REVIEW

Cereal flag leaf adaptations for grain yield under drought: knowledge status and gaps

Akshaya Kumar Biswal · Ajay Kohli

Abstract Selection for drought-tolerant cereal varieties has successfully moved to screening for grain yield under stress. Grain yield is the culmination of the process of grain filling, which in turn is closely linked to flag leaf functionalities. For grain filling to occur under drought, either a relatively uncompromised or a favorably reprogrammed functioning of the flag leaf is required. However, knowledge is limited on how effectively flag leaves can function under stress conditions or what adaptations could allow such functioning. The information on rice flag leaf function and/or adaptation under drought is critically limited, while rice continues to be the crop with the highest potential to alleviate hunger and poverty. In fact, other cereal crops are equally important in maintaining regional food baskets and these too suffer intermittently from different intensities and kinds of drought. Patchy information is available on the morpho-anatomical, physiological and biochemical aspects of flag leaves under drought; even this is dispersed within different cereals, with studies predominantly on wheat. Hence, a reasonable understanding of the function of flag leaf under drought is lacking for any cereal. Importantly, very few reports exist on the molecular and mechanistic understanding of any known adaptations of flag leaf function under drought. Here we review the existing information on cereal flag leaf function under drought and highlight the need to better understand its characteristics/adaptations, especially at the molecular level. Novel drought-tolerant breeding material generated through selection for yield under stress can be a useful resource to underpin the mechanistic basis of the contribution of flag leaves to such yield. Improved knowledge can then be used for providing dependable markers (morphological, anatomical, physiological, biochemical and/or molecular) for robust flag leaves, leading to efficient and judicious use of resources for screening broader germplasm collections.

Keywords Flag leaf · Drought · Cereals · Rice · Yield · Grain filling · Assimilates · Source–sink transition

Introduction

Frequent major crop shortfalls in the vast drought-prone areas of Asia, Africa and other continents threaten food security, human health and the livelihood of millions of people. Uncertain threats associated with climate change further aggravate the situation. At least 23 million ha of rice area (20 % of the total rice area) in Asia are subject to drought of different intensities (Pandey et al. 2007). To meet the

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ever-growing demand for rice by 2030, an increase of at least 35 % in yield is needed (Bouman et al. 2007; Ainsworth 2008). Since rice yield in the irrigated ecosystem has apparently plateaued for many years (FAO 1996), breeders are looking into new horizons of yield enhancement in the rain-fed ecosystem. However, the rain-fed ecosystem is obviously most prone to mild, moderate or severe drought. Drought of different intensities at different growth stages affects yield through separate routes (Pantuwan et al. 2002; Pirdashti et al. 2004). For example, late-season drought coinciding with the rice booting to heading stage affected plant height and the photosynthetic rate of the flag leaf, thus leading to an overall significant loss in yield (Ji et al. 2012).

To leverage untapped rice yield potential in the rain-fed ecosystem, generating drought-tolerant rice genotypes has received a new impetus. Drought tolerance is a complex trait which is a combined function of various morphological, physiological and biochemical characters (Table 1). This presents the challenge of improving multiple characters in coordination: for example, the physiological and biochemical nature of water use along with improvement in nitrogen and carbon assimilation and mobilization under drought stress (Anjum et al. 2011; Serraj et al. 2011; Farooq et al. 2009, 2010). Importantly, however, not all characteristics known to be useful for drought tolerance need to be manipulated in a particular scenario because different sets of characteristics manifest as useful in specific plants and locations (Tardieu 2012).

The approach of direct selection for yield under reproductive stage stress initiated in maize (Bolanos and Edmeades 1993; Quarrie et al. 1999; Araus et al. 2012), and its success in the identification in rice of large-effect quantitative trait loci (QTL) for yield under drought (Swamy and Kumar 2012) calls for updating of knowledge on the role of the flag leaf as a major source of assimilates for grain filling. This is especially relevant due to the lack of comprehensive information on the importance of the flag leaf in drought tolerance of cereals.

Various characteristics or their combinations in the flag leaf have been used or proposed for selecting drought-tolerant plant, for example, higher flag leaf area, relative dry weight, excised-leaf weight loss, residual transpiration, leaf glaucousness, canopy temperature depression (CTD) and chlorophyll content; late senescence; and higher carbon isotope discrimination

(CID). Despite the recognition of the importance of cereal flag leaves, little is known about hormone homeostasis in flag leaves under stress. Also, practically no information is available on flag leaf-specific gene promoters, constitutive or stress-inducible. Effectively, there is a major gap in our understanding of the biochemical pathways, genes and genetic networks that are affected in the flag leaf under stress to alter the signaling, synthesis, accumulation, transport and use-efficiency of primary resources.

In this report, we review the morpho-anatomical, physiological and biochemical adaptiveness of the cereal flag leaf for its role in grain filling under drought. We highlight particular loci-, gene- or genome-based studies conducted with particular reference to flag leaves. Finally, we assess the selection or engineering of flag leaf characteristics as a route to drought-tolerant cereals.

Flag leaf to the fore

Screening breeding lines under reproductive-stage drought is now popular and successful in rice, as demonstrated through the identification of some large-effect QTL for grain yield under stress. In cereals, grain yield is mainly dependent on the photosynthetic source–sink relationship dictating changes in carbohydrate synthesis, accumulation and partitioning. The top two leaves, including the flag leaf, are considered the primary source while the developing grains are the primary sink (Sicher 1993). In rice, the top three leaves, including the flag leaf, export assimilates to the panicle (Yoshida 1972). Photosynthesis in these three leaves contributes the major part of the total grain carbon content during grain filling. During drought, however, the culm, internode and flag leaf sheath (Fig. 1) are thought to play an increasingly important role as sources of carbohydrates for grain filling (Garcia et al. 2010; Slewinski 2012). Nakano et al. (1995) observed that when rice panicles were removed at anthesis, flag leaf senescence and the decrease in the rate of flag leaf photosynthesis was retarded. This led to an accumulation of photosynthates in shoots and roots, increasing their weight up to 200 and 150 %, respectively. Similar delayed senescence of the flag leaf was observed in wheat when spikelets were removed (Srivalli and Khanna-Chopra 1998). In panicle-removed rice, there was no appreciable rise in starch or sucrose

