Journal of
Experimental
Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Non-destructive staging of barley reproductive development for molecular analysis based upon external morphology

José Fernández Gómez and Zoe A Wilson*

School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD, UK

* To whom correspondence should be addressed. E-mail: zoe.wilson@nottingham.ac.uk

Abstract

A prerequisite to study the molecular genetic pathways of pollen and anther development is an accurate staging system for reproductive development. However in barley, floret formation occurs mainly within the pseudostem, which makes the observation of floret development and access to the floret particularly difficult without dissecting the plant. Thus selecting stages for molecular analysis cannot be done non-destructively. A staging method has therefore been developed for barley in order to define the relationship between readily detectable growth points and reproductive development, to provide a clear key to enable accurate selection of reproductive material. Initial staging followed the traditional Zadoks decimal system, with minor adaptations to stages 31–34 and stage 37. The later stages, from 37 onward, were replaced by growth staging based upon the last flag elongation (LFE) and the position occupied by the spike within the pseudostem. Spike size could be readily predicted by using the staging system incorporating Zadoks stages 31–37, supplemented with substages and by LFE staging to improve accuracy. The different spike sizes, as well as the LFE stages, showed a clear relationship to events occurring within the anther, as confirmed by light microscopy of the anthers. The defined relationship between spike size and development to anther development now makes possible the accurate prediction of anther and pollen progression by external staging. This, therefore, provides a mechanism for non-destructive selection of material for analysis that is critical for the molecular characterization of genes in anther and pollen development.

Key words: anther, barley, growth staging, last flag elongation, pollen development, Zadoks stage.

Introduction

As plants grow, they pass through a series of developmental stages, and although these phenological stages are continuous and gradual, it is possible to recognize the distinct stage that an individual plant or crop is at on a particular date (Leather, 2010). Despite this, many scientists conducting high-throughput molecular biology research that depends upon detailed and precise knowledge of gene sequences and genomic positions still describe their experimental plants by age, rather than their growth stage (Simmons *et al.*, 2006; Ninkovic and Ahman, 2009). Implementing effective methods of characterizing plant growth are critical for stage selection of material for comparative expression studies, phenotypic and molecular comparisons, and trait analysis.

The characterization of anther and pollen development and the genes involved in the regulation of this process are

vital to provide mechanisms for selective breeding and hybrid production for increased yield. Much has been learnt from the study of these pollen regulatory pathways in *Arabidopsis*, with numerous genes characterized as essential for male fertility (e.g. *AtMS1*, *AtAMS*, *AtDYT1*, *AtMYB26*) (Scott *et al.*, 2004; Ma, 2005; Wilson and Zhang, 2009). In addition, orthologue genes have been identified in rice (Li *et al.*, 2006, 2011; Wilson and Zhang, 2009) and this knowledge is being extended to barley (J Fernández Gómez and Z Wilson, unpublished). However, in many cereals, this molecular characterization has been restricted by difficulties of material collection, principally due to the localized expression and tissue specificity of these genes and the fact that the early stages of inflorescence development are inaccessible. Barley floret development occurs mainly within the pseudostem

shoot, which is a false stem composed of concentric rolled or folded blades and sheaths that surround the growing point. This makes the observation of floret development and access to the floret particularly difficult without dissecting the plant. This in turn means that it is extremely difficult to accurately collect staged floral material by a quick visual check, since it is not possible to check floral progression without destruction of the floral tissues. There is, therefore, a need for a system that defines the relationship between clear and easily recognizable external stages of barley development and the events occurring within the anther.

Spike development is a critical point in plant development and reproduction (Fischer, 1985, 1993; Slafer *et al.*, 1990; Savin and Slafer, 1991; Abbate *et al.*, 1995; Demotes-Mainard and Jeuffroy, 2004). It occurs comparatively early in floral initiation (Slafer and Rawson, 1994), during a relatively short developmental window, starting about 20 days before spike emergence, which occurs in parallel with stem elongation (Kirby, 1988). Spike elongation has often been related to the development of reproductive organs in barley (Waddington *et al.*, 1983), linking the developmental stage of barley florets to the spike size.

There have been various proposals to subdivide the life cycle of cereals into periods and stages. Landes and Porter (1989) identified two principal ways to describe cereal development: one based on the 'exterior' morphology (Large, 1954; Zadoks *et al.*, 1974) (visible to the naked eye without dissection) and the other based on the 'interior' morphology of the shoot apical (Kirby and Appleyard, 1986). Internal scales have greater potential for precision; however, such methods have the disadvantage of requiring dissection and microscopy, which are time-consuming and destructive. Furthermore, for most purposes, external staging is sufficient, and if the characteristics used for identification are chosen so that classification can be visual or tactile (using fingertips), the classification of the tillers is less time-consuming and non-destructive.

Among the most frequently used external systems for generically staging cereal development is the decimal code proposed by Zadoks *et al.* (1974), which defines specific developmental stages that are easy to identify in the field. This system originally related to individual plants, or a main shoot with tillers, but can also be used for individual tillers. Modifications to this scale have been carried out to adapt it to particular grasses: for instance, Tottman and Broad (1987) redefined some of the descriptive phases for wheat, barley, and oats. In addition, Lancashire *et al.* (1991) presented a universal development scale (also called the BBCH scale), designed to be applicable to most agricultural crops and weeds, which was largely based upon Zadoks decimal code for cereals. Developmentally similar morphological stages of different crops were given the same stage identification in order to present a generalized framework within which more specific scales for individual crops could be constructed. To define this general code, Lancashire *et al.* (1991) made some modifications to the Zadoks scale, of which the major ones were associated with late maturity stages. Examples of this staging adaptation can be seen

in the recent literature: Gustavsson (2011) adapted the universal decimal code for grasses to the perennial forage grasses, introducing modifications that facilitated the identification of constant stages. This accurate staging scale was then utilized for important managerial decisions on cultivation.

