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An alternative mechanism for cleistogamy in barley

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Abstract Cleistogamy in barley is genetically determined by the presence of the recessive allele *cly1*, but the dominant allele at the linked locus *Cly2* is epistatic over *cly1*. Although the molecular basis for *cly1* action is well understood, that of *Cly2* is not. Here we show that anther non-extrusion can occur not just when the lodicules fail to expand adequately (a trait which is fully determined by the allelic state at the *cly1* locus), but by the premature timing of anthesis before the spike has emerged from the boot. The transcription of *HvAP2* at *cly1* is unaffected by the timing of anthesis. Where this occurs prematurely, by the time that the spike has emerged from the boot, the lodicules have already become shrunken and have lost the capacity to push the lemma and palea apart. Premature anthesis appears to be governed by a dominant gene, probably *Cly2*. Of the three phases of development of a non-cleistogamous barley floret (spike emergence from the boot, floret gaping induced by lodicule expansion and anther extrusion), genetic variation is available regarding at least the former two.

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Introduction

In the typical grass flower, the swelling of the lodicules forces the florets to gape, thereby exposing the pistil to wind-borne pollen. Flowers which behave in this way are referred to as non-cleistogamous, while those which remain closed preventing anther extrusion until at least after anthesis are cleistogamous (Lord 1981). Cleistogamous barleys in which the palea and lemma remain tightly closed throughout anthesis have been described by Briggs (1978). The lodicule in a cleistogamous barley tends to be smaller than in a non-cleistogamous type (Honda et al. 2005; Nair et al. 2010), as also is the case in the rice mutant *SUPERWOMAN 1* (Yoshida et al. 2007). Non-cleistogamy is essential in the context of F_1 hybrid production (Abdel-Ghani et al. 2004), while cleistogamy provides an effective means of preventing pollen escape from transgenic plants (Abdel-Ghani et al. 2004; Daniell 2002; Ma and Wang 2004), as well as advantageous for tolerance to fungi that appear during anthesis (Dahleen et al. 2012; Hori et al. 2005, 2006).

Cleistogamy in barley is controlled by a single recessive gene *cly1* (Kurauchi et al. 1994; Turuspekov et al. 2004), which has proven to be an ortholog of the *Arabidopsis thaliana AP2* transcription factor (Nair et al. 2010). A single base change in the *HvAP2* sequence differentiates *cly1* carriers from the non-cleistogamous *Cly1* wild type. The result of this alteration is the loss of a miRNA172 target site, thus preventing its miRNA-mediated cleavage. In the non-cleistogamous type, the effect of normal mRNA cleavage is to reduce the abundance of HvAP2, a protein which acts to suppress lodicule swelling. Turuspekov et al. (2004) have, however, observed that in six cleistogamous × non-cleistogamous combinations, the F_1 plants were uniformly non-cleistogamous and their F_2 progeny segregated in the

ratio of three non-cleistogamous to one cleistogamous, whereas in the crosses, Misato Golden (MG) × Satsuki Nijo (SN) and Kanto Nakate Gold (KNG) × SN, the F_1 plants were cleistogamous and the F_2 progeny segregated in the ratio of one non-cleistogamous to three cleistogamous. Turuspekov et al. (2004) therefore proposed the existence of either two tightly linked loci or multiple alleles (*cly1* and *Cly2*) at a single locus, which were able to act epistatically on one another. The re-sequencing of *HvAP2* in a number of non-cleistogamous cultivars revealed that they all (with the single exception of cv. Morex) shared an identical coding sequence (Nair et al. 2010), raising the question why the hybrids Azumamugi (AZ) × KNG and SN × KNG differ from one another with respect to flowering type, while the SN × KNG and SN × MG F_1 phenotypes and F_2 segregation patterns are identical. To address this issue, we have here characterized the lodicule size and spike morphology of a number of critical segregants, re-sequenced the AZ, SN, KNG and MG *HvAP2* coding and regulatory regions, and derived the *HvAP2* transcription profile throughout the period of spike development. Anther extrusion was used as an indicator for flower gaping, since this trait has been used as a surrogate for resistance to FHB (Lu et al. 2012; Skinnes et al. 2010). The present study has identified that the timing of anthesis can affect the expression of cleistogamy. We demonstrate that both spike emergence from the boot prior to anthesis and lodicule development are essential prerequisites for anther extrusion, and hence for the development of non-cleistogamy in barley.

Materials and methods

Plant materials

Grain of the cultivars KNG, MG, SN and AZ was obtained from the National Institute of Crop Science, Tsukuba (accessions JP15436, JP15780, JP185755 and JP17209, respectively), while that of 'Golden Promise' (GP, JP15923) was obtained from the gene bank of National Institute of Agrobiological Sciences, Tsukuba (Table 1). RIL50 is an F_{13} line derived from the cross AZ × KNG (Mano et al. 2001). The segregating populations analyzed were RILs (F_{13}) of the cross AZ × KNG (96 plants), F_2 progeny of the crosses SN × KNG (90 plants), SN × MG (95 plants) and RIL50 × GP (62 plants). All the materials, including the parental cultivars and the various F_1 hybrids, were planted at Tsukuba 20 cm apart, in rows, with 80 cm between rows.

Assessment of lodicule size

Three spikes per individual were detached at the yellow anther stage (Kirby and Appleyard 1981), shortly before