Table 1 Various characters affecting drought tolerance

Morphological	Physiological	Biochemical
Vigor	Transpiration rate	Accumulation of proline
Leaf and flower emergence	Water-use efficiency	Accumulation of polyamine
Coleoptile length	Stomatal conductance	Accumulation of trehalose
Leaf area	Canopy temperature	Accumulation of LEA[a] and dehydrin proteins
Leaf rolling	Osmotic adjustment	Accumulation of shock proteins *hsp*[b] or *csp*
Wax content	Relative water content	Cell membrane integrity
Awns	Leaf turgor	Antioxidant actvity
Stomatal density	Photosynthetic rate	Nitrate reductase activity
Tillering		Photosynthate storage
Root characteristics		Nutrient remobilization
		Cell membrane stability

Based partly on Ali et al. (2009)

[a] *LEA* late embryogenesis proteins

[b] Heat or cold shock proteins (*hsp* or *csp*)

accumulation in the flag leaf, suggesting an active transport of assimilates out of the flag leaf. However, more recently, rice panicle removal was shown to increase the glucose content in leaves (Shimono et al. 2010). These results suggest that translocation of photosynthates away from the flag leaf is a largely irreversible default pathway and that bioconversion of assimilates is affected in the flag leaf under stress. Shoot culms, leaf sheaths and even roots becoming sinks was also observed by Kato et al. (2004) in sink-limited rice plants. In wheat, defoliation of the flag leaf blade increased the contribution of assimilates to grain from the stem and the chaff under normal conditions (Alvaro et al. 2008), confirming the observations of Garcia et al. (2010) in rice that the stem and sheath can be an important source of assimilates. Overall, removal of the flag leaf adversely affected grain yield under normal or water-limiting conditions in cereals such as rice (Nakano et al. 1995; Hirano et al. 1998; Saitoh et al. 2002), wheat (Cruz-Aguado et al. 1999; Ali et al. 2010) and barley (Jebbouj and El Yousfi 2009). Also, the net CO_2 assimilation rate (P_N) in flag leaves during water deficit displayed a strict correlation with the drought sensitivity of wheat (Saeedipour and Moradi 2011) and rice genotypes (Ji et al. 2012). Such studies emphasize that morpho-anatomical, physiological, biochemical and molecular characteristics of the cereal flag leaf could be an important component of predicting and/or generating drought tolerance in cereal crops.

Morpho-anatomical characteristics of the flag leaf under drought

Flag leaf morphology

Many studies indicate the importance of simple morphological characteristics such as shape, size, angle, color and viability of the cereal flag leaf in relation to yield. Results linking yield to various traits of the rice plant established a positive correlation with flag leaf morphology, mainly considering flag leaf area (18.5–44.1 cm^2) using 24 rice varieties (Sedeek et al. 2009) or flag leaf length to width ratio (mean = 21.2) using 50 accessions of upland rice (Sohrabi et al. 2012). High heritability and genetic advance were noted in both studies of these flag leaf traits. A positive correlation between rice flag leaf area and yield was also noted by Li et al. (1998) and Yue et al. (2006). Similarly, in barley (Yap and Harvey 1972), oat (Peltonen-Sainio 1990) and wheat (Simon 1999; Quarrie et al. 2006), flag leaf area was an important component related to yield. Yap and Harvey (1972) also noted high heritability and genetic advance of morphological traits in barley as in rice above, while Simon (1999) noted slow genetic advance in wheat. Flag leaf area was shown to be a reliable morpho-physiological marker for drought tolerance in 10 sorghum cultivars (Ali et al. 2009). Similarly, flag leaf length and width were positively correlated to rice yield under drought in 30 cultivars (Abarshahr et al. 2011). Fifty
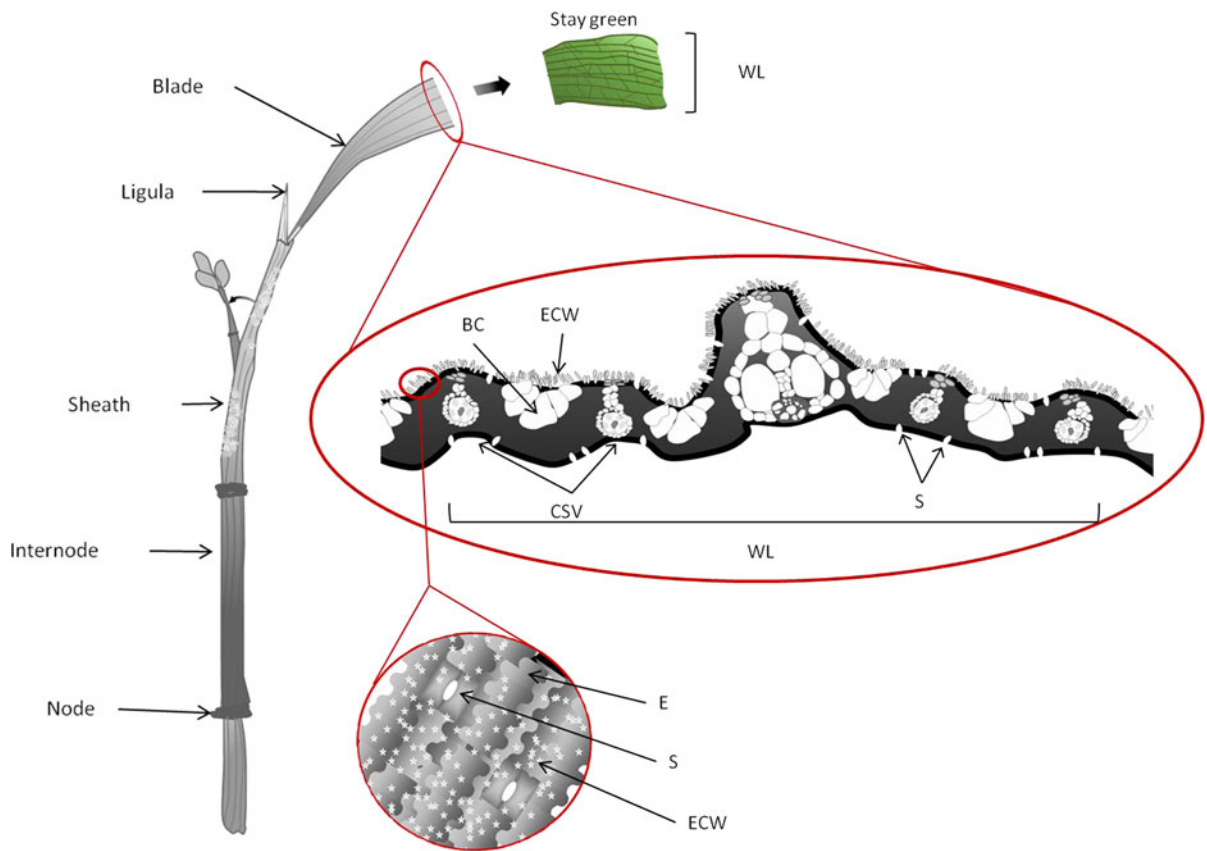
Fig. 1 Schematic details of rice flag leaf, showing traits useful for drought tolerance. A wider (*WL*), thicker, actively photosynthesizing, late-senescing (*stay-green*) flag leaf is recommended. A tall erect flag leaf growing over the panicle with a 35° angle between the panicle and the flag leaf (depicted here as the *arrow* between the sheath and the pedicle of the three grains) is recommended. The *larger inset* of the transverse section of the wide leaf (WL) *blade* shows larger bulliform cells (*BC*), higher stomatal density (*S*), more and closely spaced veins (*CSV*) and a thicker epidermis (*E*; *smaller inset*), with stellate or filamentous epicuticular wax (*ECW*) leading to glaucousness. The flag leaf sheath, along with the node and culm, is known to become an important source of carbohydrates under drought. Its length, thickness, dry weight, glaucousness etc. are now considered important characteristics for it to be a source of carbohydrates for grain filling during drought. Biochemical and molecular understanding of the morpho-anatomical and physiological characteristics of the flag leaf under drought is critically lacking. An improved knowledge of such aspects can lead to biochemical and genetic markers for selecting for effective flag leaves. Figure generated by Dr. Berta Miro of CESD, IRRI

