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Abiotic stress and control of grain number in cereals.

Rudy DOLFERUS, Xuemei JI and Richard A. RICHARDS

Address:

CSIRO Plant Industry, GPO Box 1600, Canberra ACT 2601, Australia.

Corresponding author:

Rudy Dolferus
Tel: +61-2-62465010
Fax: +61-2-62465000
E-mail: rudy.dolferus@csiro.au
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Abstract
Grain number is the only yield component that is directly associated with increased grain yield in important cereal crops like wheat. Historical yield studies show that increases in grain yield are always accompanied by an increase in grain number. Adverse weather conditions can cause severe fluctuations in grain yield and substantial yield losses in cereal crops. The problem is global and despite its impact on world food production breeding and selection approaches have only met with limited success. A specific period during early reproductive development, the young microspore stage of pollen development, is extremely vulnerable to abiotic stress in self-fertilising cereals (wheat, rice, barley, sorghum). A better understanding of the physiological and molecular processes that lead to stress-induced pollen abortion may provide us with the key to finding solutions for maintaining grain number under abiotic stress conditions. Due to the complexity of the problem, stress-proofing our main cereal crops will be a challenging task and will require joint input from different research disciplines.

Key words: cereal/abiotic stress/grain number/pollen/sterility
1. Introduction

1.1. Abiotic stress and reproductive development

Maximising crop growth and yield in regions where there is abiotic stress, which are variable in their timing and unpredictable in their intensity, is a challenge to all plant scientists engaged in this area of research. The variability in timing and intensity of stress means that there is no straightforward path towards understanding the molecular and physiological basis of growth and yield formation. To make progress in understanding the physiology of stress responses and then translate this to develop a molecular understanding and fully progress towards breeding, it is important to identify the most vulnerable aspects of growth and yield formation and focus on it. Plants are opportunistic; their genetic and developmental program is tuned by environmental signals. Substantial research has been conducted on the effect of abiotic stress during early crop growth and on survival following stress. However, the effect of abiotic stress on cereal reproductive development has received comparatively less attention, despite the dramatic effect on grain yields. Even mild abiotic stress can irreversibly affect grain yield, without affecting survival of the vegetative plant parts. There are numerous studies in cereals that have dealt with the effect of post-anthesis stress on grain-filling and grain size [1, 2], but relatively few studies have addressed the effect of stress on grain number prior to flowering. Yet, it is loss in grain number, rather than a reduction in grain size, that largely accounts for crop yield reduction when abiotic stress occurs. Grain number is the most vulnerable yield component in grain crops, yet very little is known of its control. In an evolutionary context it has made sense that under adverse conditions our crop progenitors abort important organs such as tillers and florets so that a small number of large seeds are formed to assure some fecundity.
This strategy can be important for species in the wild but it is likely to put a limit on grain yield in crops. The importance of grain number rather than grain size is also evident from historic yield studies showing that breeding has successfully increased yield. In all cases it has been grain number that has been the primary determinant of yield increases and grain size is either unchanged or reduced [3]. This has occurred in both favourable and unfavourable environments. Part of the explanation for this could be that an adequate grain size must be maintained for market acceptance and hence more selection pressure has been on size rather than number of grains. A result is that little is known about the regulation of grain number, especially for crops grown in adverse conditions. The 10-15 day period before anthesis is most important for the determination of grain number [4, 5]. At this stage the reproductive structures are still small and protected by the leaf sheaths, making them less accessible for investigation using non-destructive methods. This paper will focus only on the effect of abiotic stress on cereal reproductive development and how this affects grain number.

1.2. Mechanisms for abiotic stress avoidance and tolerance
Reproductive development starts with the transition of a vegetative meristem into a floral meristem and includes development of the floral and reproductive structures, formation of the male and female gametophytes, fertilisation and finally seed development (Fig. 1). The timing of the switch from vegetative to reproductive development is controlled by environmental cues such as photoperiod (long/short day length) and temperature (vernalisation) [6, 7]. Adjustment of flowering time is therefore an essential adaptation mechanism to the expected environmental conditions as it can result in the avoidance of abiotic stress such as frost, heat and
drought in a given environment. Breeding for short- or long-duration varieties is therefore a common breeding strategy to maximise yield and adaptation to specific environments. But, avoidance mechanisms may limit yield potential when conditions are favourable as the crop duration may be too short to achieve maximum yield. Furthermore, avoidance mechanisms do not protect the crop when unexpected abiotic stress events occur. This is particularly true if stress events are short and transient (e.g., cold, heat or drought spells). In this case, a tolerance mechanism is required to protect reproductive development and guarantee maximum grain formation. Under field conditions, it is hard to discriminate between avoidance and tolerance mechanisms. Screening for tolerant germplasm in the field has most often resulted in selection of stress-avoiding lines with an altered flowering time rather than lines with genuine stress-tolerance.