Table 1 Variation for morphological traits related to non-cleistogamy in barley

Crosses	*HvAP2* alleles	Accession no.	Row type	Lodicule size (depth)			Spike emergence at anthesis			Anther extrusion	
				Yellow anther stage (mm)	Anthesis stage (mm)	Response to 2,4–D	Emergence distance (cm)	Spike length (cm)	Emergence ratio[a]	Spikes/plant (%)[b]	Turuspekov (2005)[c]
AZ	*Cly1.a*	JP17209	Six-row	0.56 ± 0.05	1.23 ± 0.04	+	6.7 ± 0.3	8.7 ± 0.2	0.77	63	Non-cleistogamous
SN	*Cly1.a*	JP185755	Two-row	0.53 ± 0.03	1.28 ± 0.01	+	10.1 ± 0.7	8.9 ± 0.2	1.12	82	Non-cleistogamous
RIL50	*Cly1.a*	[d]	Two-row	0.54 ± 0.03	1.10 ± 0.07	+	7.5 ± 0.8	13.3 ± 0.3	0.56	60	Non-cleistogamous
KNG	*cly1.b*	JP15436	Two-row	0.29 ± 0.02	0.28 ± 0.01	−	2.2 ± 0.3	8.7 ± 0.5	0.25	0	Cleistogamous
MG	*cly1.b*	JP15780	Two-row	0.26 ± 0.01	0.26 ± 0.01	−	8.8 ± 1.4	6.8 ± 0.1	1.29	0	Cleistogamous
GP	*cly1.b*	JP15923	Two-row	0.31 ± 0.02	0.31 ± 0.02	−	−1.8 ± 0.7	9.2 ± 0.5	−0.20	0	Cleistogamous
AZ × KNG F_1			Two-row	0.49 ± 0.01	1.25 ± 0.01	+	6.6 ± 0.8	9.2 ± 0.4	0.72	66	Non-cleistogamous
SN × KNG F_1			Two-row	0.48 ± 0.02	1.10 ± 0.01	+	−1.9 ± 0.1	8.4 ± 0.9	−0.23	18	Cleistogamous
SN × MG F_1			Two-row	0.43 ± 0.02	0.97 ± 0.05	+	1.4 ± 0.5	8.5 ± 1.0	0.16	9	Cleistogamous
RIL50 × GP F_1			Two-row	0.49 ± 0.02	1.26 ± 0.14	+	−3.0 ± 0.5	9.8 ± 0.4	−0.31	18	Cleistogamous

[a] Ratio of emergence distance/spike length at anthesis

[b] % of spikes with at least one anther extruded

[c] Classification of flowering type followed the method described by Turuspekov et al. (2005)

[d] RIL 50 is an F13 line derived from the cross AZ × KNG (Mano et al. 2001)

anthesis. To allow the lodicules to be photographed and therefore for their depth to be measured, the lemmas were removed from the set of spikelets located midway between the tip and the base of the spike. The images of the lodicules were quantified using the graphical analysis program Makijaku v1.1 (http://cse.naro.affrc.go.jp/iwatah/). Keeping the spikes in a 100 mg/l 2,4-D solution for 24 h at room temperature maintained the swollen state of the lodicules for an additional 2 days, an observation which facilitated the assessment of lodicule depth.

Assessment of anther extrusion at heading

Anther extrusion was scored in two ways. The first score was based on an average % of field grown spikes (based on 10–20 spikes per plant, where maximum 47 spikes per plant were developed) showing at least one extruded anther. The second followed the method described by Turuspekov et al. (2005), in which the definition of an anther extruding spike was one spike in which 30–100 % of the spikelets displayed observable anther extrusion.

Assessment of spike emergence distance and ratio at anthesis

Three spikes per plant were sampled at anthesis (the timing of which was confirmed, where necessary, by the removal of the flag leaf sheath) with respect to both lodicule depth and a spike emergence distance. The latter parameter represented the spatial separation between the tip of the spike and the ligule. Where anthesis occurred outside the boot, the score assigned was positive, whereas when anthesis occurred within the boot, the score was negative (Electronic Supplementary Material 1). For ease of comparison, a spike emergence ratio was devised, calculated from the spike emergence distance/spike length.

HvAP2 allele designation and expression

DNA was extracted from each plant following the method described by (Komatsuda et al. 1998) for use as a PCR template for the determination of which allele was present at the marker P101AP25′. The DNA was digested by *Nmu*CI, which targets a restriction site associated with the functional variant of the *HvAP2* miR172 targeting site (Nair et al. 2010). For the purpose of sequencing *HvAP2*, each 10 µl PCR reaction comprised 40 ng template, 0.5 U ExTaq DNA polymerase (TaKaRa), 1× buffer (TaKaRa), 2.5 mM $MgCl_2$, 200 µM dNTP and 300 nM of each primer. The cycling was initiated by a denaturation step (94 °C/5 min), followed by 35 cycles of 94 °C/30 s, 55 °C/30 s and 72 °C/60 s, and ended with a 72 °C/10 min extension step. The primers used to amplify the *HvAP2* coding region were those suggested by Nair et al. (2010) the up- and downstream regions are detailed in Electronic Supplementary Material 4. For the purpose of assessing the level of *HvAP2* transcription, RNA was isolated from immature spikes at various stages of their development using an RNAqueous RNA extraction kit (Ambion, Japan), as instructed by the manufacturer. The experimental method, and the primers/probe set for qRT-PCR followed Nair et al. (2010).

Results

Lodicule depth

The variation observed for lodicule depth is summarized in Table 1. The depth of the lodicule was taken as the critical dimension, since this determines the strength of the outward pressure on the lemma. The lodicules formed in the spikelets of AZ, SN and RIL50 were well developed, reaching a depth of 0.53–0.56 mm by the end of the yellow anther stage (Table 1), which was about double that achieved by the lodicules of KNG, MG and GP (0.26–0.31 mm). At anthesis, the AZ, SN and RIL50 lodicules had swollen to a depth of 1.10–1.28 mm. The lodicules of KNG, MG and GP did not swell as anthesis approached, even when the spikes were held in 2,4-D. The lodicule depth of the AZ x KNG F_1 plants (0.49 mm) was only marginally less than that of the AZ ones (0.56 mm) at the yellow anther stage. At anthesis, they enlarged to 1.25 mm, a size which was indistinguishable from that of the AZ lodicules (1.23 mm) (Fig. 1a, b). The lodicule depth at anthesis in the SN × KNG and SN × MG F_1 plants (respectively, 1.10 and 0.97 mm) was similarly a little less than that in SN itself (1.28 mm), while the RIL50 × GP F_1's lodicule depth (1.26 mm) was slightly greater than that of RIL50 (1.10 mm) (Fig. 1c–h). The lodicules of all the F_1 plants were of sufficient depth to cause the floret to gape at anthesis (Table 1).