accessions of upland rice evaluated for 12 different traits, including growth, yield and yield components, also recorded flag leaf length to width ratio as significantly correlated to yield (Sohrabi et al. 2012).

The erect nature of the rice flag leaf as well as its stay-green trait are recommended for yield but the contribution of these two traits to drought tolerance is not clear. One of the major concepts behind the development of the rice New Plant Type (NPT) was the erectness of the flag leaf rising up over the developing panicles and a sustained photosynthesis therein (Virk et al. 2004). For rice, erect flag leaves were highly recommended for better yield by Kropff et al. (1994) but flag leaf angle was not seen to be important by Sohrabi et al. (2012). Interestingly, in the study of Yap and Harvey (1972) on barley, flag leaf angle was not associated with yield, similar to the results obtained by Sohrabi et al. (2012) for rice. However, for wheat Simon (1999) found flag leaf angle to be important in yield.

With regard to flag leaf viability, programmed flag leaf senescence of monocarpic plants is a highly regulated process during which cells undergo orderly changes in gene expression, metabolism and cell

structure. One of the early targeted and most significant changes in cell structure is the breakdown of the chloroplast through autophagy (Wada et al. 2009), resulting in a change in flag leaf color from green to pale yellow. Catabolites from the degradation of chlorophyll and other macromolecules are converted into exportable nutrients supplied to the developing florets (Lim et al. 2007). The typically oblong stroma and grana thylakoids of fully expanded flag leaves change to spherical and swollen thylakoid membranes during natural senescence (Zhang et al. 2010). Chen et al. (2011) demonstrated that, under drought stress, the chloroplast and thylakoid of drought-susceptible barley genotype Moroc9-75 changed significantly to more aberrant conformations than those of the drought-tolerant genotype HS41-1. Treatment with ABA, a commonly up-regulated hormone during drought stress, also promoted unstacking of thylakoid membranes, a decrease in chloroplast size and rupture of the chloroplast envelope in rice leaves (Hurng et al. 1988). Additional Ca^{++} was shown to change the leaf senescence in *Rumex obtusifolius* L. as a result of the role of calcium in maintaining cellular membranes (Poovaiah and Leopold 1973). Similarly, the mitochondrial membrane and cristae are affected under drought stress. Flag leaf stay-green trait, which is the delay in or reduced rate of senescence as it approaches maturity, was not seen to have any particular effect on yield in barley (Yue et al. 2006). However, delayed senescence of the flag leaf was recommended by Kropff et al. (1994) for better yield because of a concomitant prolonged photosynthetic activity. Nitrogenous fertilizer application later in the growing season adds to the stay-green trait through protection of RuBisCO from degradation which contributes to yield increase from greater grain weight (Cassman et al. 1992) in an irrigated environment. Earlier, high and stable yield under drought in stay-green germplasm was reported for sorghum (Rosenow et al. 1983). A positive correlation between flag leaf stay-green and yield under drought was also shown for maize (Banziger et al. 1999) and wheat (Hafsi et al. 2000; Verma et al. 2004). The positive correlation of flag leaf stay-green traits with yield was confirmed in wheat by Blake et al. (2007).