1.3. The importance of timing: different effects at different stages
While abiotic stress affects grain size from anthesis onwards, grain number is determined in the period before anthesis (Fig. 1). The potential grain number is fixed early during spike differentiation. Abiotic stress affects the number of spikelet and flower initials that are formed on the spike. The potential grain number can still be further adjusted during stress situations, as developing florets can abort prematurely later in development (Fig. 1). This can be easily observed in rice; the rice panicle has an open structure compared to the compact structure of wheat and barley ears, making it easier to assess the effect of stress on grain number (Fig. 2). From meiosis onwards, reproductive development becomes irreversibly committed to male and female gametogenesis. Meiosis occurs approximately at the same time in the ovary and anther to form the male and female gametophyte [8]. The grain
number that was fixed before meiosis can be dramatically reduced when abiotic stress coincides with meiosis and the first stage of gametogenesis. In self-fertilising cereals such as rice and wheat, cold and drought stress at the young microspore stage of pollen development have the highest impact on grain number as a result of pollen sterility (Fig. 2) [9, 10]. Conversely, the ovary is much more resilient to the effect of abiotic stress [10, 11].

Grain number can also be affected later when abiotic stress coincides with anther dehiscence (Fig. 1). Failure to release pollen from the anther locules prevents pollination in self-fertilizing species such as rice and wheat. Longer stress events that overlap with the young microspore and dehiscence stage are expected to show the cumulative effect of both stages. Therefore, sterility that has been attributed to stress at anthesis may actually have been caused at the young microspore stage of pollen development. Pollen viability in self-fertilising cereals is very short, further preventing the chance of cross-pollination. Flowering in rice and wheat is not synchronised and proceeds from the top of the panicle to the bottom in rice, or from the middle of the ear towards the top and bottom in wheat. This asynchrony in flowering provides limited protection to shorter stress events. The length and timing of the stress period can therefore determine the number of florets that abort. In contrast, the maize ovary was shown to be most sensitive to reproductive stage drought stress [12]. Maize is different from temperate cereals due to the physical separation of male and female flower structures and is therefore less dependent on self-pollination than self-pollinating cereals.
The stage-specificity (timing), combined with the length and severity of a stress event during reproductive development determines the severity of the reduction in grain number. It is unclear whether all these events that lead to reduction in grain number are under the same or different (stage-specific) genetic controls. Focusing our understanding on the most sensitive stage of reproductive development (young microspore stage of pollen development) may result in reducing the impact of abiotic stress and provide obvious advantages for germplasm and population screening and therefore for breeding. However, once a genetic control mechanism has been identified, its contribution to tolerance at other stages of reproductive development will need to be assessed, as well as its overall effect on yield when there is no stress.

2. Genetic improvement in tolerance to abiotic stress

2.1. How to screen for abiotic stress tolerance?
Screening for tolerance to abiotic stress under field conditions is notoriously difficult due to variability in severity, timing and duration of the stress. In the field, plants usually experience several stresses at the same time (e.g., nutrient stress, pathogens), resulting in screening for the cumulative effect of different stresses and the interaction between these stress responses [13]. In addition, variability within populations for such factors as flowering time, plant height, water use and rooting depth further confound results. This is a particular problem in mapping populations. Attempts have been made to control the timing and incidence of stress conditions (e.g., rainout shelters for drought, variation of sowing time for cold and heat stress, deep-water irrigation for cold treatments in rice, artificial heat and flooding treatments, etc.), but treatments are often longer than necessary to allow capture of a maximum number of plants going through a particular stage of development.
resulting in differential levels of stress applied to the population. Furthermore, these managed conditions do not overcome other variability in populations, particularly in flowering time, that may mask the critical variation and therefore the critical QTL identification.

For the identification of genomic regions responsible for grain number a solution is to develop a fast and effective screening method under managed conditions that focuses on a particular stage of reproductive development such as the most sensitive young microspore stage. To achieve this, it is necessary to move screening from the field to a controlled environment infrastructure and develop more accurate, stage-specific screening methods for germplasm discrimination. Screening approaches in controlled environments can exclude most of the variability that cannot be controlled under field conditions, but it can also introduce new challenges that need to be carefully considered in phenotyping approaches [14]. These screening methods may require specialised, expensive growth facilities that allow screening of larger numbers of plants (e.g., populations) under accurately controlled conditions (temperature, humidity, lighting). In addition, since screening includes the flowering stage, plants will need to spend their entire growth cycle in these facilities, as opposed to the shorter periods required for vegetative stage screenings. However, this may not need to be the case as plants can be moved at a critical growth stage into the managed stress condition and then after the stress moved back to the original conditions. Ultimately, field evaluation will remain essential to demonstrate the tolerance of selected lines and the value of the gene/QTL for stress tolerance. This is more easily done using near-isogenic lines (NIL) or populations differing in the trait, gene or QTL [15].
young microspore-stage specific screening methods for cold tolerance in rice and for drought-and shading-tolerance in wheat. As an easier alternative to drought stress for QTL mapping we are using osmotic stress [14, 16]. Applying osmotic stress using a hydroponics system makes it possible to apply the stress at the young microspore stage and to apply similar levels of stress to a large population of plants – something that is impossible to control using soil drought conditions.