Despite the different analysis strategies that have been described, there are currently no clear systems that accurately define barley anther and pollen development based upon non-destructive phenotypic analysis. This paper therefore presents a new external barley staging system to achieve this by predicting barley anther and pollen development by external visualization of plant growth. This staging system was used for spike size prediction, which was in turn used to predict anther and pollen developmental progression. Accurate prediction of anther and pollen development stages is essential for the study of genetic regulation of this process, for the characterization of genes involved in reproductive development, which exhibit spatial and temporal specificity, and for management decisions associated with crop maintenance.

The barley variety Optic was adopted as the model for this study since it is widely used in barley molecular analysis and also because of the availability of molecular genetic tools such as Optic Tilling populations (Caldwell *et al.*, 2004). These make this cultivar an ideal candidate for molecular and genetic research and make the application of the prediction model extremely useful for gene characterization. However, since this staging prediction analysis is based upon easily defined growth characteristics, this method can also be easily adapted to other varieties and other growth conditions.

Materials and methods

Plant material

Two double-rowed spring barley, Optic, was grown under controlled conditions of 16/8 h 15/12 °C light/dark cycle (80% relative humidity, 500 μmol m^{-2} s^{-1} metal halide lamps supplemented with tungsten bulbs). Seeds were sown in 36-well pots using John Innes No. 3 compost. After 2–3 weeks (Zadoks stage 11–12: one shoot with one or two unfolded leaves), plants were transferred to 5-l pots containing Levington C2 compost (four plants each).

Barley morphological development was studied from the onset of the elongation stages to heading in order to gain a better knowledge of barley external development and establish clear stages corresponding to reproductive development. Initially, the Zadoks decimal code was used to identify the key developmental points. Spike appearance and size was measured from the base of the most basal floret to the tip of the most apical floret. Anther development and the floral score within a particular spike size was determined following the scale of Waddington *et al.* (1983). The material used for data collection was restricted to shoots that were already within the elongation stage when the main shoot had finished its elongation phase. The florets analysed were always those in central positions within the spike.

For Zadoks stages 30–55, 15–58 shoots per stage (the number depending on the availability/transient nature of certain stages) were split open and spike sizes were measured in order to establish the relationship between the barley external stages and spike size.

Histological analysis of anther development

Florets from the middle zone of the spike (from three to five per stage) were collected from different-sized spikes and fixed in resin in order to study the anther and pollen development from the beginning of the elongation phase to heading. Floral buds from different spike sizes and stages were fixed overnight at 4 °C in 4% (v/v) paraformaldehyde. Tissues were washed twice (30 min each) with $1 \times$ phosphate-buffered saline. Fixed panicles were immediately dehydrated with ethanol prior to embedding, using a mixture of ethanol/resin (Technovit 710; Heraeus Kulzer, Wehrheim, Germany) at increasing proportions of resin (2:1, 1:1, and 1:2) for 1 h, finishing with 100% resin. Sections were stained with 0.05% (w/v) toluidine blue prior to imaging. To detect nuclei, dissected anthers were treated with 0.05% (w/v) DAPI and imaged under UV illumination.

Results

Development of barley staging scale

Barley (Optic) was grown under controlled environmental conditions to determine the link between reproductive development and vegetative growth. Initially the Zadoks scale (Zadoks *et al.*, 1974), which utilizes external features for staging of barley growth, was used for material collection. During Zadoks stages 30–55, three main developmental phases can be seen: elongation, booting, and heading. Barley development was studied in detail over these stages in order to establish constant and easily recognizable points that could be used for accurately staging growth and development. However, the Zadoks scale, although providing a basic model for stage identification, did not provide sufficient sensitivity to enable accurate external identification of the key stages associated with reproductive development. Therefore, some of the later Zadoks stages were substituted by new and easily identifiable points based upon barley vegetative growth characteristics.

Developmental stages during the early growth stages, from the onset of the elongation phase to Zadoks stage 34, were clearly defined by the appearance of nodes, following Tottman's node-counting system (Tottman and Broad, 1987), and their elongation. These stages, from 31 to 34, followed consecutively without overlapping and were easily recognizable (Table 1 and Fig. 1). However, the last flag emerged completely following stage 34, often not giving sufficient time for nodes 5 and 6 to elongate (Fig. 1). Stages 35 and 36 were frequently absent, with development proceeding directly to last flag emergence (stage 37).

Accurate characterization of barley growth stages beyond stage 37 proved to be very difficult using the Zadoks system since many stages overlapped, which made it difficult to establish continuous sample collection points. For instance, using the Zadoks system, stage 37 was followed by stage 39 (the stage at which point the flag leaf ligule is visible); however, plants normally reached booting stage without showing this ligule. In addition, at booting stages, the first awns, which were proposed as being visible at stage 49, occurred as early as stages 41–43, when booting was still not obvious. This method was extremely complicated and a sequential staging method for barley was needed.

The last flag elongation (LFE) scale was created as an alternative approach to Zadoks stages 39–53. Each of the four new LFE stages was identified by a combination of novel features based upon developmental observations, such as last flag sheath elongation and the spike position within the pseudostem (Table 1 and Fig. 1). LFE1 followed stage 37, with the last flag sheath elongating 0.5–5 cm. The tip of the inflorescence was palpable above the leaf blade base of the second leaf below the flag leaf. At LFE2, the last flag sheath elongated 5–10 cm and was slightly swollen. The tip of the inflorescence had grown into the flag leaf sheath, leaving the rest remaining within the previous sheath. By LFE3, the last flag sheath elongated >10 cm and the boot was visibly swollen, the spike was between the previous sheath and the last flag sheath. During LFE4, the spike within the last flag sheath started to emerge.