Flag leaf glaucousness

A morphological characteristic that leads to differences in traits such as reduced epidermal conductance and surface reflectance of light is leaf glaucousness (Fig. 1). Although associated with epicuticular waxes, degree of glaucousness is not strictly associated with the amount of wax per se. For example, there was no direct relationship between flag leaf glaucousness and the amount of epicuticular waxes in barley (Larsson and Svenningsson 1986; Febrero et al. 1998), oat (Svenningsson and Liljenberg 1986) or wheat (Araus et al. 1991). In glaucous barley lines, the flag leaf waxes tended to increase from irrigated to rain-fed conditions while the opposite happened in non-glaucous lines (Febrero et al. 1998). Reflectance as well as the amount of epicuticular wax of the flag leaf in wheat increased under drier conditions (Johnson et al. 1983) and glaucous wheat lines exhibited more favorable physiological traits for drought tolerance than non-glaucous lines (Richards et al. 1986). Glaucous flag leaves may afford an advantage for drought tolerance through a still unresolved quantitative combination of a reduction in both transpiration rate and photosynthesis (Richards et al. 1986; Febrero et al. 1998). Leaf glaucousness is associated with an increase in yield in wheat (Johnson et al. 1983; Richards et al. 1986) and barley (Febrero et al. 1998) but the mechanisms invoking changes in the rate of transpiration and photosynthesis remain to be better understood. A QTL with a positive correlation between wheat flag leaf glaucousness and yield was recently identified (Bennett et al. 2012). Differences in wax load pattern and degree of crystallization as related to surface reflectance and in turn related to carbon and water use/uptake efficiency may be explored further. The structure of waxes lining the surface of the flag leaf seems to be important since filamentous structures of the waxes were seen to increase reflectance more than the classical plate-like structures (Juniper and Jeffree 1983; Blum 1988). Epicuticular wax shapes associated with glaucousness were explored at the ultrastructural level in sorghum cultivars that exhibited stellate or filamentous wax deposition (Tarumoto 2005). Wax crystal morphology such as stellate, rods, ribbons, tubes, platelets or branched versions of these structures was recently reviewed in relation to their chemical composition and the effect of wax morphology on surface reflectance was discussed (Shepherd and Griffiths 2006).

It is important to distinguish between leaf glossiness and glaucousness. Leaf glossiness is due to intracuticular waxes while glaucousness is due to epicuticular waxes on the leaf surface. Buschhaus and

Jetter ([2011](#)) provide an extensive review of the molecular differences between the two kinds of waxes in a number of plants; however, only one example of cereals (*Secale*) is reviewed, indicating a knowledge gap. Within cereals, more information is available on leaf glossiness, including the characterization of non-glossy mutants that lack intracuticular waxes (Kunst and Samuels [2009](#)), than on leaf glaucousness. For example, in rice, there is practically no information on flag leaf glaucousness while non-glossy mutants have been well characterized (Qin et al. [2011](#)). However, the correlation between epicuticular wax and a reduction in epidermal conductance, in relation to drought tolerance in rice, was recently explored in genotypes of *Oryza sativa* and *O. glaberrima* and in their interspecific progeny (Saito and Futakuchi [2010](#)). In flag leaves of wheat, the wax deposits on the adaxial side covered the surface of the leaf more densely and uniformly than those on the abaxial side (Araus et al. [1991](#)). Sufficient genotypic variation was found in epidermal conductance for it to be recommended as a breeding trait (Babu et al. [2003](#); O'Toole [2004](#)). However, information on the direct relationships between the amount, composition and structure of surface waxes and epidermal conductance is still limited (Shepherd and Griffiths [2006](#)). This is especially so for the flag leaf and flag leaf sheath.

Flag leaf rolling

Leaf rolling is another morphological trait easily scored and closely linked to drought response. Leaf rolling is hydronasty that leads to reduced light interception, transpiration and leaf dehydration (Kadioglu and Terzi [2007](#)). Although leaf rolling occurs under abiotic as well as biotic stress conditions (Kadioglu et al. [2012](#)), it is mostly under the former conditions that it has been used as a screen for tolerant genotypes. It may help in maintaining internal plant water status (Subashri et al. [2009](#)). If cell turgor is maintained under drought stress, it results in delayed leaf rolling. However, increased leaf rolling under severe stress would have the advantage of preventing water loss and radiation damage. Variation in leaf rolling among genotypes has a genetic basis and QTL associated with leaf rolling were reported for pearl millet (Sehgal et al. [2012](#)) and rice (Price et al. [1997](#); Subashri et al. [2009](#); Salunkhe et al. [2011](#)). Flag leaf rolling as a drought-adaptive mechanism was also

seen in wheat (Sarieva et al. [2010](#)). Anatomical and molecular aspects of leaf rolling were recently reviewed by Kadioglu et al. ([2012](#)). Anatomically, leaf rolling occurs through changes in the water status of bulliform, hypodermal and schlerenchyma cells.

Flag leaf anatomy

Water stress during flag leaf development distinctly affects flag leaf anatomy. Leaf area and thickness were decreased but, interestingly, increased stomata, bulliform cells and total cell wall were noticed along with fewer intermediate veins. The importance of smaller intermediate veins in maintaining high photosynthetic rates was highlighted in a comparison of leaf anatomy of plants with C_3 and C_4 photosynthesis (Ueno et al. [2006](#)). A decrease in leaf thickness was due to a reduction in both the number and size of mesophyll cells. These changes in flag leaf anatomy were irreversible and indicated the development of leaf xerophily. Certain aspects of this phenomenon could be directly related to water conservation; that is, an increase in cell wall due to increased intermediate veins may reduce transpiration. Hence, a reduction in size of the flag leaf through such anatomical changes might lead to drought tolerance. However, it was argued that higher flag leaf area is correlated with drought stress tolerance and is an important component of yield. Enlarged flag leaves were also seen to increase grain size and yield per plant in rice, although through a minor delay in heading (Yan et al. [2011](#)).

The mesophyll cells of the rice flag leaf sheath were characterized anatomically to show an active photosynthetic apparatus, including chloroplast number per cell, comparable with that in the leaf blade (Guo et al. [2011](#)). However, a clear decrease in photosynthetic capacity was noticed, going from the top (attached to the blade) to the bottom (attached to the culm) along the sheath (Ishimaru et al. [2004](#)). The vascular bundle elements were more compact and smaller at the bottom of the sheath, with mesophyll cells also arranged compactly around the vascular bundles. The top part of the sheath exhibited larger vascular elements and relatively looser and fewer mesophyll cells. Metabolically, there was a clear transition from the top part being a source organ to the lower part being a sink organ (Ishimaru et al. [2004](#)).