2.2 QTL mapping and marker-assisted breeding

A major culprit for the lack of success in QTL mapping for reproductive stage abiotic stress tolerance has been the screening procedure [13] and the confounding factors we highlight above. A detailed account of the problems generally associated with QTL mapping for abiotic stress tolerance in crops has recently been published [13, 17]. QTL mapping experiments have often led to identification of multiple QTLs each with minor contributions to the stress-tolerance phenotype. It is not clear whether this is a direct consequence of the screening method (e.g., covering a multitude of developmental stages), the inherent complexity of the genetic control mechanisms or a combination of both.

When screening procedures have been optimised QTL mapping approaches have led to major breakthroughs in the case of submergence tolerance in rice [18] and salt tolerance in wheat [19], resulting in identification of candidate genes and ultimately a better understanding of the molecular and physiological basis of the tolerance mechanism. Once isolated, candidate genes can be used in transgenic approaches to improve reproductive stage abiotic stress tolerance in other crops or in other varieties of the same crop. Such breakthroughs have so far not been made for traits
during the reproductive stage for a variety of reasons. A major reason has been that the underlying determinants of grain number under abiotic stress are largely unknown. This results in the absence of an effective selection target other than final grain number. Another reason is that, for convenience, existing mapping populations developed for other traits are typically used. These are unlikely to be important for gene discovery. Instead, populations specifically developed from tolerant and sensitive parents are required.

2.3. Breeding priorities and choice of germplasm
For commercial reasons, breeding programs are inherently focused on factors such as high yield potential and grain quality and not on insurance against stress, which may or may not occur. While many landraces seemingly have better abiotic (and biotic) stress tolerance than commercial germplasm, they have too often been excluded from breeding programs because of their negative impact on yield and grain quality and because of the difficulties of trait selection. Landraces and wild ancestors have gradually started to find their way into breeding programs for essential traits such as cold tolerance in temperate climate rice [20, 21], and synthetic wheat lines have become a valuable resource for abiotic stress tolerance traits [22]. Other approaches that may also lead to trait understanding and gene discovery may come from mutagenised populations [23] or from the study of model species such as Brachypodium [24], or from drought-tolerant species found in the indigenous flora – for example from dry regions. However, little is known about reproductive mechanisms under stress of model species or of the indigenous flora found in dry environments. Ideally, abiotic stress tolerance will need to be confirmed throughout the breeding process of new cereal varieties. But the lack of
knowledge of the most critical traits, the lack of reliable screening methods, the
inherent genetic complexity and the absence of closely-linked genetic markers
creates a stumbling block to take full advantage of new genetic resources. It will
also need to be established whether there are interactions between specific traits for
abiotic stress tolerance and other desirable traits. For instance, it is not known
whether the control of grain number under normal and stress conditions actually
involves the same genes or regulatory processes and whether expression of these
genes is affected by abiotic stress. High-yielding varieties are often selected under
close-to-ideal growing conditions. It is possible that this selection process has
affected the sensitivity or capacity to respond to abiotic stress. Addressing these
questions will unavoidably require a better molecular understanding of grain
number control in cereals.

2.4. Unravelling the genetic complexity of abiotic stress tolerance
The genetic complexity of reproductive stage abiotic stress tolerance may be
something that we need to accept and exploit to unravel the genetic basis of grain
number control in cereals. Provided that a focused and reliable screening method is
available, QTL mapping may be the method of choice to study the genetic and
molecular basis of abiotic stress tolerance. One of the few known genes that
regulate grain number in cereals was identified via QTL mapping: the rice Gnl gene
encodes the cytokinin catabolic enzyme cytokinin oxidase [25]. However, it remains
unknown whether Gnl expression is affected by abiotic stress and what other genes
are involved in relaying the cytokinin signal to the grain development pathway.
Mutagenesis is also a technique that could be considered: selection for maintenance
of grain number under stress conditions in a mutagenised stress-sensitive population
could be used as phenotype to identify mutants (or revertants, in case abiotic stress sensitivity is due to a loss-of-function mutation that has been inadvertently selected) and then identify the corresponding gene. The availability of genome sequences for most of the important cereals will greatly facilitate the identification of candidate genes and comparative genomics can be used to match the genomic sequences of different cereal species. A better understanding of the molecular and physiological basis of abiotic stress tolerance will allow targeting genes within QTL regions and subsequently using TILLING (Targeting Induced Local Lesions IN Genomes) [26] to identify and characterise the phenotype of natural or induced mutations in these candidate genes.