Spike, anther, and pistil development during the last flag elongation stages indicated the consistency of these new growth features as developmental staging points (Fig. 2). From stages LFE1 to LFE4, spike appearance changed

Table 1. Barley external development stages

Stages 31–37 follow Zadoks decimal code (Zadoks *et al.*, 1974). The later stages have been replaced by the last flag elongation (LFE) stages to provide a clear system for developmental staging linked to reproductive development. See also Fig. 1.

Stage	Characteristics
31	One node detectable.
32	Two nodes detectable.
33	Three nodes detectable.
34	Four nodes detectable.
35	Fifth node detectable. This stage was normally absent as last flag emerged completely before the fifth node was detectable. Stage 36 was never observed before stage 37 appears.
37	Last flag completely emerged, still rolled. This stage followed directly from stage 34, when stages 35 and 36 were not present.
LFE1	Flag leaf emerged completely and unrolling, ligule may be visible; last flag sheath extended 0.5–5 cm.
LFE2	Last flag sheath extended 5–10 cm. Boot swelling obvious. Awns may be visible. Spike still inside the sheath, rachis has not started elongating.
LFE3	Flag leaf opening and awns clearly visible. Last flag sheath extended over 10 cm. Rachis starts elongation, moving the spike upwards towards the last flag sheath.
LFE4	Spike has completed its upward movement and was entirely localized within the last flag sheath. Heading is imminent.
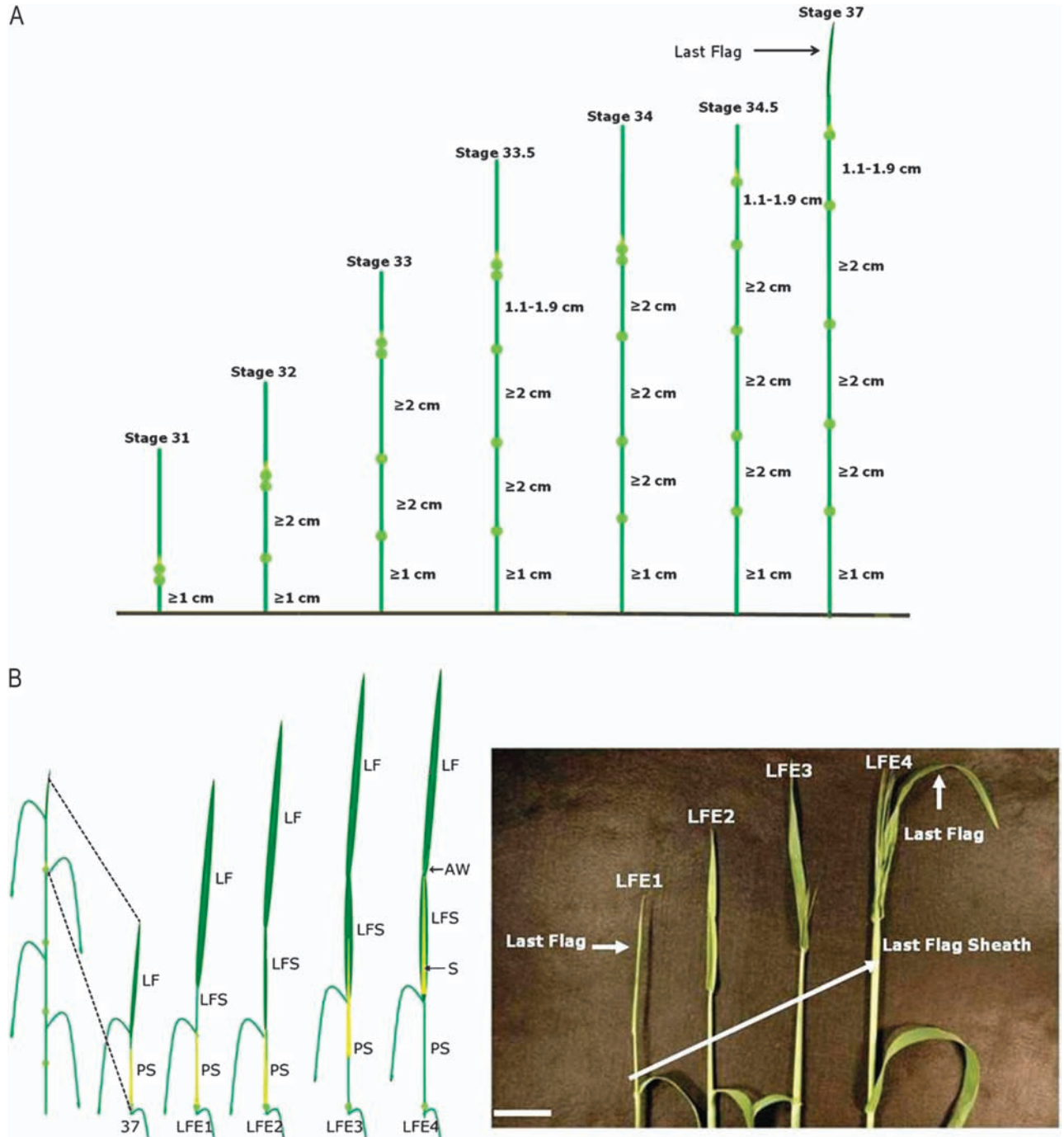
Fig. 1. Barley external development. (A) Staging based upon Zadoks stages 31–37 (Zadoks *et al*., 1974). Substages 33.5 and 34.5 were introduced to facilitate the prediction of spike sizes. These substages were based upon the elongation of the internode above the third and the fourth nodes, respectively and were identified when the internodes had elongated 1.1–1.9 cm. (B) Last flag elongation scale (LFE1–4). LFE stages are based upon the elongation of the last flag sheath in combination with the spike position during these stages, which moves upward until heading occurs (Table 1). LF, Last flag; LFS, last flag sheath; PS, previous sheath; AW, awns; S, spike. Bar, 4 cm. See also Table 1.