Investigation of winter wheat genotypes with contrasting drought stress tolerance revealed that the

total number of stomata per leaf was lowest in drought-tolerant varieties (Deák et al. 2011). Among three *Triticum* species, the flag leaves with the highest rates of photosynthesis were also the thickest (Parker and Ford 1982) and had closely-spaced veins near the mesophyll cells. Relatively more air-filled spaces were present in these leaves, providing the highest ratio of mesophyll cell surface exposed to such air-filled spaces per unit leaf area. Wheat flag leaf net photosynthesis decreased with a decrease in leaf area, leaf width and mean area per mesophyll cell. However, it increased with increased number of veins and stomatal frequency per mm of leaf. The relative importance of these anatomical features for rates of photosynthesis was not determined separately. Wheat genotypes known for their drought resistance had more veins per unit area in the flag leaf (Ahmed et al. 2004). Diploid, tetraploid and hexaploid species of *Triticum* and *Aegilops* were investigated for the relationship between flag leaf anatomy and photosynthetic rates per unit leaf area at light saturation. The diploid species that showed maximum photosynthetic rates had thinner leaves, which contained less chlorophyll and less dry matter per unit area (Kaminski et al. 1990). However, the ratio of mesophyll cell surface area to per unit leaf area was not substantially different between the species in this study. The diploid species were seen to be comparatively more drought-tolerant (Molnár et al. 2005), perhaps due to higher compactness of mesophyll cells in the leaf blade (Sasahara 1982), which may reduce the average diffusion path length to carboxylation sites (Kaminski et al. 1990). Study of a wheat QTL revealed a functional association of flag leaf width with yield under drought. It was demonstrated that cell number rather than cell size of the leaves was responsible for the increase in flag leaf width (Quarrie et al. 2006). Although flag leaf width increased in QTL near-isogenic lines in comparison with QTL-negative lines, the increment was variable across multiple locations, which is indicative of a genotype × environment (G × E) interaction for flag leaf anatomy. Other QTL for wheat yield under drought were also seen to exhibit G × E interaction (Snape et al. 2007). In contrast, rice lines with a large culm and increased flag leaf length and width were associated with increased bundle sheath and vascular bundle area. Larger xylem and phloem vessels were present as well as increased interveinal distance (Wu et al. 2011c). The plants with a large culm and flag leaf

exhibited fewer tillers, larger panicles and more and heavier grains per panicle but lower seed-set and a higher number of blighted grains. The total yield of such plants was not significantly different from that of normal-culm plants. Although no data were presented for their performance under drought, the increased interveinal distance may not be a suitable characteristic, while increased leaf photosynthetic rate and favorable culm transport characteristics may be suitable. However, it was shown that, despite similar photosynthetic rates and flag leaf area, the difference in carbohydrate storage capacity of the flag leaf sheath contributed substantially to a yield increase (Ishimaru et al. 2004). Soluble carbohydrate reserves in the wheat stem were also positively correlated with yield under drought conditions (Snape et al. 2007).

Physiological traits of flag leaves under drought

Photosynthesis serves as the main metabolic process contributing to grain filling, but photoassimilates stored pre-anthesis are the resource for grain filling (Gebbing and Schnyder 1999). Over 80 % of the total carbohydrate accumulated in rice grains is produced by the top two leaves, which includes the flag leaf (Gladun and Karpov 1993). Under water stress at tillering stage, stem internodes and the flag leaf sheath also become a source of photoassimilates for grain filling (Garcia et al. 2010). The leaf sheath as a source of carbohydrates for rice grain filling was also invoked when a relationship could not be established between the rate of grain filling and the rate of photosynthesis during the rapid grain-filling phase (RGFP). However, the flag leaf itself could be the major source of photoassimilates accumulated in stem culms and leaf sheaths. This was evident when a female sterile rice line was used to measure the rate of photosynthesis in the flag leaf during the ripening stage, in comparison with a normal variety (Kato et al. 2004). In wheat, too, the transport of assimilates from vegetative organs to kernels increased under water stress and was higher in the drought-tolerant genotype (Plaut et al. 2004). Estimation of the relative contribution of pre- and post-anthesis assimilates to grain yield in wheat indicated that stability in yield and drought tolerance was characterized by substantial mobilization of pre-anthesis assimilates. In the drought-resistant wheat cultivar Hongwangmai, remobilization of pre-anthesis

assimilates to the grain declined under drought while a higher net photosynthetic rate was maintained in the flag leaf. In contrast, remobilization increased in the drought-susceptible cultivar Haruhikari, suggesting an early senescence of the flag leaf, which in turn led to lower yield under drought (Inoue et al. 2004). The role of flag leaf sheaths in storing and later transporting assimilates to the developing grains seems to be more important than functioning as photosynthetic organs after the onset of senescence. It is suggested that the accumulation of carbohydrates in leaves might some-how trigger senescence simultaneously in the flag leaf blade and sheath. An effective translocation system in/ of the flag leaf could avoid carbohydrate accumulation, in turn avoiding onset of senescence. The question that remains to be addressed is: which is better for grain filling under drought, an increased rate of flag leaf photosynthesis or an increased rate of assimilate delivery from the sheath?

The source for grain filling under drought may be rerouted from the flag leaf to other vegetative organs, but the flag leaf suffers distinctly under stress, not least through reduced photosynthesis. Water deficit led to a reduction in the net CO_2 assimilation rate in flag leaves of the susceptible variety earlier than in the tolerant variety (Saeedipour 2011). Signs of water deficit-associated senescence, that is, loss of chlorophyll and soluble proteins, were also more and earlier in the susceptible variety. In the tolerant variety, fructose content increased in the culm and peduncle but fructan content fell, apparently due to rerouting assimilates to the grain in the form of fructans (Saeedipour 2011). Another study using the young flag leaves of fully-grown wheat recombinant inbred lines (RILs) showed that drought stress significantly decreased the photosynthesis rate while net photosynthesis and stomatal conductance were found to be more sensitive than intercellular CO_2 concentration of the flag leaf. In rice, Guóth et al. (2009) did not find a strict correlation between drought sensitivity of the genotypes and net CO_2 assimilation rate or chlorophyll a fluorescence parameters in flag leaves during soil water deficit. However, a physiological study in two contrasting rice genotypes with respect to drought stress susceptibility at the reproductive stage revealed a more severe reduction in the osmotic potential of leaves in drought-susceptible Zhenshan97B than in drought-tolerant IRAT109 (Ji et al. 2012). The photosynthetic and transpiration rates along with stomatal conductance of the rice flag leaf declined significantly under severe drought stress while the canopy temperature was significantly higher.