3. Abiotic stress and plant reproductive development

3.1. Pollen formation: the Achilles tendon of reproductive development
Abiotic stress has its biggest impact on grain number when it occurs during the early stage of pollen development. Drought stress has long been known to affect pollen development in wheat [27, 28]. An increased frequency of out-crossing is associated with poor grain set in drought-stressed wheat crops [3]. Male gametophyte sterility in wheat is induced even under moderate water stress conditions [29]. Both drought stress in wheat and cold stress in rice inflict their highest levels of sterility at the time the tetrads separate after meiosis when forming the uni-nucleate microspores [9, 10]. Similarly, greatest sensitivity to cold in sorghum occurred between the pollen mother cell stage and the leptotene stage of meiosis, yet female fertility was not affected [30]. Drought stress often occurs together with heat stress in the field and the regulatory system for both stresses may have co-evolved [31]. Heat stress in cereals affects both pollen dehiscence and
grain-filling [32, 33], but a stage of high sensitivity responsible for loss in grain number occurs before the ear or panicle emerges from the leaf sheath [34]. Low light conditions (shading) before anthesis also reduces grain number in wheat [35]. This problem is due to pollen sterility induced at the young microspore stage and again the ovary showed higher resilience to stress, as measured by their ability to be fertilised by pollen from unstressed plants [36]. Interestingly, in the absence of stress conditions, high nitrogen levels boost grain number but have an adverse effect on pollen fertility and abiotic stress tolerance such as cold in rice [37]. The high sensitivity of young microspore-stage pollen to a variety of abiotic stress provides opportunities for germplasm screening. Pollen fertility and sterility are also distinct phenotypes that provide an entry point to determine the molecular basis of the problem.

3.2. Why the male gametophyte?
The higher sensitivity of male gametophyte development to abiotic stress may have something to do with the unique properties of the innermost layer of the anther wall, the tapetum. The tapetum in anthers is to some extent similar to the placenta in mammals; it is a highly specialised sporophytic secretion cell layer that is dedicated to feeding the nascent microspores. After fulfilling this function the tapetum undergoes a natural, pre-programmed cell death response and its cellular content is consumed by the young microspores (Fig. 3). This nursing function of the tapetum takes extreme proportions in plants with an amoeboid tapetum, where the microspores are completely engulfed by the tapetum layer after meiosis [38, 39] The tapetum deposits the intine and exine layers of the pollen cell wall and it secretes the locule fluid which contains nutrients for pollen development [40, 41]. During
meiosis and at the young microspore stage the tapetum is metabolically extremely active and the anther is the floral organ with the highest sink strength [42]. To satisfy the high energy requirements, the number of mitochondria per tapetum cell is increased 20-40-fold [43]. The exact timing of the cell death response in the tapetum is also critical for pollen viability. Both cold and drought stress have been shown to cause a pre-mature cell death response in the tapetum [44, 45], suggesting that abiotic stress interferes with the important functions of the tapetum. In tropical grasses such as maize, the nucellus is supported by the pedicel. The placento-chalaza cell layer separates the nucellus from the pedicel. Under drought conditions, the placento-chalazal cells undergo a senescence response, thereby restricting sugar flow to the nucellus [46]. This situation is reminiscent of the tapetum and may explain why the maize ovary is highly sensitive to drought stress.

3.3. Tolerant germplasm is available
The identification of tolerant and sensitive germplasm for reproductive stage stress tolerance should allow the introduction of the trait into commercial germplasm. Comparing the response of tolerant and sensitive germplasm will also enable us to identify the molecular basis of the tolerance mechanism. A screening method based on the high sensitivity of young microspore-stage pollen development was used to identify cold-tolerant rice germplasm in a collection of rice varieties from the Yunnan province in China, where rice is traditionally grown at high altitudes (2,000-2,400m) [9]. A high altitude sorghum line also had a strong tolerance at the young microspore stage [47]. A wheat line derived from a CIMMYT synthetic wheat was shown to have strong young microspore-stage drought tolerance [10]. These examples show that abiotic stress tolerance is present in cereals where the trait may have been maintained out of necessity, often imposed by topographic
circumstances (e.g., high altitude rice for cold-tolerance). This observation raises the question whether tolerance mechanisms may have been inadvertently lost from current commercial germplasm, either through direct/indirect selection or due to negative selection drag while selecting for other traits. For example, in wheat germplasm from CIMMYT which has been selected under irrigated conditions has been very successfully adopted in semi-arid countries such as Australia. This may have resulted in the loss of tolerance in current Australian wheat cultivars [48]. Genes responsible for maintenance of grain number under drought may also have been lost due to selection pressure in breeding programs for grain size and hectoliter weight which is demanded by industry. If there is a terminal drought then genotypes that are conservative in number of grains they set may be selected as these are also likely to have larger grains at maturity.