significantly, not only in size, but also in rigidity. At stage LFE1, spikes showed a feeble appearance and were unable to remain upright. During the subsequent developmental stages, spikes continued growing and gaining in resistance. By stage LFE3, spikes and awns presented an upright and compact conformation (Fig. 2). Increases in spike strength were accompanied by floret changes. Anther and pistil

development underwent significant changes, culminating in anthesis and fertilization. Anthers were green from stage 37 to stages LFE3–4, at which point they became dry and yellow and were about to release the pollen (Fig. 2). At stage LFE1, the pistil stigmatic branches were elongating and presented differentiating hairs on the ovary wall. From this point, the stigmatic branches and style continued

Fig. 2. (A) Barley spike development during the last flag elongation stages (LFE1–4). During these stages, spikes changed from a feeble appearance at stage LFE1, to a mature and upright one at stage LFE4. (B) Anther and pistils changed rapidly during the final stages. Anthers become dry and yellow and release pollen at stage LFE4. Stigmatic branches spread, reaching maximum (w.10 pistil development scale; Waddington *et al.*, 1983) at about stage LFE4. Bars, 2 cm (A), 1 mm (B).

elongating (w.8.5 pistil development scale; Waddington *et al.*, 1983). Spreading of the stigmatic branches occurred around LFE4, indicating that they were ready for pollination, (w.10; Fig. 2).

Spike size prediction

During the stem elongation phase, the spike started a rapid growth period that commenced around stage 31 and lasted until approximately stage LFE3 (Fig. 3 and Table 2). Spike sizes measured at different stages of barley external development confirmed a constant spike growth pattern from stages 30 to LFE2 (0.5 ± 0.18 cm to 9.38 ± 1.27 cm), reaching the maximal growth rate between stages LFE1 and LFE2. Finally, from stages LFE2 to LFE3, the variation in spike size was very small until it reached its maximum size (Fig. 3).

To reduce the overlap in spike sizes observed at certain stages and gain more accuracy in spike size prediction, substages 33.5 and 34.5 were introduced between stages 33 and 34 and between stages 34 and 37. These substages were identified by the partial elongation of the internodes above the third and the fourth nodes (Fig. 1). Substage 33.5 was constant and easily recognizable after stage 33 due to the partial elongation of the fourth internode (1–1.9 cm; Fig. 1). However, stage 35 was not always detectable. Although the fifth node was frequently detected at stage 34, the fifth internode rarely elongated before the emergence of the last flag. Thus, stage 35 was rarely achieved before the emergence of the last flag leaf (stage 37). Therefore, substage 34.5 was only detected when the fifth internode extended, but did not reach the 2 cm required to be defined as stage 35 (Tottman and Broad, 1987). Only when stage 35 was observed were spike sizes at this stage considered as the substage between 34 and 37 (Fig. 1); thus facilitating a more accurate spike size prediction between these stages.

The results showed that spike sizes varied between stages 33 (2.46 ± 0.97) and 33.5 (3.14 ± 0.72). Substage 34.5 (4.77 ± 0.889) allowed a more accurate spike size prediction between stages 34 (3.90 ± 1.15) and 37 (6.11 ± 1.12). Therefore, stages up until 37 can be used to predict spike size; however, beyond this point the developmental progress does not directly correlate with spike growth and these stages cannot be used for spike size prediction.
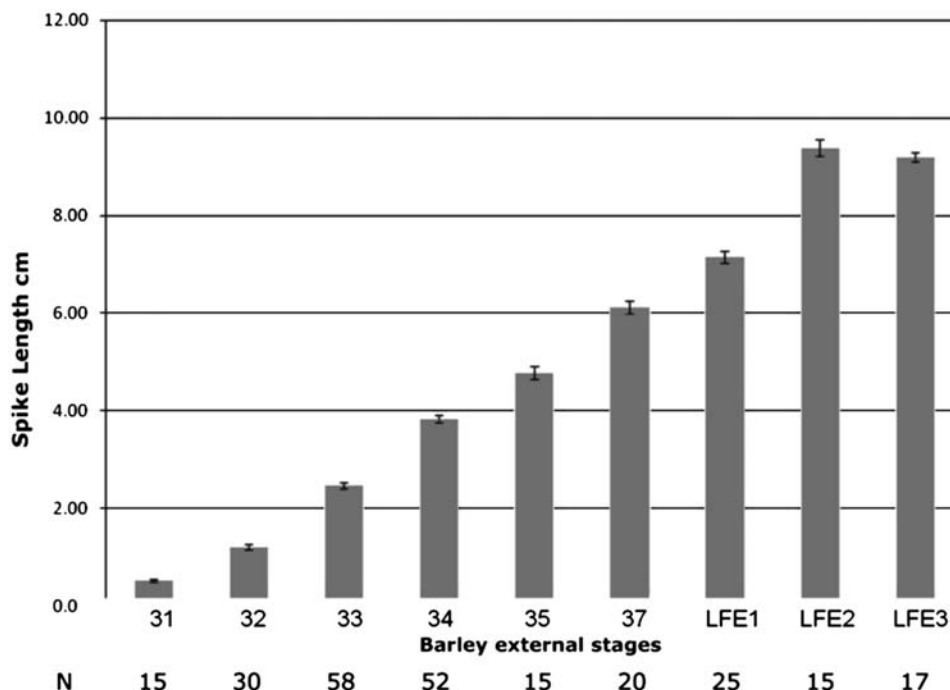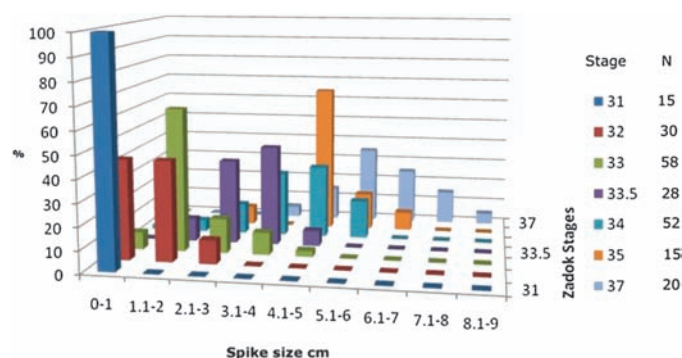
Fig. 3. Spike sizes in relation to barley external stages. Spikes showed a constant growth pattern from stages 31 to LFE1–2 and reached maximum size at LFE2–3. See also Table 2.

