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Evidence for an evolutionarily conserved interaction between cell wall biosynthesis and flowering in maize and sorghum

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Abstract

Background: Factors that affect flowering vary among different plant species, and in the grasses in particular the exact mechanism behind this transition is not fully understood. The *brown midrib (bm)* mutants of maize (*Zea mays* L.), which have altered cell wall composition, have different flowering dynamics compared to their wild-type counterparts. This is indicative of a link between cell wall biogenesis and flowering. In order to test whether this relationship also exists in other grasses, the flowering dynamics in sorghum (*Sorghum bicolor* (L.) Moench) were investigated. Sorghum is evolutionarily closely related to maize, and a set *of brown midrib (bmr)* mutants similar to the maize *bm* mutants is available, making sorghum a suitable choice for study in this context.

Results: We compared the flowering time (time to half-bloom) of several different *bmr* sorghum lines and their wild-type counterparts. This revealed that the relationship between cell wall composition and flowering was conserved in sorghum. Specifically, the mutant *bmr7* flowered significantly earlier than the corresponding wild-type control, whereas the mutants *bmr2*, *bmr4*, *bmr6*, *bmr12*, and *bmr19* flowered later than their wild-type controls.

Conclusion: The change in flowering dynamics in several of the *brown midrib* sorghum lines provides evidence for an evolutionarily conserved mechanism that links cell wall biosynthesis to flowering dynamics. The availability of the sorghum *bmr* mutants expands the germplasm available to investigate this relationship in further detail.

Background

Defining which factors affect flowering is important for a better understanding of plant growth and development and offers an opportunity to study the interactions of environmental cues, chemical signals, and gene expression. This is also relevant from an agronomic perspective. A plant needs to flower in order to set seed, and in the case of many crop plants, including legumes and cereals, the seed serves as a main source of food in large parts of the world. Flowering time – defined as the time that elapses between planting and the emergence of functional reproductive structures – affects the yield. Early flowering can be beneficial to prevent loss due to frost or other adverse weather conditions towards the end of the season. Signif-

icant reduction of flowering time could even enable the production of an additional crop per year.

Recent advances in genetics have made it possible to study the genetic control of flowering. In the model plant *Arabidopsis thaliana* many genes affecting flowering time or the transition from the vegetative to the reproductive phase have been identified and a number of them have been cloned (for recent reviews, see [1,2]). This has resulted in a model in which meristem identity genes activate a developmental program that enables the shoot apical meristem to produce reproductive structures. The meristem identity genes can be activated via three different pathways. One pathway involves a set of autonomous genes (which are turned on at a specific developmental stage), a second pathway involves genes that are responsive to the photoperiod, and a third pathway includes genes that respond to the phytohormone gibberellin.

Orthologs of Arabidopsis flowering genes have been identified in several other species, including pea and snapdragon [3], rice [4], maize [5], pine [6] and ryegrass [7]. In addition, transformation of one plant species with constructs resulting in the over-expression of flowering genes from another plant species resulted in effects on flowering dynamics [3,7,8]. This indicates that the function of several flowering genes is conserved between plant species. On the other hand, there is evidence that the transition to flowering is not governed by a universal set of signals. Many plant species, including maize and other grasses are much less dependent on the photoperiod and gibberellin than Arabidopsis, suggesting that different signals may be required for flower development. This is conceptualized in the "multifactorial control" hypothesis [9] in which a combination of chemicals, including phytohormones, assimilates and minerals interact with genetic components in the process of floral initiation. The recent cloning of the *Indeterminate (Id1)* gene from maize also illustrated incomplete understanding of the floral transition process. The *Id1* gene is expressed in developing leaves prior to their transition from sink to source tissue. Its sequence shows similarity to a transcriptional regulator, and the gene product may play a role in the movement of a flowering signal in developing leaves [10,11]. The *Id1* gene is different from any of the flowering genes isolated from Arabidopsis so far.

We recently reported changes in flowering dynamics in some of the *brown midrib* mutants of maize (*Zea mays* L.) [12]. Compared to wild-type maize, near-isogenic lines carrying the *brown midrib1* and *brown midrib2* mutations flowered at a different time from their wild-type counterparts.

The maize *brown midrib* mutants have a reduction in lignin content and an altered lignin subunit composition [13]. Lignin is a complex hydrophobic polymer made of substituted *p*-hydroxy-cinnamyl alcohol units that differ in their extent of hydroxylation and methoxylation (for recent reviews see [14,15]). Lignin is important for the transport of water and plant rigidity and it serves as a defense mechanism against pests and pathogens. In maize the relationship between cell wall composition and flowering time appeared independent of lignin content, but instead seemed to depend on lignin subunit composition [12].

Life history theory and experiments have repeatedly demonstrated trade-offs between current survival and future reproduction in both animals and plants [16,17]. According to this theory there is an overall energy budget for an organism, with a finite amount available to divide between these two evolutionarily important processes. Such a trade-off may well be at the basis of the genetic correlation between flowering time and cell wall biosynthesis observed in maize. If this is indeed the case, a similar relationship might exist in other species. Sorghum (*Sorghum bicolor* (L.) Moench) is a good species for explorations of this nature. Maize and sorghum diverged approximately 15–20 million years ago and are considered closely related [18]. This is reflected by the degree of synteny between maize and sorghum [19] and the high degree of DNA sequence homology of coding regions between the two species [20]. In addition, *brown midrib* mutants exist in sorghum [21], making a study on the relationship between cell wall composition of sorghum and flowering directly comparable to the maize study. Despite these similarities the morphology of the sorghum floral structure is very different. Unlike maize, which is monoecious (separate male and female flowers), the panicle of sorghum is hermaphroditic. The sorghum florets contain two spikelets, one of which contains both the anthers and the ovary, whereas the other is often sterile.