Drought stress impaired photosystem II (PS II) activity in the flag leaf under high light/energy conditions in both rice (Pieters and El Souki 2005) and wheat (Yang et al. 2006). Studies with different species have shown that PS II is rather tolerant of water deficit. This implied that, with high solar energy input in tropical environments, down-regulation of PS II could be an important factor limiting photosynthetic capacity and yield. A decrease in photosynthetic activity with the onset of water deficit includes decreased photochemical efficiency of PS II, diffusional limitations due to early stomatal closure, a reduction in amounts and activities of key photosynthetic enzymes, altered carbon metabolism shown by changes in leaf sugar content and composition, and changes in activities of key enzymes of carbohydrate metabolism, especially when drought is combined with light stress. Total flag leaf photosynthesis and its duration appear to be more relevant than net photosynthesis of the plant for grain yield under drought. The flag leaf possesses higher tolerance or avoidance of dehydration than the lower leaves. The photosynthesis rate of the flag leaf was found to be significantly higher and less affected by drought in resistant wheat cultivar Hongmangmai (Inoue et al. 2004). The intercellular CO_2 concentration in the flag leaf of the resistant cultivar was lower than that of the susceptible cultivar in spite of a similar rate of reduction in stomatal conductance, which might be due to higher photosynthetic rate. The flag leaf has a lower water potential, solute potential and turgor pressure than the lower leaves but has a high rate of photosynthesis, nitrogen assimilation and dry matter per unit area. In a study of the yield of 30 wheat genotypes across three irrigation regimes of severe, moderate and no water deficit, three physiological traits of the flag leaf, carbon isotope discrimination (CID), flag leaf senescence (FLS) and canopy temperature (CT), together explained 92 % of the total phenotypic variation in grain yield. Flag leaf CID was positively correlated with grain yield whereas FLS and CT were negatively correlated (Li et al. 2012).

Biochemical changes in flag leaves under drought

Hormones

Extensive literature is available on hormonal transitions in plants under drought, but limited information

exists on such changes specifically within the flag leaf. Considering its importance in source–sink transition under drought, it can be predicted that flag leaves would show substantial, and most likely dynamic, changes in the level of different hormones.

The differential response of cereal genotypes to water stress was associated with differential abscisic acid (ABA) concentration in the flag leaves. Between drought-tolerant and susceptible wheat genotypes, Nayyar and Walia (2004) saw higher content of ABA in flag leaves of the tolerant genotype while Saeedi-pour and Moradi (2011) saw higher ABA content in flag leaves of the susceptible one. The discrepancy may be the result of an underlying dynamic ABA homeostasis within and between the flag leaf and the developing grain under drought (Seiler et al. 2011; Govind et al. 2011). Such homeostasis may be influenced by source–sink transition at the developmental stage at the time of drought application and at the time of sampling the source/sink tissues for analysis. Progressive accumulation of leaf ABA with more severe drought was also shown in wheat (Ali et al. 1999).

Under terminal drought stress, ABA and its degradation products (phaseic acid and diphaseic acid) increased in barley flag leaves and 19 of the 41 ABA metabolism genes exhibited differential regulation in flag leaves (Seiler et al. 2011). For rice under drought stress, increased ABA in the peduncle was shown to be detrimental to peduncle elongation, thus leading to sterility (Muthurajan et al. 2011). The adverse effect of ABA on the peduncle could be reversed with external application of the growth hormone gibberellic acid. ABA is known to positively regulate abiotic stress tolerance by affecting stomatal guard cells, but it negatively affects growth by impairing photosynthesis. Such effects of ABA on plant growth, development and stress response were recently reviewed (Sreenivasulu et al. 2012), stressing the contrapuntal role of ABA during drought stress.

Cytokinins regulate many important aspects of plant development in aerial and subterranean organs and also have roles in the response to abiotic stress. A reduced level of cytokinins in the leaf might induce the onset of senescence (Noodén et al. 1990). Hormone-induced changes in source–sink relationships have been proposed to improve the drought tolerance of rice (Peleg et al. 2011). Cytokinin-mediated source–sink modifications using the cytokinin biosynthesis gene

isopentenyltransferase (*IPT*) were reported to improve drought tolerance and increase grain yield in rice under water stress (Peleg et al. 2011). Song et al. (2012) proposed a coordinated regulation of cytokinin gene expression during flag leaf development. Cytokinins act antagonistically to ABA and their content goes down under drought. Hormonal cross-talk and hence effects of the differential content of one hormone on another are becoming increasingly recognized; for example, the cytokinin–auxin cross-talk and the effect of ethylene on auxin response factors is known (Peleg and Blumwald 2011; Kohli et al. 2013).

Evolution of the gaseous hormone ethylene increased under drought in the growing flag leaves of wheat and concomitantly polyamine content of the flag leaf also increased (Zhenzhu et al. 1995). Ethylene reinforces the drought effect of shortening the grain-filling period and reducing grain weight (Beltrano et al. 1999). Such effects could be reversed by application of the ethylene synthesis inhibitor amino-ethoxyvinylglycine. Song et al. (2012) proposed that the high β-glucosidase gene activity at the anthesis stage could lead to the release of cytokinins from their conjugated molecules in the leaf and provide a source of cytokinins for the developing carpels/seeds.

Osmolytes, antioxidants and proteases

Osmoregulators such as polyamines, proline, glycine-betaine, total sugars and reducing sugars increase in the flag leaf under drought and such increases were greater in tolerant genotypes of wheat (Szegletes et al. 2000; Nayyar and Walia 2004). With such osmoregulators, the tolerant genotypes had higher water content in their flag leaves and grains. Wheat flag leaves of tolerant genotypes exhibited relatively increased sucrose and proline accumulation under chemically induced drought (Sawhney and Singh 2002). Water stress also had a significant effect on flag leaf soluble sugar and proline content in rice (Yan et al. 2012). Drought-resistant sorghum genotypes accumulated a higher concentration of proline, which contributed to osmotic adjustment and turgor maintenance under water stress (Castro Nava et al. 2003). Similarly, the resistant sorghum lines accumulated more glycine-betaine in their flag leaves (Monyo et al. 1992). Foliar application of glycine-betaine could maintain higher photosynthetic efficiency in wheat flag leaves under drought (Ma et al. 2006; Zhao et al.