3.4. Anther sink strength and abiotic stress tolerance
The investigation of the effects of cold and drought stress on the young microspore stage of pollen development revealed that there is a major difference in the control of sink strength between tolerant and sensitive germplasm in both rice and wheat [9, 10]. Plasmodesmata between the tapetum and the outer cell layers of the anther wall disappear at meiosis, and callose deposition isolates the meiocytes from the rest of the anther. Uptake of sugars into the tapetum therefore has to occur via the apoplast, requiring an active uploading process involving cell wall invertase and monosaccharide transporters [49, 50]. While sensitive germplasm has reduced tapetum cell wall invertase levels under cold and drought, tolerant germplasm maintains sink strength [51, 9, 10]. Subsequently, a premature cell death response is observed in the tapetum of sensitive varieties, leading to abortion of pollen development [44, 45]. Depletion of sucrose and starch was also observed in tomato
and capsicum pollen following heat stress [52, 53] and heat stress affects cell wall invertase activity in sorghum pollen [54, 55]. A critical difference between tolerant and sensitive germplasm is the capacity to control and maintain sink strength and sugar supply to sustain pollen development. The regulatory pathway that controls anther sink strength and cell wall invertase activity remains unknown. A second peak in sink strength occurs during flowering and grain filling and cell wall invertase is known to be equally important in this step [56, 57]. In drought-stressed maize ovaries, cell wall invertase activity is repressed in the placento-chalazal cell layer [46]. The mechanism of ovary abortion under drought conditions in maize may be similar to cold- and drought-induced pollen abortion in rice and wheat. However, drought-tolerant wheat germplasm that is able to maintain sink strength when stressed at the young microspore-stage fails to maintain sink strength when stressed during anthesis and grain-filling [10]. This indicates that the genetic control of sink strength is different during pollen and grain development.

3.5. Male sterility and pollen development
Cytoplasmic male sterility (CMS) in plants is a gain-of-function mutation caused by complex rearrangements in essential mitochondrial genes. These mitochondrial dysfunctions only affect the anthers and not the other plant parts. Some of the CMS mutations are known to disturb mitochondrial metabolism in the tapetum of anthers. This may result in poor respiratory performance and an inability of the mitochondria to meet energy requirements for successful gametogenesis [58, 59]. The result is pre-mature tapetal cell death and abortion of pollen development. The mitochondria play an important role in programmed cell death and CMS has been important in demonstrating the role of plant mitochondria in CMS [59]. Although CMS and
abiotic stress at the young microspore stage in anthers both trigger an accelerated programmed cell death response in the tapetum, the cell death response may be a convergence point of two totally different upstream triggering pathways. CMS has been exploited in plant heterosis breeding for the production of hybrid seed. An alternative method that is simpler to reverse is the use of gametocides or chemical hybridisation agents (CHA) [60, 61]. A wide variety of chemicals have been used to target male fertility at various stages of anther development. Although some of these components have been shown to interfere with pollen meiosis and the early stages of microspore development, in most cases it is not clear what their cellular targets are. It is however possible that some chemical agents, through interference with tapetal function, have a similar result to the effect of abiotic stress on pollen development. Many transgenic approaches that affect metabolism and cellular function in anthers and particularly the tapetum have resulted in the induction of male sterility. These approaches range from the introduction of a bacterial ribonuclease gene [62] to the manipulation of genes involved in callose deposition during meiosis [63] and inhibition of respiratory enzymes [64]. All these examples further illustrate the vulnerability of male gametophyte development and the important role the tapetum plays in controlling pollen fertility.

4. Is it all in the hormones?

4.1. ABA: the stress hormone

ABA was a prime suspect as a regulator of sink strength. In the case of cold- and drought-induced sterility in rice and wheat ABA represses cell wall invertase expression and cold and drought-tolerant germplasm does not accumulate ABA [65, 16]. ABA plays a role in male sterility in tomato [66] and may also be involved in
response to heat stress [67]. Reduction in anther ABA levels during chilling of transgenic rice over-expressing the ABA catabolic gene ABA 8’-hydroxylase resulted in maintenance of sink strength and improved cold tolerance, suggesting that regulation of ABA catabolism in anthers is important for regulating ABA homeostasis [16]. ABA homeostasis in anthers is different in tolerant and sensitive germplasm, but it remains unknown how ABA homeostasis is regulated. Cold and drought tolerant rice and wheat lines were more sensitive to ABA treatment of the reproductive structures than sensitive lines [65, 16]. Selection of ABA sensitivity mutants was used to identify drought-tolerant wheat [68] and QTLs for ABA sensitivity have been identified in wheat with the intention to improve ABA-mediated abiotic stress responses [69]. In maize, a QTL that affects root traits and leaf ABA concentration was shown to affect grain yield [70]. However, using ABA sensitivity as an indirect selection method for abiotic stress tolerance may have secondary effects, as ABA plays a role in many important aspects of plant development (seed dormancy, regulation of stomatal closure). Although increased ABA levels are negatively correlated with abiotic stress tolerance at the reproductive stage, the opposite appears to be true at the vegetative stage. There is plenty of evidence that ABA treatment at the vegetative stage can improve acclimation to a variety of abiotic stress conditions [71-74]. The cold and drought-tolerant lines we are currently investigating in rice and wheat are not noticeably disadvantaged at the vegetative stage. ABA has different roles in the vegetative and reproductive plant parts. Unlike the vegetative plant parts, the reproductive parts are sink tissues for sugars. Regulation of ABA homeostasis is likely to involve interaction with other plant hormones that regulate plant development (auxin,
cytokinins, gibberellic acid, ethylene …) and these interactions may function differently in the reproductive and vegetative tissues.