The pattern of segregation for lodicule depth in the RIL (F_{13}) and F_2 generation is shown in Fig. 2. In each population, two distinct classes could be recognized at the yellow anther stage ("large" and "small") (Fig. 2a, c, e, g), and the former group had enlarged further by anthesis (Fig. 2b, d, f, h). The segregation ratio was compatible with that of a monogenic dominant gene (three large to one small) (Table 2). The segregation among the RILs appeared skewed (as similarly noted by Turuspekov et al. (2004). No recombination between *HvAP2* and lodicule depth was identified, consistent with the notion that the allelic status of *HvAP2* is responsible for lodicule depth.

The transcription level of *HvAP2* was consistent for AZ, KNG, SN, and MG throughout the development of the immature spike (Fig. 3). However, transcript abundance

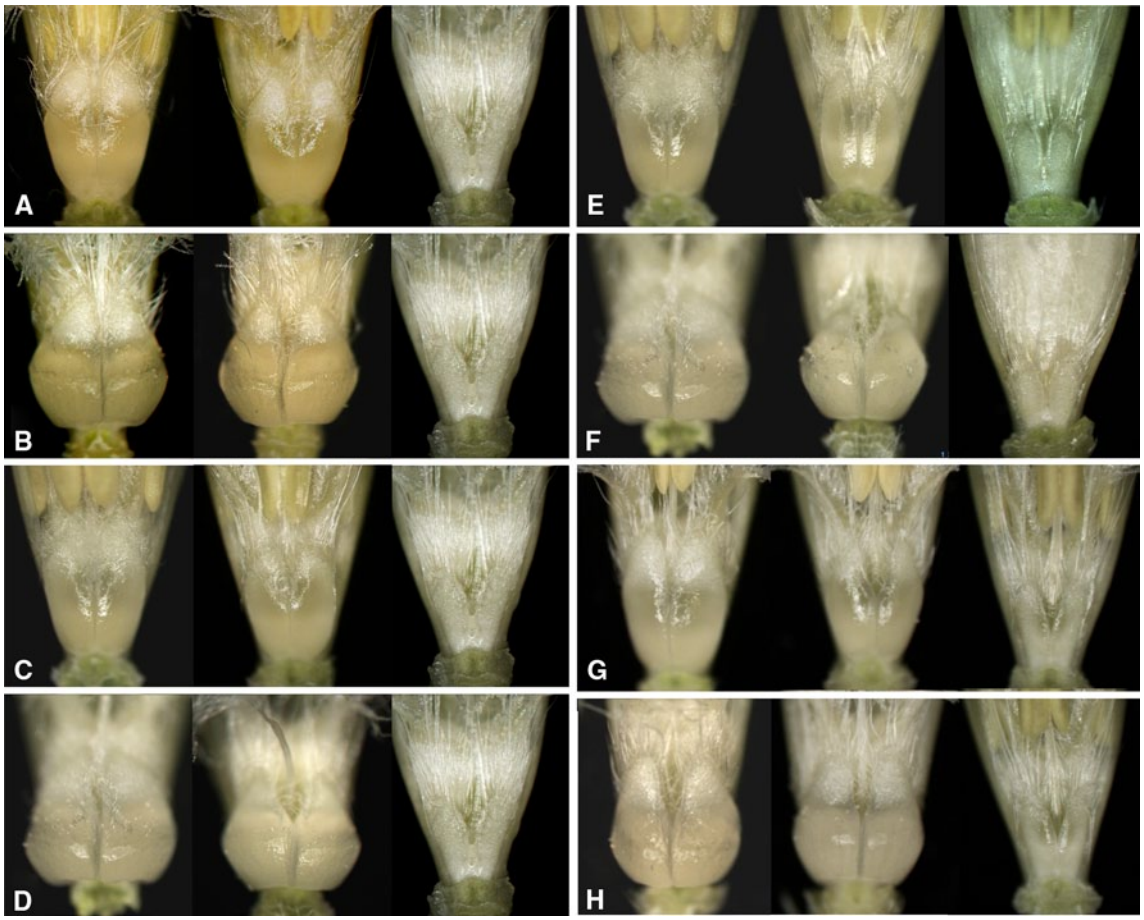

Fig. 1 Variation in lodicule size. The images shown in **a**, **c**, **e** and **g** were taken at the yellow anther stage, and those in **b**, **d**, **f** and **h** from spikes held in 2,4-D for 24 h after anthesis. (**a**, **b**, from *left* to *right*): AZ, AZ × KNG F_1, KNG. (**c**, **d** from *left* to *right*): SN, SN × KNG F_1, KNG. (**e**, **f** from *left* to *right*): SN, SN × MG F_1, MG. (**g**, **h** from *left* to *right*): RIL50, RIL50 × GP F_1, GP

varied somewhat among these cultivars throughout the development of the immature spike. However, in none of the cultivars was there either no, or a particularly high abundance of *HvAP2* transcription at any specific developmental stage.

Anther extrusion at anthesis

Anther extrusion was used to assess flower opening as it serves as a good indicator of this trait (Ceccarelli 1978; Turuspekov et al. 2004). It was treated as a quantitative character (% of spikes per field grown plant showing extrusion in at least one floret at anthesis), as shown in Table 1. The extent of extrusion in AZ, SN and RIL50 ranged from 60 to 82 %, whereas the rate for KNG, MG and GP was zero, in agreement with a previous observation of the behavior of these cultivars. The rate for the AZ × KNG F_1 plants was 66 %, indicating dominance for the AZ allele(s). The SN × KNG, SN × MG and RIL50 × GP F_1 plants had rates of 9–18 % (giving the impression of cleistogamy). The F_2 segregation for extrusion in the three populations fitted the expected monogenic 1:3 ratio (Table 2), as also noted by Turuspekov et al. (2004). The trait was strongly linked with *HvAP2* (Table 3), with both homozygotes (small lodicules) and heterozygotes (large lodicules) always being non-extruders. Most, but not all, of the *HvAP2* homozygous F_2 individuals (large lodicules) were extruders.