Table 2. Spike sizes in relation to barley external stages

Stages 31–37 follow Zadoks decimal code (Zadoks *et al.*, 1974). The later stages have been replaced by the last flag elongation (LFE) stages to provide a clear system for developmental staging linked to reproductive development. See also Fig. 3.

Stage	Spike size (cm)	Number of samples
31	0.52 ± 0.18	15
32	1.17 ± 0.54	30
33	2.46 ± 0.97	58
33.5	3.14 ± 0.72	28
34	3.90 ± 1.15	52
34.5 (35)	4.77 ± 0.89	15
37	6.11 ± 1.12	20
LFE1	7.14 ± 1.29	25
LFE2	9.38 ± 1.27	15
LFE3	9.19 ± 0.77	17

Fig. 4. Histogram showing the spike size prediction in relation to barley external development from stages 31–37 (substages 33.5 and 34.5 included). Vertical columns show the probability of finding a specific spike size within any stage.

Fig. 4 shows the final stages and substages used for spike size prediction and the probability of finding the expected spike size. Vertical columns represent the probability of finding specific spike sizes within any stage. Spike sizes followed a clear pattern where bigger spikes were found, as plants got closer to stage 37. For instance, the probability of finding spikes 0–2 cm was higher in stages 31–33, however, spikes 0–1 cm were more likely in stage 31–32, whereas spikes 1–2 cm were more likely in stages 31–33. Moreover, spikes 2–4 cm were observed more often in stages 33.5 and 34, and spikes 4–6 cm in stages 34–37 (Fig. 4). Accuracy was gained by using the substages that helped to provide a higher degree of differentiation between the closely related stages. In substage 33.5, spike collection of 2–4 cm was

more likely than 3–4 cm. Spikes 3–5 cm were found between stages 33 and 35; however, spikes 3–4 cm and 4–5 cm were more likely between stages 33.5–34 and 34–35, respectively. Finally, in stage 37, the last stage used for spike size prediction, spikes 5–7 cm were frequently found, although some spikes 4–5 cm long were also observed.

Anther and pollen staging

Prediction of the timing of barley anther and pollen developmental events based upon external plant development was the target of this study. A relationship between external staging and the spike size was observed at specific stages during barley development (Table 1). Staging points were established in order to study key changes within the

anthers alongside spike size and the final stages of barley development. From stages 31 to 37, spike samples were collected at different sizes, in intervals of 1 cm, whereas from stage LFE1, last flag elongation stages were used for floret collection and light microscopy (Table 3).

Fig. 5 shows a series of transverse sections through barley anthers at different spike sizes and stages taken from the central region of spikes, from spike 0.6 cm to stage LFE4. Anther and pollen development is correlated to spike size (0.6–5.7 cm, stages 31–37) and also to the last flag elongation stages (Table 3).

Spikes 0–1 cm showed sporogenous cells and three anther cell layers (Fig. 5 and Table 3). Four cell layers were seen when the spikes were 1–2 cm (Fig. 5). Spikes 2–3 cm showed

entry of pollen mother cells into meiosis and by 3–4 cm microspore release had occurred. Spikes 4–5 cm had released microspores and the middle layer was being crushed. Tapetum degeneration was evident in spikes 5–6 cm, at which stage, microspores become vacuolated. From stage LFE1, anthers and pollen development entered into the final stages before anthesis. Tapetum degeneration finished and microspores entered into two mitosis cycles (mitosis I, LFE1–2; mitosis II, LFE3), which concluded at stage LFE4 with tri-nucleated pollen and anthesis taking place (Table 3 and Fig. 5). The developmental progression observed through these late stages was reinforced by changes in stomium development. Stomium area increased in size and the cells got thinner as anthers approached dehiscence, finally breaking and releasing the

Table 3. Anther and pollen development in relation to spike size and the last flag elongation stage

The later stages of Zadoks decimal code (Zadoks *et al.*, 1974) have been replaced by the last flag elongation (LFE) stages to provide a clear system for developmental staging linked to reproductive development. See also Fig. 5.