4.2. Importance of adaptation to abiotic stress in reproductive tissues.
While ABA in anthers plays a role in controlling sink strength and sugar supply to the young microspores, the response to abiotic stress also involves ABA-independent components. In the case of cold and drought stress, the ABA-independent response is regulated by the CBF/DREB1 family of transcription factors and is thought to activate the acclimation and adaptation response that allows longer term survival of abiotic stress conditions [75, 76, 77]. However, the fact that ABA treatment can improve adaptation to abiotic stress suggests that the two pathways are not strictly independent. Analyses of abiotic stress responses using various “-omics” technologies revealed that plants activate a variety of defence responses to protect the cellular machinery against damage [78]. These adaptations include synthesis of lipids to protect membranes, antifreeze proteins that protect against frost, osmotic components to maintain water status in the cell (e.g., sugars such trehalose and fructans), chaperone proteins that protect protein folding and a variety of other protective components (e.g., proline, polyamines) [78]. However, a dangerous side-effect of all abiotic stress conditions is the overproduction of reactive oxygen species (ROS), resulting in oxidative stress and irreversible damage to various cellular components. Plants activate an antioxidant scavenging mechanism such as the ascorbate-glutathione pathway to inactivate ROS [79, 80]. The oxidative stress response in particular shows that adaptation to various abiotic stress situations in plants is shared by different types of environmental injuries. Little is known about the adaptation to abiotic stress at the reproductive stage, but it is expected that a metabolically very active and vulnerable cell layer
such as the tapetum will benefit from some of these protection mechanisms against stress. Adaptation responses to abiotic stress have so far been studied in model plants or crop plants with unknown ranking in terms of stress tolerance/sensitivity. Our rice and wheat lines with known tolerance and sensitivity to cold and drought will allow investigating the quantitative and qualitative differences in the adaptation response between tolerant and sensitive germplasm.

4.3. A common regulatory pathway for abiotic stress tolerance?
The similarity that exists between the effects of abiotic stress on pollen development suggests that there may be an overlap in the regulatory pathways that control stress tolerance (Fig. 4). It has been shown that there is a great deal of overlap between the genes that are recruited by both cold and drought stress in plants [81] This is an important observation, as it may provide the basis for alternative screening methods for identifying tolerant germplasm. Tolerance genes that play a role in response pathways that overlap between different stresses are expected to provide protection against various stresses (Fig. 4). One of our drought-tolerant wheat lines (Halberd) is also tolerant to heat stress [10, 82] and cold-tolerant rice is also more tolerant to reproductive stage drought stress (Dolferus, unpublished result). In addition, our drought-tolerant germplasm is also shading-tolerant [36]. Alternative screening methods may therefore provide a powerful way to identify QTLs and genes for an abiotic stress tolerance mechanism that is shared between different stress responses.
5. Future directions of reproductive stage abiotic stress research

5.1. Use of alternative screening methods
Conventional breeding and selection has been very successful in steadily increasing crop yields, even without having knowledge of the underlying traits. However, breeding in stress prone environments is challenging and progress is slow. This is primarily due to the high degree of seasonal variability in temperature and rainfall, resulting in large genotype x environment interactions. In order to further improve abiotic stress tolerance in cereals it is necessary to increase our knowledge of the current bottlenecks that constrain yield [15]. In the case of the control of grain number we require a better understanding of cereal reproductive development, how it is affected by stress and what are the underlying mechanisms responsible for the variation in grain number. Reliable screening methods are a major bottleneck for improvement of reproductive stage stress tolerance. A better understanding of the effect of stress on reproductive development will lead to development of more reliable screening alternatives that are targeted to the very root of the problem. The similarity that exists between different abiotic stress conditions that cause pollen abortion may allow us to use alternative stress treatments. This may be particularly useful for difficult to control stress treatments such as drought. Osmotic stress treatments using hydroponics and polyethylene glycol as osmoticum have been used in the past to impose water stress conditions [14, 83]. However, it is also possible to replace drought stress with an easier to apply abiotic stress that has the same effect on pollen development (e.g., cold, shading). We have recently shown that drought-tolerant wheat germplasm is also tolerant to shading conditions [36], suggesting that we could replace drought stress treatment by a more simple shading treatment at the young microspore stage. The QTL mapping work that we are currently carrying out in wheat for osmotic stress and shading will serve as a test case to prove the validity
of this approach. Once the knowledge about the molecular and physiological basis of the problem has progressed to the stage where we can target specific components of the abiotic stress signalling pathway we may be able to design alternative screening methods based on chemical treatments (e.g., hormones or hormone inhibitors, signal transduction inhibitors…). Although alternative screening methods could initially facilitate the screening of large population for QTL mapping – and make this process more reliable – the ultimate test will remain the confirmation of selected material under field conditions. QTL mapping and marker identification will therefore require the input and close collaboration between various research disciplines.