Spike emergence and anthesis

The observation that some of the spikes of both SN × KNG and SN × MG F_1 plants reached anthesis within the boot prompted a more detailed study of premature anthesis. Anther extrusion occurred in these spikes when they were freed from the leaf sheath, implying that lodicule swelling must have taken place within the boot, and that by the time that the spike emerged, the lodicules had already begun to shrink. Once this shrinkage had been initiated, the lodicules were no longer able to exert sufficient pressure to

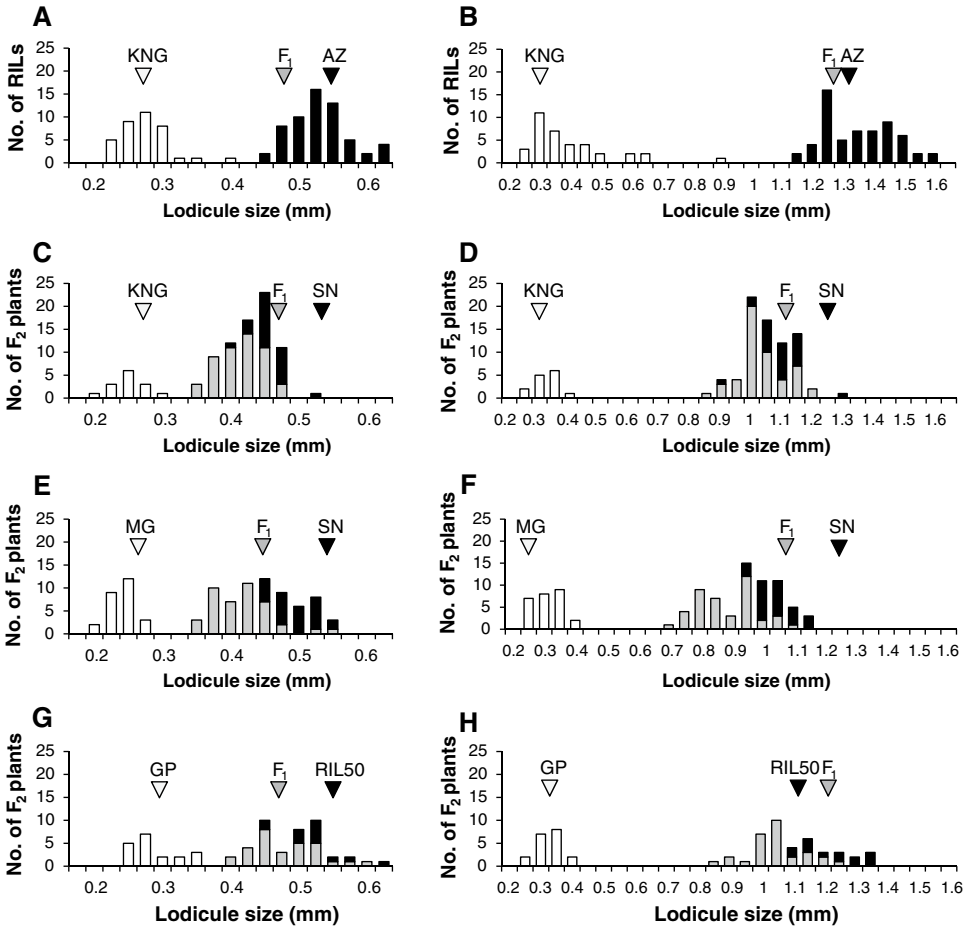

Fig. 2 Frequency distribution of lodicule depth expressed in the RIL and various F_2 populations. Parental values reflect the mean of six individuals. The data shown in **a**, **c**, **e** and **g** were collected from florets at the yellow anther stage, and those in **b**, **d**, **f** and **h** from spikes held in 2,4-D for 24 h after anthesis. **a**, **b**: AZ × KNG RIL population ($n = 96$). *White bars* KNG *HvAP2* allele homozygotes, *black bars* AZ allele homozygotes. **c**, **d**: SN × KNG F_2 population ($n = 90$). *White bars* KNG *HvAP2* allele homozygotes, *black bars* SN allele homozygotes, *light gray bars* heterozygotes. **e**, **f**: SN × MG F_2 population ($n = 95$). *White bars* MG *HvAP2* allele homozygotes, *black bars* SN allele homozygotes, *light gray bars* heterozygotes. **g**, **h**: RIL50 × GP F_2 population ($n = 62$). *White bars* GP *HvAP2* allele homozygotes, *black bars* RIL50 allele homozygotes, *light gray bars* heterozygotes

push apart the lemma and palea. (In AZ, SN and RIL50, the majority of the spikes had emerged fully from the boot by anthesis.) The spike emergence distances for MG, AZ, SN and RIL50 were, respectively, +8.8, +6.7, +10.1 and +7.5 cm, compared to the rather smaller distances for KNG and GP of, respectively, +2.2 and −1.8 cm. In the SN × KNG, SN × MG and RIL50 × GP F_1 plants, the distances ranged from −3.0 to +1.4 cm, rather less than that shown by AZ × KNG F_1 plants (Fig. 4b, d). The spike emergence ratio for AZ, SN and RIL50 ranged 66–100 %, considerably greater than that seen in either KNG or GP (Table 1). The ratio for the AZ × KNG F_1 was 72 %, while for SN × KNG, SN × MG and RIL50 × GP F_1s it was at most only 17 %. Low spike emergence ratios were associated with a low % anther extrusion. After anthesis, the culms of both the F_1 hybrids and their parental lines continued to elongate, so that grain set and maturation took place outside the boot. The conclusion was that the spike emergence distance depended on the timing of anthesis rather than on the length of the culm. The length of the mature spike was not correlated with either spike emergence distance or the extent of anther extrusion (Table 1).

