water deficit condition, plants have two different mechanisms to combat the stress, drought avoidance and dehydration tolerance [125]. Moreover, plants' responses to drought stress can be affected by multiple factors such as plant genotype, growth stage, severity and duration of stress, mixtures of drought and other natural abiotic stresses, different patterns of the activity of respiration [69]. It is not difficult to predict that the responses of wheat plants to drought stresses in different periods can be dramatically different. For instance, plants treated with drought stress at their early stage still have a theoretical chance to adjust their growth period to avoid exposing critical growth stages under stress. However, plants suffering the stress at a late stage can only count on their dehydration tolerance. Thus, the timing of the drought treatment is crucial throughout the entire experiment.

As the root system is in direct contact with soil, this hidden part of the plant usually responds to reduced soil moisture actively. Larger root biomass grants the plant better water use efficiency under drought stress. The root biomass of wheat has also been reported to be correlated with final grain yield under water deficit [327]. Therefore, studying the root traits is crucial to evaluate the plant's drought tolerance. Additionally, combining wheat root parameters with above-ground traits can be of great interest in stress adaptation analysis.

However, as mentioned before, the root system is a hidden part of the plant. It is not easy to observe or measure any parameters at maturity as getting rid of the soil without damaging the roots is scarcely possible. The loss of any single root can lead to a great error on root traits such as root length and root biomass. Thus, the most suitable approach for root assay is the hydroponic culture. Scientists can easily measure and compare the root traits of different genotypes. It is even feasible to observe and record the growth parameters of roots every day. The disadvantage of growing wheat plants in water is also very sharp, which lies in the difficulty of imposing drought stress on plants. Even though scientists have successfully applied PEG-induced drought stress to water cultured wheat plants, there are still some remaining obstacles to overcome [328]. It is not possible to fully simulate natural drought by adding a chemical to the solution. Adding PEG to the nutrient solution is in fact inducing a higher osmotic potential which is similar to the soil water osmotic potential in drought condition. It affects roots water uptake in the same way as water-limited soil. But natural drought is a slow process which means that the soil moisture reduces gradually when precipitation decreases. On the contrary, the osmotic potential changes immediately after adding the PEG. It can further cause a greater shock on seedlings than the natural drought does and so far it is not clear whether this will activate other response mechanisms in plants. Another drawback is that wheat plants cannot grow to maturity under hydroponic conditions. It means that researchers are only allowed to investigate root responses under early drought stress. To see the correlation between root traits and final grain yield, scientists have to carry out one additional soil-cultured trial. Plant's behavior at early stages does not reflect its drought tolerance throughout the entire growth period due to the lack of critical root developmental detail.

In recent decades, researchers have always been seeking an optimal approach to investigate root-soil interactions with damaging the roots. Fortunately, with the profound advancement of technology exchange between scientists in different domains, plant scientists realized to adopt X-Ray computed tomography (X-Ray CT) as a non-invasive way to study plant-soil interactions [329, 330]. Subsequently, researchers went one step further rapidly in this field. They found neutron imaging (NI) could effectively visualize the root architecture in soil [331]. NI technology has been successfully exploited to capture water movement between root and soil [332]. Early plant experiments with NI mainly adopted 2-dimensional neutron radiography (NR) to study the root structure properties in situ [333, 334]. Later on, the neutron computed tomography (NCT) was introduced as another powerful tool to study root-soil water dynamics with even more detailed 3D visualization of the root system compared to NR. The NCT has been utilized to map 3D wheat root architecture under drought stress and successfully monitor soil water distribution after the short drought treatment [335].

The expression of genes in plants has been proven to be influenced by drought stress. Therefore, the detection of genes during stress is crucial to study plants' drought-tolerant mechanisms at molecular level [123]. However, as described in section 5.1.1, wheat has a very large genome size estimated at approximately 17 Gb in accordance with IWGSC's announcement. The genome sizes of maize and rice, the other two of the world three major cereal crops, are only one-seventh and one-fortieth of that of wheat respectively [224, 226]. Furthermore, plants' tolerance to drought stress is a complex trait regulated by multiple genes and each gene contributes differentially to this trait [124]. To thoroughly dissect the wheat plant's drought tolerance at the genetic level, researchers should not ignore those minor genes when trying to identify the major genes. One of the inspiring news is that through the unremitting efforts and cooperation of scientists from all over the world, the massive wheat genome was finally mapped in 2018 [242]. Researchers have finally been allowed to conduct the whole-genome scan for candidate genes.

8. References

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