5.2. Markers or transgenic approaches?
The identification of candidate genes is critical for improving our understanding of the molecular and physiological basis of abiotic stress tolerance. These genes, together with the knowledge of how they fit into the tolerance pathway will enable us to use transgenic approaches to improve abiotic stress tolerance in cereal crops. Transformation methods have been established for cereals but they may not work with all elite breeding lines. However, transgenic approaches that target abiotic stress tolerance will need to be carried out judiciously, considering the fact that many manipulations of genes that interfere with tapetum metabolism and function have resulted in male sterility (see above). The choice of promoter to drive expression of the transgene will also be critical. Constitutive promoters that express the transgene ectopically in all plant parts may lead to negative side-effects and compromise plant yield. This was illustrated using the CBF/DREB1 transcription factors that lead to improved vegetative cold and drought-tolerance, but also stunted
plant growth [84, 85]. CBF/DREB1 transcription factors are normally expressed only in the vascular parenchyma cells [86]; they are normally induced by stress conditions and there is obviously a penalty associated with expressing a stress adaptation response all the time and in all plant parts. An attempt to reduce anther ABA levels by expressing the ABA catabolic gene ABA 8’-hydroxylase using a strong tapetum-specific promoter has led to reduced anther ABA levels, improved sink strength and improved cold-tolerance in rice [16], suggesting that transgenic approaches are indeed feasible. The availability of candidate genes for stress tolerance in reproductive structures will enable us to design perfect markers that can be used for marker-assisted breeding.

5.3. The benefits of using a model system
The availability of a model plant system with a small, uncomplicated and sequenced genome like *Arabidopsis thaliana* has been critical in advancing our general knowledge about plants. Rice is generally considered as a model for cereal research, but *Brachypodium* has been proposed as a model for the Triticeae cereals because of its phylogenetic position between rice and wheat. The advantage of *Brachypodium* over rice is a smaller genome size (advantage for mutagenesis), shorter generation time, simpler growth requirements and easy/faster transformation [24]. *Brachypodium* is also the only wild grass with a sequenced genome, allowing us to assess the effects of domestication based on co-linearity with other cereal genomes [87]. Unfortunately for rice, a crop plant that was domesticated more than 10,000 years ago, we have the genome sequence of the two most commonly grown subspecies (indica and japonica) but not the blue-print sequence of the original wild rice genome (*Oryza rufipogon*). *Brachypodium* may naturally be more resilient to
abiotic stress compared to domesticated cereals, making this wild grass less suitable to study the effect of abiotic stress on grain production. The huge size and hexaploid nature of the wheat genome will always be an obstacle to make fast progress, even with an available genome sequence. But important genetic variability for reproductive drought tolerance has been identified in wheat [10]. Rice is still the most developed cereal research model and there are many tools to mine the available genome sequence. Rice is a diploid species with a relatively small genome and tools are available to mine the genome information. In addition, as mentioned earlier, rice offers advantages compared to other cereals for studying grain number control by abiotic stress (Fig. 2). The co-linearity that exists between the rice and wheat genome sequences will make it possible to identify wheat genes based on known genes or map positions in rice (synteny mapping), an approach which has already proven its merits [88, 89]. Using the combination of rice and wheat will be useful for map-based cloning and identification of candidate genes for reproductive stage abiotic stress tolerance. A laborious and time-consuming part of fine-mapping is the development of large populations to fine-map the QTL regions, especially the identification of polymorphic QTL-region-specific markers [90, 91]. This requires large targeted populations (e.g., NILs) to provide the genetic resolution required for fine-mapping QTLs to a mendelian factor. This approach can also benefit from meta-analysis to improve resolution of QTLs [92]. The availability of SNP (single nucleotide polymorphism) markers in recent years has facilitated this work. Although SNP markers are available for both rice and wheat, the rice SNP platform is particularly well developed and can provide resolution to the gene level [93, 94]. Knowledge about the molecular and physiological basis of reproductive stage abiotic stress tolerance will also help to identify potential candidate genes within
QTL regions and design gene- or allele-specific markers for these genes based on the rice genome annotation and SNP information. TILLING [26] can then be used as a reverse genetic approach to identify mutants in candidate genes and study the effect of these mutations on abiotic stress tolerance. TILLING is a technique that has been shown to work well in both rice and wheat [95, 96].