The correlation between the identity of the allele of *HvAP2* present and spike emergence distance was significant ($8.44 \times 10^{-8} < p < 9.61 \times 10^{-5}$) among the F_2 population bred from the cross SN × KNG, SN × MG and RIL50 × GP (Table 4). The result indicated that a major gene affecting the latter trait must be located close to *HvAP2*, which explains, respectively, 23, 31 and 26 % of the total variance present in the three populations. In the RIL50 × GP population, the significant Spearman correlation ($\rho = +0.68$, $p < 0.01$, see Electronic Supplementary

Table 2 Segregation in various F_2 populations with respect to traits related to flowering type

Crosses $P_1 \times P_2$	Population type	Traits	No. plants for each class			*P* value			
			P_1	F_1	P_2	χ^2 (1:1)	χ^2 (1:2:1)	χ^2 (3:1)	χ^2 (1:3)
AZ × KNG	RIL (F_{13})	*HvAP2*/*Nmu*CI	60	0	36	0.01	–	–	–
		Lodicule size (yellow anther stage)	60		36	0.01	–	–	–
		Lodicule size (anthesis stage)	60		36	0.01	–	–	–
		Anther extrusion[a]	60		36	0.01	–	–	–
SN × KNG	F_2	*HvAP2*/*Nmu*CI	25	51	14		0.05	–	–
		Lodicule size (yellow anther stage)	76		14		–	0.04	–
		Lodicule size (anthesis stage)	76		14		–	0.04	–
		Anther extrusion[a]	24		66		–	–	0.72
SN × MG	F_2	*HvAP2*/*Nmu*CI	27	42	26		0.52	–	–
		Lodicule size (yellow anther stage)	69		26		–	0.59	–
		Lodicule size (anthesis stage)	69		26		–	0.59	–
		Anther extrusion[a]	28		67		–	–	0.31
RIL50 × GP	F_2	*HvAP2*/*Nmu*CI	13	30	19		0.56	–	–
		Lodicule size (yellow anther stage)	43		19		–	0.30	–
		Lodicule size (anthesis stage)	43		19		–	0.30	–
		Anther extrusion[a]	18		44		–	–	0.46

[a] Classification followed the method described by Turuspekov et al. (2005)

Material 2) between the spike emergence distance and % anther extrusion also suggested that anther extrusion was heavily influenced by the spike emergence distance. The extent of dominance with respect to spike emergence was hardly detectable.

Re-sequencing of *cly1*

An 11 kb stretch of the *HvAP2* region, including 4 kb up- and 3 kb downstream of the coding sequence was re-sequenced (KF261342–KF261346). The data revealed the presence of three SNPs (Table 5), two of which had already been identified by Nair et al. (2010). The site of the third variable base was at −379 nt, distinguishing SN from AZ (both non-cleistogamous), but the SN type was present in all of the cleistogamous cultivars.

Discussion

The elongation of the filament is the most common means by which grasses force anther extrusion at anthesis (Cheignon 1972; Cheignon et al. 1973; Heslop-Harrison and Heslop-Harrison 1996; Koevenig 1973). No mutations for this trait in barley have yet been identified, so that the anther extrusion can be used as a surrogate for gaping of the floret, at least for male fertile plants. (Note that fertilized florets close shortly after the formation of the zygote, making the scoring of floret gaping somewhat unreliable.) Gaping of the floret is achieved by the swelling of the lodicule (Honda et al. 2005; Zeng et al. 1999), but here we have shown that in barley, the timing of spike emergence from the boot also has an important influence over floret gaping. Thus the occurrence of anthesis in the boot furnishes a second means of ensuring cleistogamy.

Lodicule depth is determined by allelic status at the *cly1* locus

Insufficient swelling of the lodicules is assumed to be the prime mechanism underlying cleistogamy in the grasses (Briggs 1978; Heslop-Harrison and Heslop-Harrison 1996; Yoshida 2012). It is well established that anthers are not extruded in the *cly1cly1* homozygote (Turuspekov et al. 2004). The demonstration of a perfect association between

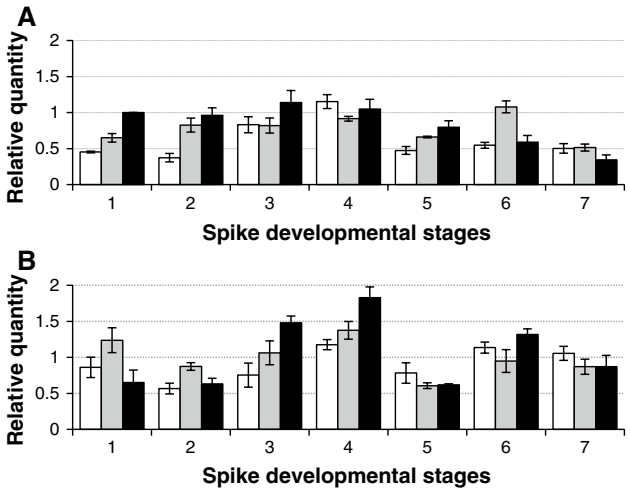

Fig. 3 *HvAP2* transcript abundance (mean and standard error from three biological replications) during spike development. **a** AZ (white bars, *Cly.a*), AZ × KNG F_1 (*gray bars*, *Cly.a* × *cly.b*) and KNG (*black bars*, *cly.b*). **b** SN (*white bars*, *Cly.a*), SN × MG F_1 (*gray bars*, *Cly.a* × *cly.b*) and MG (*black bars*, *cly.b*). *1* lemma primordium stage, *2* stamen primordium stage, *3* awn primordium stage, *4* white anther stage, *5* green anther stage, *6* yellow anther stage, *7* anthesis. Relevant data are presented as the fold difference in transcript abundance normalized against that of the constitutively expressed *actin* gene, and related to the expression of a calibrator sample (KNG at the lemma primordium stage)

small lodicules and cleistogamy in a barley germplasm collection suggested that the major effect of *cly1* is on lodicule depth rather than on anther extrusion (Nair et al. 2010). This suggestion was strongly supported by three F_2 recombinant plants which narrowed down *cly1* candidate window to a single gene, *HvAP2*. Anther extrusion was completely associated with lodicule size in each of the three recombinants, and this evidence was used to identify *HvAP2* as the gene responsible for lodicule in size. Here, crosses and segregation behavior were used to verify that this association does indeed have a genetic basis, resulting from either close linkage or pleiotropy, since none of the F_2 segregants which carried the cleistogamy associated allele at *HvAP2* developed large lodicules. Despite the suggestion that anther non-extrusion is under the control of *Cly2* acting in a dominant manner in populations derived from either of the crosses SN × KNG or SN × MG, here the conclusion from the analysis of the same crosses was that the development of a small lodicule was due only to the inheritance of *cly1*. The lack of concordance between lodicule depth and anther extrusion in these crosses must therefore be explained by the influence of *Cly2* on the timing of anther extrusion. RIL50 is a derivative of the cross AZ × KNG, carrying *Cly1*. Lodicule depth and anther extrusion segregated in the RIL50 × GP F_2 population in the same manner as occurred in both the SN × KNG and SN × MG populations (Fig. 2).