| Spike range (cm)	Anther and pollen development stage
0–1	Primary sporogenous cells. Three cell layers surrounding the anther locule.
1–2	Secondary sporogenous cells to pollen mother cells. Four layers surrounding the anther locule: epidermis, endothecium, middle layer, and tapetum.
2–3	Pollen mother cells undergo meiosis. Tapetum layer is prominent.
3–4	Microspores released from the tetrad. Tapetum vacuolated.
4–5	Free microspores. Middle layer undergoes crushing. The prominent tapetum layer starts to degenerate.
5–6	Microspores become vacuolated. Tapetum degenerating.
LFE1	Mitosis I. Tapetum degenerating, but still present.
LFE2	
LFE3	Binuclear pollen. Mitosis II occurs.
LFE4	Trinuclear pollen. Septum breakage.
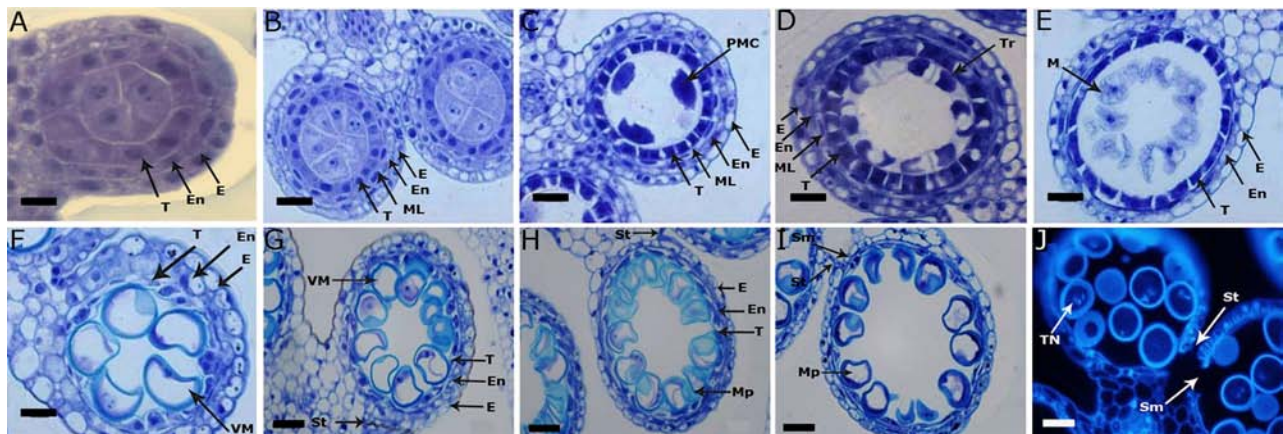
Fig. 5. Barley anther and pollen development relative to spike size: transverse sections of barley anthers from spike size 0.6–5.7 cm (A–F) and the last flag elongation stages (G–J). These samples were collected from within the spike ranges specified in Table 3. (A) Primary sporogenous cells; three layers surrounding the anther locule (spike 0.6 cm). (B) Secondary sporogenous cells to pollen mother cells; four cell layers surround the anther (spike 1.7 cm). (C) Pollen mother cells enter into meiosis; tapetum layer is prominent (spike 2.8 cm). (D) Microspores released from the tetrad; tapetum becomes vacuolated (spike 3.5 cm). (E) Free microspores; middle cell layer undergoes crushing. The prominent tapetum layer starts to degenerate (spike 4.9 cm). (F) Microspores become vacuolated; tapetum is degenerating (spike 5.7 cm). (G–H) Mitosis I; tapetum is degenerating, but still present. (I) Binuclear pollen; mitosis II occurs. (J) Trinuclear pollen; septum breakage. Sections represented are examples of the mentioned ranges; A–I, toluidine blue; J, DAPI). BN, binuclear cells; E, epidermis; En, endothecium; ML, middle layer; Mp, microspores; N, nucleus; PMC, pollen mother cells; Sm, septum; St, stomium; T, tapetum; TN, trinuclear cells; Tr, tetrads; VM, vacuolated microspores. Bars, 40 μm (this figure is available in colour at *JXB* online).

pollen at stage LFE4 (Supplementary Fig. S1, available at *JXB* online).

The correlation between barley external development and spike size and LFE stages on one hand, combined with the relationship between spike size/LFE stages and anther and pollen development on the other, confirms the ability to predict anther and pollen development by observations of external development. In addition, the four developmental stages based on last flag leaf elongation provided greater clarification of the Zadoks scale description of barley external development. The ability to predict spike size and developmental stage indicates that this approach provides a reliable key vegetative feature which predicts corresponding anther and pollen development stages and, therefore, facilitates genetic and molecular studies.

Stage-specific analysis of anther gene expression

The non-destructive staging system was used to identify samples for expression analysis of anther- and pollen-specific genes. Expression of a variety of barley targets that are putative orthologues of characterized *Arabidopsis* pollen development genes were tested for expression in staged spike tissue samples. This allowed further validation of the identity of these putative orthologous sequences, but also in some instances facilitated cloning, since cDNA samples derived from all spike stages were not able to generate fragments by PCR using degenerate primers. In particular *HvMS1*, the *AtMS1* putative orthologue, has been characterized using this staging system (J Fernández Gómez and Z Wilson, unpublished). In *Arabidopsis* and rice this gene shows a highly localized expression in the tapetum from late tetrad stage to early microspore release (Yang *et al.*, 2007; Li *et al.*, 2011). Therefore expression analysis (reverse-transcription PCR or quantitative real-time PCR) or *in situ* hybridization and subsequent mutant analysis required very detail staging. Samples (from both Optic and Golden Promise plants) were collected using the prediction model and expression was seen only in spikes 2–3 cm and 3–4 cm, corresponding to pollen mother cell meiosis through to microspore release (Fig. 6).
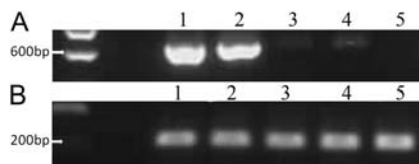
Fig. 6. Stage-specific reverse-transcription PCR of the *HvMS1* anther-related gene. (A) PCR using primers based on the rice *MS1* gene sequence (*PTC1*; Li *et al.*, 2011) and RNA extracted from different spike sizes from barley variety Golden Promise. (B) Alpha tubulin PCR normalization of samples. Lane 1, 2–3 cm; lane 2, 3–4 cm; lane 3, 4–5 cm; lane 4, 5–6 cm; lane 5, >6 cm. Expression of the putative *HvMS1* gene is clear in lanes 1 and 2; however, this expression is reduced or completely absent in lanes 3–5.