5.4. Can we fix it?
Breeding and selection over decades have dramatically increased crop yield and grain marketability of all cereals in both favorable and unfavorable conditions. Increases in yield have always been associated with an increase in grain number whereas grain size is generally stable. Most of the increase in grain number under favorable conditions has been associated with an increase in partitioning of carbon to the developing reproductive structures just before flowering. This additional carbohydrate has probably come from the reduction in stem growth in semi-dwarf cereals [97]. Corresponding flow-on effects of yield increases to less favorable conditions where abiotic stress is not severe has occurred. This knowledge has resulted in breeders being able to target specific genes associated with semi-dwarf stature in wheat and rice and it has resulted in a greater grain number and yield. However, knowledge of the causes of a reduction in grain number in the presence of abiotic stress such as drought, low and high temperature are still rudimentary and further research is required to better understand the fundamental processes responsible for sterility. Also, it is important to understand whether these processes are common across different abiotic stress conditions and to understand the hormonal and metabolic changes responsible. With this knowledge better screening methods will be developed, improved parental material discovered and ultimately
genomic regions and the key genes involved will be identified. This in turn will lead
to molecular markers and fast and efficient screening methods for use by breeders in
their target environments. It will also help answer questions about the specific role
of hormones in the determination of grain number in the presence of stress, whether
there are common genes for tolerance to different stresses and whether intensive
breeding this century may have resulted in a loss of important genes for tolerance to
abiotic stress. An understanding will also be achieved about possible trade-offs
between grain number and grain size. For example, some stresses that reduce grain
size and where water is not a limitation, there is unlikely to be a trade-off in grain
number. However, where drought reduces fertility at the young microspore stage
and drought is not relieved then genes that maintain fertility are likely to be
undesirable as more grains will be produced that are small in size and therefore
unacceptable to markets. Crop simulation models using historic weather data for a
region can be useful here to assess the importance of traits and possible trade-offs
[98].

Our understanding of the control of grain number in cereals that experience abiotic
stress has increased in recent times. The most vulnerable period during meiosis is
common in such diverse species as rice grown in the tropics and wheat grown in
more temperate environments. Mechanisms of protection for different stresses, be
they cold or drought or heat, also appear to be similar. Recent advances in the
development of fast, efficient screening methods and the identification of highly
sensitive and tolerant germplasm within species are expected to lead to important
new discoveries in future years. It is also expected that these advances will also shed
light on the control of grain number under favorable conditions, which in turn could lead to genetic advances in yield potential.

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References


Figure legends:

Figure 1: Schematic overview of the reproductive cycle in cereals and the effect of abiotic stress on different stages of reproductive development.

Figure 2: Abiotic stress and grain number control in cereals.
A. Grain number is strongly affected by abiotic stress at the young microspore-stage. The figure shows the effect at the young microspore stage of cold (12°C, 4 days) in rice (left) and drought stress (no water for 5 days) in wheat (right). Control and stress-treated inflorescences are shown on the left and right respectively.

B. Comparison of the inflorescence of rice and wheat. The rice panicle consists of primary and secondary branches (inset), each containing spikelets. The rice panicle has a determinate growth, with terminal spikelets at the end of each branch. The wheat ear has a much denser structure. Spikelets (inset, bottom right) are arranged on a central rachis (inset, top right). The spikelets have an indeterminate growth, with only 2-4 florets developing into a grain per spikelet.

C. Abortion of grain number in rice can be caused by premature abortion of spikelets before reaching meiosis. This is easy to discriminate from abortion caused at the young microspore stage.

Figure 3: Cross sections of rice anthers at the early stage of male gametogenesis.
The top picture shows an anther just before meiosis. The anther wall consists of four layers. From outside to inside, these layers are the epidermis (Ep), the endothecium (En), the middle layer (M) and the tapetum (T). In the centre are the meiocytes (Me) that will undergo meiosis and give rise to the microspores. The bottom picture shows an anther cross section at the late uni-nucleate stage. The middle layer has degenerated and young microspores are still attached to the tapetum layer (dark blue layer) which is now reduced in thickness and will disappear completely towards pollen maturity.
**Figure 4:** Effect of abiotic stress on pollen development.

Cold, drought and other abiotic stress conditions (heat, salinity, shading …) induce a number of responses (arrows) in reproductive organs such as anthers. Some of these responses are specific but others (indicated with asterisk) are shared by different stresses. The control of pollen fertility appears to be part of a stress response that is shared by different abiotic stress conditions: tolerance to drought stress can cross-protect against other stresses such as cold and shading conditions.
Effect of Abiotic Stresses:

Reduction Spikelet Initials

Pre-mature Floret Abortion

Pollen Abortion

Anther indehiscence

Grain Number ↓

Grain Size ↓

Figure 1
Figure 2
Figure 4