Neither the structure nor the transcription of *HvAP2* is disturbed in SN

Re-sequencing of the *HvAP2* coding sequence revealed that the SN allele was identical to that present in both AZ and RIL50, but different to that present in KNG, MG and GP (Table 5). Presumably therefore miR172 directed cleavage occurs in SN, just as it does in AZ. miR172- mediated cleavage would have been expected to have had a similar effect on lodicule development in each of the cross combinations tested. Thus it is reasonable to infer that lodicule size among the SN × MG and SN × KNG F_2 segregants is under the control of *HvAP2* as was the case in AZ × KNG populations (Nair et al. 2010).

Anther non-extrusion cannot be ascribed to the presence of *cly1*; rather the presence of *Cly2* needs to be invoked. The specific function of the *HvAP2* gene product appears to be restricted to the control of lodicule development (Nair et al. 2010), while anther non-extrusion can be additionally influenced by *Cly2*. Although the AZ and SN *HvAP2* coding sequences are identical, the two cultivars differ with respect to their upstream sequence at one base (−379 nt) (Table 5). The SNP allele carried by SN was the same as is present in the cleistogamous cultivars KNG, MG and

Table 3 Correlation between the allelic state of *HvAP2* and anther extrusion estimated from joint segregation in three F_2 populations

Cross	Genotype	No. of F_2 plants		χ^2_{linkage} [a]	*P* value
	*HvAP2/Nmu*CI	Anther extrude	Not-extrude		
SN × KNG	*Cly1.a* homo. (SN)	14	11	17.39	1.67E−04
	Heterozygous (F_1)	10	41		
	cly1.b homo. (KNG)	0	14		
SN × MG	*Cly1.a* homo. (SN)	20	7	38.90	3.57E−09
	Heterozygous (F_1)	8	34		
	cly1.b homo. (MG)	0	26		
RIL50 × GP	*Cly1.a* homo. (RIL50)	12	1	53.22	2.78E−12
	Heterozygous (F_1)	6	24		
	cly1.b homo. (GP)	0	19		

Data of anther extrusion were collected in 2011

[a] χ^2 linkage = χ^2 total − χ^2 (1:2:1 for *HvAP2/Nmu*CI) − χ^2 (1:3 for anther extrusion)

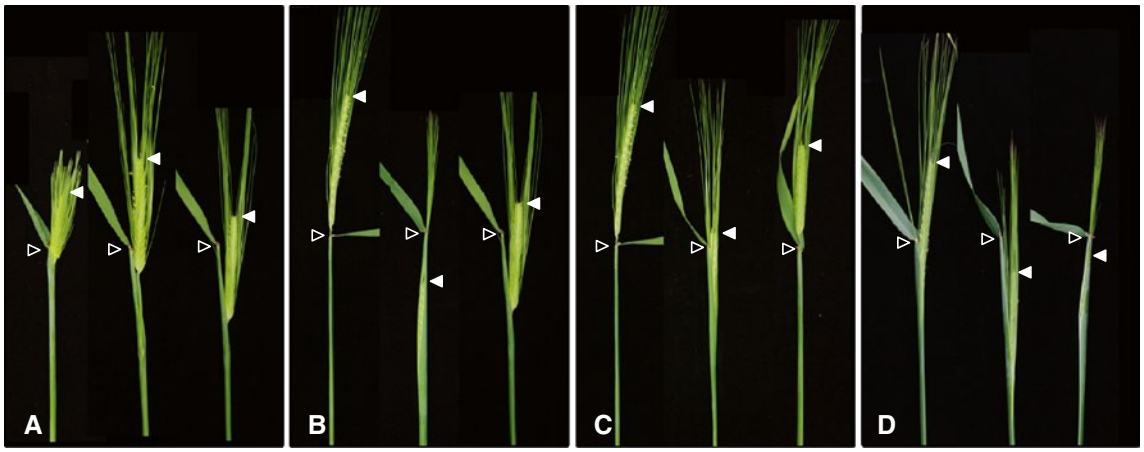

Fig. 4 Spike emergence at anthesis. (**a** from *left* to *right*): AZ, AZ × KNG F_1, KNG. (**b** from *left* to *right*): SN, SN × KNG F_1, KNG. (**c** from *left* to *right*: SN, SN × MG F_1, MG. (**d** from *left* to *right*): RIL50, RIL50 × GP F_1, GP. *White triangles* indicate the uppermost spikelets and *black* ones the junction between the flag leaf blade and leaf sheath. The distance between *white* and *black arrow* was taken as the spike emergence distance

Table 4 Correlation between the allelic state of *HvAP2* and spike emergence distance estimated from joint segregation in the F_2 population

Cross	Genotype *HvAP2/Nmu*CI	No. of F_2 plants	Mean (cm)	S.D. (cm)	*P* value ANOVA	R^2
SN × KNG	*Cly1.a* homo. (SN)	32	5.94	6.85	8.10E−06	0.23
	Heterozygous (F_1)	43	4.64	6.95		
	cly1.b homo. (KNG)	19	2.04	6.40		
SN × MG	*Cly1.a* homo. (SN)	26	10.00	12.01	8.44E−08	0.31
	Heterozygous (F_1)	49	7.80	4.55		
	cly1.b homo. (MG)	17	5.16	1.85		
RIL50 × GP	*Cly1.a* homo. (RIL50)	13	0.55	2.29	9.61E−05	0.26
	Heterozygous (F_1)	30	−1.03	3.19		
	cly1.b homo. (GP)	19	−2.26	2.56		

Data of spike emergence for SN × KNG and SN × MG crosses were collected in 2012, and RIL50 × GP cross in 2011

R^2 contribution ratio

Table 5 The location of variable nucleotides with the 11 kb genomic region around *HvAP2*