2007) and such leaves also maintained higher antioxidative enzyme activities and suffered less oxidative stress. Under drought, flag leaves exhibit up-regulation/accumulation of antioxidative enzymes such as superoxide dismutases, catalases, peroxidases and dehydroascorbate reductases in wheat (Osipova et al. 2011), rice (Ji et al. 2012) and millet (Dai et al. 2011). When flag leaves of seven different wheat genotypes were examined for four separate antioxidant enzyme activities under four water regimes, the results revealed up-regulation of at least two antioxidant enzymes under low water conditions in every genotype (Hameed et al. 2011). Drought-tolerant genotypes exhibited enhanced Cu/Zn-superoxide dismutase and peroxidase activities while the susceptible ones had enhanced Mn-superoxide dismutase and catalase activity (Simova-Stoilova et al. 2009).

The antioxidant system generally protects membranes by avoiding lipid peroxidation, as judged by the content of malondialdehyde (MDA), a degradation product of polyunsaturated membrane lipids. In rice, it was shown that the severity of drought directed the kind and extent of antioxidant enzyme/system, including ascorbic acid, tocopherol and glutathione, thus affecting hydrogen peroxide and MDA content, total membrane lipids and index of unsaturated fatty acids (IUFA) (Liu et al. 2011). These, in turn, were a measure of membrane damage under drought. Such membrane damage is typical of tissue senescence, which is also accompanied by protein breakdown by proteases. Indeed, water stress enhanced proteolytic (endopeptidase and exopeptidase) activities in the wheat flag leaf (Srivalli and Khanna-Chopra 1998; Martinez et al. 2007). A decrease in soluble protein content is a measure of senescence and this was shown to occur under drought in the flag leaves of wheat (Hameed et al. 2011; Saeedipour and Moradi 2011) and millet (Dai et al. 2011). The drought-susceptible wheat genotype exhibited comparatively more protein degradation (Saeedipour and Moradi 2011). Proteolytic action of specifically up-regulated root proteases under drought was proposed to be linked to nutritional requirements and drought-responsive protein processing under stress (Kohli et al. 2012).

Additional biochemical changes in flag leaves under drought are associated with the effect of drought on photosynthesis. Post-anthesis drought substantially accelerated the loss of chlorophyll and RuBisCO in wheat (Martinez et al. 2003). In rice, orthophosphate dikinase, glycine dehydrogenase, ribulose 1,5-bisphosphate carboxylase (RuBisCO), glycine hydroxymethyltransferase and ATP synthase were down-regulated under drought in the drought-susceptible cultivar Zhenshan97B. This suggested a reduction in carbon assimilation capacity while in the drought-tolerant variety IRAT109, transketolase and RuBisCO were down-regulated but RuBisCO activase and peptidyl-prolyl *cis–trans* isomerase, which might alleviate damage to RuBisCO by drought stress, were up-regulated (Ji et al. 2012). Severe water stress during grain filling decreased the flag leaf photosynthesis rate through aggravation of the adverse effect on nitrogen metabolism, such as the decreased concentrations of N, free amino acid and soluble protein, as well as the activities of nitrate reductase and glutamine synthetase, and increased malondialdehyde (MDA) accumulation and endopeptidase activity in the wheat flag leaf. The activities of nitrate reductase and glutamine synthetase were positively correlated with photosynthetic rate, but those of endopeptidase were negatively correlated.

QTL, genes and omics of flag leaf traits under drought

Flag leaf QTL

A number of QTL have been identified for flag leaf traits in cereals (Table 2). Genes on the homoeologous group 2 chromosomes of wheat were associated with flag leaf senescence (stay-green) variation and were the most significant in drought interactions (Foulkes et al. 2007). QTL for flag leaf temperature of a highly drought-tolerant wheat genotype were reported recently, along with additional QTL for other traits important for drought tolerance (Kumar et al. 2012). QTL for flag leaf senescence under normal and drought conditions were also reported (Verma et al. 2004). Similarly, a flag leaf width QTL as well as the underlying candidate gene were identified for rice (Ding et al. 2011).

A rice curly flag leaf mutant was used to identify the gene *Cfl1*, which negatively regulated leaf cuticle development through interaction with a class IV homeodomain gene, *HDG1* (Wu et al. 2011b). *HDG1*, in turn, regulates two cuticle development genes (*BDG* and *FDH*) downstream, by binding to a specific *cis*-element L1 box in the promoters of these two genes. Rice histone deacetylases (*HDACs*) were seen to have a

Table 2 QTL identified for better flag leaf morphology/physiology

Species	Cross	Chromosomes	Environment	Main traits	References
Rice	Pus1266/Jaya	1–4, 6–8, 10, 12	Field	Flag leaf length	Marathi et al. (2012)
Rice	IRAT109 × Zhenshan 97	4	Field	Flag leaf width	Ding et al. (2011)
Rice	Zhenshan 97 × IRAT109	3–6, 7, 10	PVC pipe	Flag leaf length/size	Yue et al. (2006, 2008)
Rice	Lemont (J) × Teqing (I)	1–5, 6, 8, 12	Field	Flag leaf length, width	Mei et al. (2003, 2005)
Rice	M23/AK	1-6, 8, 10, 11, 12	Field	Flag leaf length, width	Kobayashi et al. (2003)
Wheat	Kukri × RAC875	3A	Field	Flag leaf glaucousness	Bennett et al. (2012)
Wheat	Halberd × Karl92	1B, 2D, 2D, 3B, 5A	Field	Flag leaf length, width, temperature depression	Mason et al. (2011)
Wheat	Hanxuan 10 (H10) × Lumai 14 (L14)	1A, 2B, 3B, 5A, 7A, 7B	Field	Flag leaf CID	Wu et al. (2011a)
Wheat	Beaver × Soissons	2B, 2D	Field	Flag leaf senescence	Verma et al. (2004)
Barley	Yerong × Franklin	2H, 3H, 6H	Field	Flag leaf chlorophyl content, length, width, length/width	Xue et al. (2008)
Barley	Harrington × OUH602	2H, 4H, 5H, 7H	Field	Flag leaf length, width	Gyenis et al. (2007)
Sorghum	Inbred line 654 × LTR108	1, 2, 3, 4, 6, 7, 10	Field	Flag leaf length, width	Zou et al. (2012)