Other genes that are critical for *Arabidopsis* fertility, such as *AtAMS* and *AtMYB26* (Sorensen et al., 2003; Steiner-Lange et al., 2003), are also being characterized by this approach. These transcription factors show a highly localized expression; therefore, the accurate prediction of anther and pollen development is essential for material collection and analysis. Moreover, this staging method is being used to select putative orthologues for further study depending on their expression tissue/stage specificity, thus facilitating the amplification of these sequences for gene silencing, over-expression constructs, and further analysis.

Discussion

An easy method to accurately predict anther and pollen development in barley throughout external staging of the plant has been developed. This staging system is designed for the classification of individual tillers. However, as noted by Simon and Park (1983), there is great developmental variation between tillers in cross-pollinated grass species. Therefore, only tillers that followed immediately to the main stem and were already within the elongation stage when the main stem had reached the end of this phase were used in this study. Moreover, to minimize spike developmental variation within different tillers and plants, controlled growing conditions were adopted. These controlled conditions were essential to confer the accuracy required for molecular and genetic characterization of barley flower development, since it enables reproducible generation and analysis of material between laboratories worldwide. However, this study has also shown this method to be highly adaptable and used it for the staged collection of material from the barley variety Golden Promise.

A modification of the traditional Zadoks decimal code was used as a base to design a barley-specific external staging scale under specific growing conditions (Tottman and Broad, 1987; Lancashire *et al.*, 1991; Gustavsson, 2011). These external growing stages were essential in order to compare the external changes observed in plant development with the events taking place within the anthers. Barley external development was studied from stages 30 and 31, since at these stages, most of the barley plants were at the maximum number of primordia stage (Hay, 1986; Arduini *et al.*, 2010). From this point, spikes started a fast and constant developmental progression that ended in anther dehiscence. Spike development runs parallel to pseudostem development during the elongation stages (Kirby, 1988; Reynolds *et al.*, 2009). In addition, spike increases in size during this developmental window have been correlated with floret developmental scores (Waddington *et al.*, 1983).

A clear relationship between spike size and external stages could be seen (Fig. 3). Furthermore, the spike growth confirmed that spike elongation occurs in parallel to stem elongation (Kirby, 1988; Reynolds *et al.*, 2009), showing the same sinusoidal growth pattern reported by Reynolds *et al.* (2009). Spike growth lasted until approximately stage LFE2; from this point, no significant changes were observed

in relation to the spike size, and therefore, no developmental features could be attributed to spike size changes. The utility of the spike size prediction scale was therefore restricted to stages identified by the node and internode elongation, including stage 37 and the substages 33.5 and 34.5, which helped to increase accuracy in spike size prediction (Fig. 1 and Table 2). This prediction was sufficiently accurate to provide a key to enable the collection of the expected spike size by simple external observation (Fig. 4).

Cytological analysis (Fig. 5) confirmed the relationship between spike size and the events occurring within the anthers. This relationship allowed prediction of anther and pollen development based upon spike size by the external appearance of the plant. Moreover, LFE stages were also validated as predictors of anther and pollen development, since a clear sequence of stages were observed in samples LFE1–4. The transition from spike size prediction stages (31–37) and the last flag elongation stages (LFE1–4; Table 1) was observed between stages 37 and LFE1, where both anther samples showed vacuolated pollen and the start of tapetal degeneration (Fig. 5).

The prediction of anther and pollen development throughout the external visualization of the barley plant development is highly effective. This developmental prediction method has proved to be essential for the amplification and characterization of barley genes involved in anther and pollen development such as *HvMS1* (J Fernández Gómez and Z Wilson, unpublished), *HvAMS*, and *HvMYB26*. The amplification of unknown genes using primers designed using the putative equivalent gene is facilitated using staged cDNA templates. In addition, this approach has been applied to the molecular analysis of other barley varieties such as Golden Promise and also for the analysis of mutants and transgenic lines carrying RNAi constructs for targeted gene for silencing. Therefore, although the prediction model has not been fully characterized for other varieties, by making small modifications it can be effectively adapted to other varieties.

This system, therefore, provides a non-destructive method for selecting appropriately staged material for reproductive analyses, which is vital for gene cloning approaches, staged expression analyses, microscopy, and phenotyping.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Transverse sections through barley anthers at last flag elongation stages.

Acknowledgements

References

Abbate PE, Andrade FH, Culot JP. 1995. The effects of radiation and nitrogen on number of grains in wheat. *Journal of Agricultural Science* **124,** 351–360.

Arduini I, Ercoli L, Mariotti M, Mason A. 2010. Coordination between plant and apex development in *Hordeum vulgare* ssp. *distichum*. *Comptes Rendus Biologies* **333,** 454–460.