Cultivars	*HvAP2* alleles	Flowering types	Upstream (−3836 to −1)	5′ UTR (1–480)	CDS (481– 3,171)		3′ UTR (3,172–3,561)	Downstream (3,562–7,223)
			−379	[a]	1st exon	miR172 site	[a]	[a]
					626	3,084		
AZ	*Cly1.a*	Non-cleistogamous	G	–	C	A	–	–
SN	*Cly1.a*	Non-cleistogamous	C	–	C	A	–	–
KNG	*cly1.b*	Cleistogamous	C	–	T	G	–	–
MG	*cly1.b*	Cleistogamous	C	–	T	G	–	–
GP	*cly1.b*	Cleistogamous	C	–	T	G	–	–

[a] No SNPs were detected in the UTRs and downstream region

GP. So the inference is that the full SN *HvAP2* sequence comprises a cleistogamous-type regulatory sequence and a non-cleistogamous-type coding sequence. The unique combination of regulatory and coding sequences of SN *HvAP2* might be related to the timing of anthesis in the F_1 hybrid plants.

Anthesis prior to spike emergence results in anther non-extrusion

A side effect of premature anthesis is the failure of the anthers to extrude, independent of the size of the lodicule. When the lodicules swell prior to the spike's emergence from the boot, floret gaping is prevented by the physical restriction imposed by the leaf sheath (Fig. 4b, c). The lodicules already have begun to shrink by the time that such spikes emerge from the boot, and they are no longer able to push the lemma and palea apart. As a result, the proportion of non-cleistogamous flowering was much reduced in the SN × KNG and SN × MG F_2 populations (Table 1). Removal of the leaf sheath prior to anthesis did allow for anther extrusion, demonstrating that lodicule development was normal; rather, it was precocious anthesis which prevented anther extrusion. This accounts therefore for the epistatic action of *Cly2* over *cly1*.

The recognition of this phenomenon explains why the MG × SN F_2 population appeared to segregate one large to three small lodicules when the spikes were treated with 2,4-D after heading (Honda et al. 2005). In our hands, the same F_2 population segregated in a 3:1, not in a 1:3 ratio, by releasing spikes from the leaf sheath prior to anthesis and applying 2,4-D. All progeny producing well developed lodicules responded to 2,4-D treatment (Figs. 1, 2). Once a lodicule had already expanded within the boot, and subsequently shrunk, it is no longer responsive. For this reason, if spikes are tested too late (as we believe was the case in the Honda et al. (2005) study) a misleading conclusion with regard to 2,4-D responsiveness is likely to be drawn.

Genetic models for cleistogamy in barley

A plausible genetic model would be that *HvAP2* exerts a pleiotropic effect on both lodicule size and timing of anthesis. Spike emergence was normal in *Cly1Cly1* or *cly1cly1* F_2 segregants, and the anthers were fully extruded at anthesis; in contrast, it was less complete in *Cly1cly1* segregants, (Fig. 4) and anther extrusion was compromised at anthesis. The location of *cly1* coincides with a known locus determining rachis internode length (Sameri et al. 2006, 2009; Turuspekov et al. 2005). However, no substantial influence of *cly1* on peduncle length was detectable in the present materials, so a likelier scenario is that it is heterozygosity at *cly1* which accelerates anthesis. *HvAP2* has been reported to co-segregate with the *Flowering time-2L* QTL (Chen et al. 2009). The gene is a member of the *euAP2* family and is a homolog of *A. thaliana APETALA2* and a paralog of the wheat domestication gene *Q* (Nair et al. 2010). *AtAP2* and *Q* are both implicated in the determination of flowering time (Aukerman and Sakai 2003; Chen 2004; Simons et al. 2006). The suggestion is that *HvAP2* acts jointly on both lodicule size and the timing of anthesis and in this pleiotropic model the SN allele is designated *Cly1.a2*: The coding sequence of *Cly1.a* and *Cly1.a2* is identical but their expression is differentially regulated and anther extrusion is compromised at anthesis in *Cly1.a2cly1* segregants.

In an alternate model the correlation between the allelic state of *HvAP2* and spike emergence suggested the presence of a major QTL determining the latter trait in the vicinity of *HvAP2* (Table 4) that *cly1* (control lodicule size) and *Cly2* (control premature timing of anthesis) are tightly linked. In the heterozygous *Cly2cly2* state, anthesis occurs earlier than in either homozygote. The two *Cly* genes are sufficiently closely linked to one another (Table 4) to assuming zero recombination between them, so in the F_2 generation bred from a cross *cly1cly1Cly2Cly2* × *Cly1Cly1cly2cly2*, 50 % of the individuals will be *Cly1cly1Cly2cly2* (spikes fail to emerge at anthesis, therefore the anthers are not extruded even after their final emergence), 25 % will be *clycly1Cly2Cly2* (spikes emerge at the anthesis, but anthers are not extruded because of homozygosity for *cly1*) and 25 % will be *ClyCly1 cly2cly2* (spikes emerge at the anthesis and anthers are extruded). The expected F_2 segregation ratio for anther extrusion is therefore 1:3, as observed experimentally (Electronic Supplementary Material 3). The hypothetical ratio was reasonable under consideration of single gene (*HvAP2*) or two tightly linked genes, while other factors such as minor QTL or environmental effect may result distortion.

It has been assumed to date that cleistogamy in barley requires the repression of lodicule development. Here we have shown that there is an alternative route to cleistogamy, based on the selection of types which reach anthesis before the spike has emerged from the boot. This accelerated timing ensures that the florets remain closed during anthesis, and is particularly effective in preventing access of non-self pollen while the pistil remains receptive. Anthesis within the boot did not result any reduction in grain set. Honda et al. (2005) have reported that the level of the auxin indole-3-acetic acid (IAA) is higher in the anther than in either the lodicule or the pistil. Auxin treatment promotes anther extrusion by inducing a rapid swelling of the lodicules, but also by lengthening the filament. The transport of IAA from the anthers to filament and then lodicule may be responsible for the timing of anthesis (Honda et al. 2005). The relevant experiments were conducted using a non-cleistogamous cultivar, and were based on measurements of the concentration of the decarboxylated form of IAA. It may be that the mode of action of *Cly2* is to trigger IAA transport through the filament to the lodicule and thereby to control the timing of anthesis.