specific spatio-temporal expression pattern. Suppression of a rice *HDAC* (*HDA740*) affected plant height and also flag leaf morphology, whereby the flag leaf was severely twisted (Hu et al. 2009). The stress-associated NAC (*SNAC*) gene, which promotes stomatal closing (Hu et al. 2006), was reported to be predominantly expressed in the root and flag leaf (Nuruzzaman et al. 2012), thereby reducing water loss from the flag leaf. Expression of two genes, *TaeIF3g* involved in protein translation initiation and *TaVAP* involved in protein sorting in response to drought stress, was modulated in a coordinated manner in the flag leaf of the two wheat cultivars, which may contribute to homeostasis under stress (Singh et al. 2007). Sharoni et al. (2012) reported the flag leaf-specific expression of an AP2 domain-containing protein gene. Similarly, a grain protein content gene (*Gpc*-B1) plays a role in early flag leaf senescence and nitrogen remobilization to ears during grain filling (Kade et al. 2005; Uauy et al. 2006; Distelfeld et al. 2007). *Gpc*-B1 belongs to the group of NAC transcription factors and it regulates an extensive list of GPC-regulated genes, including transporters, hormone-regulated genes and transcription factors (Cantu et al. 2011).

Flag leaf omics

Microarray-mediated analysis of changes in gene expression under drought revealed up-regulation of 582 genes and down-regulation of 795 genes in rice (*O. sativa* L. ssp. *indica* cv. Minghui 63) flag leaves (Zhou et al. 2007). This was comparable to the change in shoot and panicle. However, nearly four times more genes were up-regulated in the flag leaf than in the shoot or panicle after 48 h of rehydration. These data indicated the importance of the flag leaf in response to changing water availability. Drought stress altered the expression pattern of a significant number of genes involved in transcription and cell signaling in a largely organ-specific manner in the flag leaf. Similarly, Xu et al. (2011) observed that about 65 % of all transcription factors identified in rice were preferentially expressed in the flag leaf. Some transcription factors such as a C_2H_2-type zinc finger were up-regulated

more than two-fold only in the flag leaf, suggesting a role for it in drought response. The promoter regions of induced genes possessed relative enrichment of two *cis*-elements (ABRE core and DRE core) known to be associated with water stress. Studies on barley under terminal drought demonstrated that there was a difference between the regulation of transcripts of plastidic enzymes and cytosolic enzymes (Seiler et al. 2011).

Apart from the analysis of transcripts of known genes, differential expression of small RNA molecules and their targets can also inform on the role of flag leaves under drought. Certain miRNAs preferentially or specifically expressed in flag leaves have been noticed under non-drought conditions. Yao et al. (2007) reported that the expression of miR156 was higher in wheat flag leaves than in spikes. A small RNA, miR172, had maximum accumulation in the rice flag leaf during the late vegetative and panicle development stage, suggesting a possible role in regulating the transition between vegetative and reproductive tissue development (Zhu et al. 2009). Overexpression of OsmiR393 led to expression of auxin receptor gene homolog OsTIR1 only in rice flag leaves (Xia et al. 2012). Transgenic plants overexpressing miR393a/b displayed a deformed phenotype, including enlarged flag leaf inclination (Bian et al. 2012). Further detailed exploration of miRNA may provide insight into engineering flag leaves with improved drought-tolerance traits.

Conclusions

Cereal flag leaf traits have been much better studied under normal irrigated conditions and that understanding has led to appreciating their possible role in grain filling under drought conditions. The review of the literature presented above indicates that, despite a central role for the cereal flag leaf in grain filling, an understanding of flag leaf functionality under drought is critically lacking. The limited information available is predominantly from studies on wheat flag leaves under drought. As a first step, therefore, functionality of additional cereal flag leaves under drought must be explored, especially for rice. Studies on flag leaves under drought indicate that achieving increased leaf width and thickness without reducing the number of transverse veins and longitudinal vein density would

be a highly useful trait. Though exploring such properties of the flag leaf blade has attracted the attention of many researchers, the flag leaf sheath, along with the stem internode and culm, apparently performs the important function of remobilizing stored assimilates for grain filling under stress. More research on the flag leaf sheath is called for, especially on its source-to-sink transition and the nature of carbohydrates along its length. Screening the germplasm for favorable sheath characteristics may facilitate the identification of tolerant varieties. Leaf glaucousness has been shown to be useful in avoiding photo-damage due to high light intensity, which occurs simultaneously with drought. Further studies on the overall contribution of this trait to drought tolerance would be useful. Combining useful sheath traits with delayed senescence and an appropriate number of stomata per unit leaf area of the blade could lead to a better assimilate source for grain filling. The role of changes in the amount of fructans, reducing sugars and non-structural carbohydrates in the flag leaf under drought must be explored in detail along with the expression of transporters, in order to understand the dynamics of carbohydrate mobilization for grain filling under drought. An extensive understanding of the kind and amounts of antioxidants and osmoregulators specifically in the flag leaf is needed, as these can affect flag leaf survival and photosynthesis therein. Effects of hormonal cross-talk are only just starting to become clear. Some of these aspects are shown in Fig. 1. Finally, the identification of flag leaf-specific genes and promoters or control elements will be important in validating the role of candidate genes relevant to flag leaf expression or for silencing genes specifically in the flag leaves.

There is a trend to move away from using so-called secondary traits, such as flag leaf or root characteristics, as screening methods for identifying (or generating) tolerant rice varieties. However, yield under drought, as a primary screen, is largely a manifestation of adaptations in such secondary traits. Screening for yield under stress is no doubt the most effective approach and the only one that has been successful in terms of developing new drought-tolerant varieties. However, understanding the mechanism will involve the identification of the most highly contributing factors. Flag leaf and sheath characteristics such as stay-green, assimilate transport, and water and radiation use efficiency under stress, along with root characteristics, are among the foremost

such factors. Using the genetic material generated by yield under stress as the primary screen, detailed studies on flag leaves and roots may help to identify characteristics that would reduce screening time and resources required. Since such material was not readily available previously, limited knowledge has been generated. However, with novel drought-tolerant cereal lines being generated, a rapid understanding, facilitated by high-throughput approaches, can be expected. This would, in turn, lead to the judicious use of resources to screen for better and more tolerant varieties suited to specific eco-geographies.

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