Caldwell DG, McCallum N, Shaw P, Muehlbauer GJ, Marshall DF, Waugh R. 2004. A structured mutant population for forward and reverse genetics in barley (*Hordeum vulgare* L.). *The Plant Journal* **40,** 143–150.

Demotes-Mainard S, Jeuffroy MH. 2004. Effects of nitrogen and radiation on dry matter and nitrogen accumulation in the spike of winter wheat. *Field Crops Research* **87,** 221–233.

Fischer RA. 1985. Number of kernels in wheat crops and the influence of solar radiation and temperature. *Journal of Agricultural Science* **105,** 447–461.

Fischer RA. 1993. Irrigated spring what and timing and amount of nitrogen fertilizer. II. Physiology of grain yield response. *Field Crops Research* **33,** 57–80.

Gustavsson AM. 2011. A developmental scale for perennial forage grasses based on the decimal code framework. *Grass and Forage Science* **66,** 93–108.

Hay RKH. 1986. Sowing date and the relationship between plant and apex development in winter cereals. *Field Crops Research* **14,** 321–337.

Kirby EJM. 1988. Analysis of leaf, stem and ear growth in wheat from terminal spikelet stage to anthesis. *Field Crops Research* **18,** 127–140.

Kirby EJM, Appleyard M. 1986. *Cereal Development Guide*. Stoneleigh, Warwickshire, UK: National Agricultural Centre, Arable Unit.

Lancashire PD, Bleiholder H, Van Den Boom T, Langeluddeke P, Stauss R, Weber E, Witzenberger A. 1991. A uniform decimal code for growth stages of crops and weeds. *Annals of Applied Biology* **119,** 561–601.

Landes A, Porter JR. 1989. Comparison of scales used for categorizing the development of wheat, barley, rye and oats. *Annals of Applied Biology* **115,** 343–360.

Large EG. 1954. Growth stages in cereals: illustration of the Feeke's scale. *Plant Pathology* **3,** 128–129.

Leather SR. 2010. Precise knowledge of plant growth stages enhances applied and pure research. *Annals of Applied Biology* **157,** 159–161.

Li H, Yuan Z, Vizcay-Barrena G, Yang C, Liang W, Zong J, Wilson ZA, Zhang D. 2011. PERSISTENT TAPETAL CELL1 encodes a PHD-finger protein that is required for tapetal cell death and pollen development in rice. *Plant Physiology* **156,** 615–630.

Li N, Zhang DS, Liu HS, et al. 2006. The rice tapetum degeneration retardation gene is required for tapetum degradation and anther development. *The Plant Cell* **18,** 2999–3014.

Ma H. 2005. Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. *Annual Review of Plant Biology* **56,** 393–434.

Ninkovic V, Ahman IM. 2009. Aphid acceptance of *Hordeum* genotypes is affected by plant volatile exposure and is correlated with aphid growth. *Euphytica* **169,** 177–185.

Reynolds M, Foulkes MJ, Slafer GA, Berry P, Parry MA, Snape JW, Angus WJ. 2009. Raising yield potential in wheat. *Journal of Experimental Botany* **60,** 1899–1918.

Savin R, Slafer GA. 1991. Shading effects on the yield of an Argentinean wheat cultivar. *Journal of Agricultural Science* **116,** 1–7.

Scott RJ, Spielman M, Dickinson HG. 2004. Stamen structure and function. *The Plant Cell* **16.** Suppl 1, S46–S60.

Simmons AT, Nicol HI, Gurr GM. 2006. Resistance of wild *Lycopersicon* species to the potato moth, *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae). *Australian Journal of Entomology* **45,** 81–86.

Simon U, Park BH. 1983. A descriptive scheme for stages of development in perennial forage grasses. In: JA Smith, VW Hayes, eds, *Proceedings of the XIV International Grassland Congress, Lexington, Kentucky, USA, 15–24 June 1981*. Boulder, CO: Westview Press, 416–418.

Slafer GA, Andrade FH, Satorre EH. 1990. Genetic-improvement effects on pre-anthesis physiological attributes related to wheat grain yield. *Field Crops Research* **23,** 255–263.

Slafer GA, Rawson HM. 1994. Sensitivity of wheat phasic development to major environmental factors: a re-examination of some assumptions made by physiologists and modellers. *Australian Journal of Plant Physiology* **21,** 393–426.

Sorensen AM, Krober S, Unte US, Huijser P, Dekker K, Saedler H. 2003. The *Arabidopsis* ABORTED MICROSPORES (AMS) gene encodes a MYC class transcription factor. *Plant J* **33,** 413–423.

Steiner-Lange S, Unte US, Eckstein L, Yang C, Wilson ZA, Schmelzer E, Dekker K, Saedler H. 2003. Disruption of *Arabidopsis thaliana* MYB26 results in male sterility due to non-dehiscent anthers. *The Plant Journal* **34,** 519–528.

Tottman DR, Broad H. 1987. The decimal code for growth stages of cereals, with illustration. *Annals of Applied Biology* **110,** 441–454.

Waddington SR, Cartwright PM, Wall PC. 1983. A quantitative scale of spike initial and pistil development in barley and wheat. *Annals of Botany* **51,** 119–130.

Wilson ZA, Zhang DB. 2009. From *Arabidopsis* to rice: pathways in pollen development. *Journal of Experimental Botany* **60,** 1479–1492.

Yang C, Vizcay-Barrena G, Conner K, Wilson ZA. 2007. MALE STERILITY1 is required for tapetal development and pollen wall biosynthesis. *The Plant Cell* **19,** 3530–3548.

Zadoks JC, Chang TT, Konzak CF. 1974. A decimal code for the growth stages of cereals. *Weed Research* **14,** 415–421.