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References

Abdel-Ghani AH, Parzies HK, Omary A, Geiger HH (2004) Estimating the outcrossing rate of barley landraces and wild barley populations collected from ecologically different regions of Jordan. Theor Appl Genet 109:588–595

Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. Plant Cell 15:2730–2741

Briggs D (1978) Barley. Chapman and Hall, London

Ceccarelli S (1978) Single-gene inheritance of anther extrusion in barley. J Hered 69:210–211

Cheignon M (1972) Structural modifications of cell-walls during elongation of stamen filament of *Zea mays* L. Cr Acad Sci D Nat 275:549

Cheignon M, Schaever J, Cornier N (1973) Elongation of stamen filament of graminaceae as growth phenomenon. Cr Acad Sci D Nat 276:319–322

Chen X (2004) A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science 303:2022–2025

Chen A, Brûlé-Babel A, Baumann U, Collins NC (2009) Structure–function analysis of the barley genome: the gene-rich region of chromosome 2HL. Funct Integr Genomics 9:67–79

Dahleen LS, Morgan W, Mittal S, Bregitzer P, Brown RH, Hill NS (2012) Quantitative trait loci (QTL) for Fusarium ELISA compared to QTL for Fusarium head blight resistance and deoxynivalenol content in barley. Plant Breed 131:237–243

Daniell H (2002) Molecular strategies for gene containment in transgenic crops. Nat Biotechnol 20:581–586

Heslop-Harrison Y, Heslop-Harrison JS (1996) Lodicule function and filament extension in the grasses: potassium ion movement and tissue specialization. Ann Bot-Lond 77:573–582

Honda I, Turuspekov Y, Komatsuda T, Watanabe Y (2005) Morphological and physiological analysis of cleistogamy in barley (Hordeum vulgare). Physiol Plant 124:524–531

Hori K, Kobayashi T, Sato K, Takeda K (2005) QTL analysis of Fusarium head blight resistance using a high-density linkage map in barley. Theor Appl Genet 111:1661–1672

Hori K, Sato K, Kobayashi T, Takeda K (2006) QTL analysis of fusarium head blight severity in recombinant inbred population derived from a cross between two-rowed barley varieties. Breed Sci 56:25–30

Kirby EJM, Appleyard M (1981) Cereal development guide. Cereal Unit, Kenilworth

Koevenig JL (1973) Floral development and stamen filament elongation in *Cleome hassleriana*. Am J Bot 60:122–129

Komatsuda T, Nakamura I, Takaiwa F, Oka S (1998) Development of STS markers closely linked to the vrs1 locus in barley, Hordeum vulgare. Genome 41:680–685

Kurauchi N, Makino T, Hirose S (1994) Inhieritance of cleistogamy-chasmogamy in barley. Barley Genet Newsl 23:19

Lord EM (1981) Cleistogamy—a tool for the study of floral morphogenesis, function and evolution. Bot Rev 47:421–449

Lu Q, Lillemo M, Skinnes H, He X, Shi J, Ji F, Dong Y, Bjornstad A (2012) Anther extrusion and plant height are associated with Type I resistance to Fusarium head blight in bread wheat line 'Shanghai-3/Catbird'. Theor Appl Genet 126:317–334

Ma SM, Wang YF (2004) Molecular strategies for decreasing the gene flow of transgenic plants. Yi Chuan 26:556–559

Mano Y, Kawasaki S, Takaiwa F, Komatsuda T (2001) Construction of a genetic map of barley (*Hordeum vulgare* L.) cross 'Azumamugi' × 'Kanto Nakate Gold' using a simple and efficient amplified fragment-length polymorphism system. Genome 44:284–292

Nair SK, Wang N, Turuspekov Y, Pourkheirandish M, Sinsuwongwat S, Chen GX, Sameri M, Tagiri A, Honda I, Watanabe Y, Kanamori H, Wicker T, Stein N, Nagamura Y, Matsumoto T, Komatsuda T (2010) Cleistogamous flowering in barley arises from the suppression of microRNA-guided HvAP2 mRNA cleavage. Proc Natl Acad Sci USA 107:490–495

Sameri M, Takeda K, Komatsuda T (2006) Quantitative trait loci controlling agronomic traits in recombinant inbred lines from a cross of oriental- and occidental-type barley cultivars. Breed Sci 56:243–252

Sameri M, Nakamura S, Nair SK, Takeda K, Komatsuda T (2009) A quantitative trait locus for reduced culm internode length in barley segregates as a Mendelian gene. Theor Appl Genet 118:643–652

Simons KJ, Fellers JP, Trick HN, Zhang ZC, Tai YS, Gill BS, Faris JD (2006) Molecular characterization of the major wheat domestication gene Q. Genetics 172:547–555

Skinnes H, Semagn K, Tarkegne Y, Maroy AG, Bjornstad A (2010) The inheritance of anther extrusion in hexaploid wheat and its relationship to Fusarium head blight resistance and deoxynivalenol content. Plant Breed 129:149–155

Turuspekov Y, Mano Y, Honda I, Kawada N, Watanabe Y, Komatsuda T (2004) Identification and mapping of cleistogamy genes in barley. Theor Appl Genet 109:480–487

Turuspekov Y, Kawada N, Honda I, Watanabe Y, Komatsuda T (2005) Identification and mapping of a QTL for rachis internode length associated with cleistogamy in barley. Plant Breed 124:542–545

Yoshida H (2012) Is the lodicule a petal: molecular evidence? Plant Sci 184:121–128

Yoshida H, Itoh J, Ohmori S, Miyoshi K, Horigome A, Uchida E, Kimizu M, Matsumura Y, Kusaba M, Satoh H, Nagato Y (2007) superwoman1-cleistogamy, a hopeful allele for gene containment in GM rice. Plant Biotechnol J 5:835–846

Zeng XC, Zhou X, Zhang W, Murofushi N, Kitahara T, Kamuro Y (1999) Opening of rice floret in rapid response to methyl jasmonate. J Plant Growth Regul 18:153–158