The sorghum *brown midrib (bmr)* mutants, generated via chemical mutagenesis with di-ethyl sulfate (DES), are similar to the *bm* mutants of maize in that they have brown vascular tissue in the leaves and stem. The content and composition of the cell wall polymer lignin is also altered in the sorghum *bmr* mutants [21]. The *bmr6* mutation was shown to result in an accumulation of coniferaldehyde [22] and sinapaldehyde [23] and seems to be most similar to the maize *bm1* mutant [24]. The *bmr12* and *bmr18* mutants are allelic [25]. These mutants have lignin with lower amounts of syringyl residues. Instead, the lignin contains more 5-hydroxyguaiacyl residues and therefore resembles the lignin composition of the *bm3* mutant of maize [26,27]. Little is known about the lignin composition of the other *bmr* mutants.

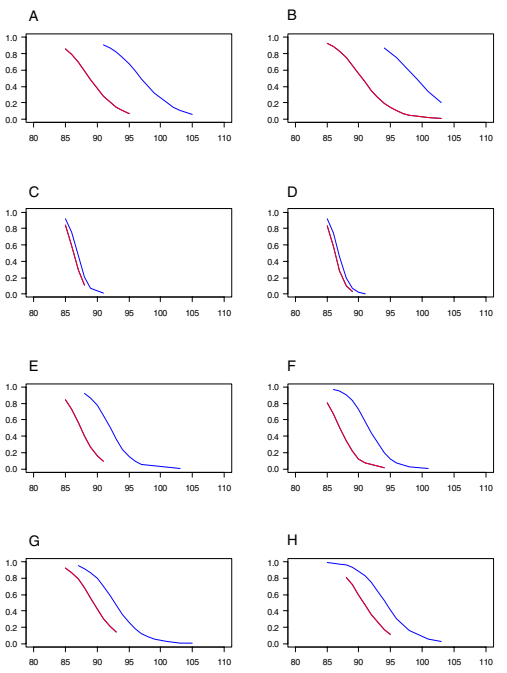

Figure 1
Modeling of flowering dynamics using a log-logistic survival model with data from both replicates at location A. The horizontal axis displays the flowering time (in days after planting), the vertical axis displays the predicted probability of not flowering (a value of 1.0 indicates none of the plants are flowering). In each graph the *bmr* mutant is represented by a blue line and the corresponding N-line by a red line. **A, B** *bmr2-N2* **C, D** *bmr3-N3* **E, F** *bmr4-N4* **G, H** *bmr6-N6*. For each pair of mutant and wild type the panel on the left represents replicate 1, and the panel on the right represents replicate 2.

In this study the sorghum *bmr* mutants are compared to their wild-type counterparts in terms of their flowering dynamics.

Results

Variation in flowering time

Table 1 lists the mean and median flowering times of the different lines at the two locations. There was considerable variation in the flowering time when the bmr lines were compared to their normal counterparts, and when the bmr lines were compared to each other. In general, the bmr mutants flower later then their wild-type counterparts, except in the case of bmr7, which flowers earlier than the N7 line.

Linear regression was performed on the data from location A using the model $Y_{ijk} = \beta_0 + \beta_1 X_{1i} + \beta_2 X_{2j} + \varepsilon_{ijk}$, where X_1 is the replicate (i = 1, 2), X_2 is the genotype (j = *bmr*, normal), and Y_{ijk} is the time to half-bloom for individual k in replicate i for genotype j. Based on the linear regression, the flowering times of the mutants *bmr3* and *bmr18* do not differ significantly from those of their wild-type counterparts. In all other cases the difference in flowering time was statistically significant ($P < 0.0001$). Differences between genotypes were consistent in direction across replicates, although the estimate of the distance between genotypes varied to some extent. In the cases of *bmr3-N3*, *bmr7-N7* and *bmr12-N12* residuals showed departures from the normality assumptions. Linear regression was then conducted on the data from location A using the model $^{10}\log(Y_{ijk}) = \beta_0 + \beta_1 X_{1i} + \beta_2 X_{2j} + \varepsilon_{ijk}$. This log-transformation did not change the conclusions or improve residual behavior.

The data were also analyzed using a survival analysis. Compared to linear regression, survival analysis does not require the data to be normally distributed, and early data on plants that do not survive until the end of the experiment due to diseases or other damage can be included in the analysis [12]. Furthermore, survival analysis allows the comparison of modeled flowering dynamics, as opposed to a comparison of just the mean flowering times.

Figure 1 and Figure 2 show the flowering dynamics of the plants in the two replicates in location A, obtained using survival analysis. A parametric log-logistic model [12] gave the best fit across genotypes. Based on these figures it is apparent that most *bmr* mutants flower later than their wild-type counterparts, with the exception of *bmr7*, which flowers earlier than N7, and *bmr18*, which flowers at the same time as N18. There is very good agreement between the two replicates. Differences between *bmr2* and N2, *bmr4* and N4, *bmr6* and N6, *bmr7* and N7, *bmr12* and N12, *bmr19* and N19 were statistically significant ($P < 0.001$). As with the linear regression, there was no statistically significant difference between *bmr3* and N3, and between *bmr18* and N18.

The slopes of the lines represent the rate at which flowering progresses. A steep slope (such as in *bmr3*/N3) is indicative of a more rapid progress in flowering than in cases where the slope is more gradual (such as in *bmr2*/N2). Note that for each combination of mutant and wild type the lines are parallel, which suggests that the *bmr* mutations affect the time to flowering, but not the rate of flowering.

In both the two-dwarf (*bmr2*, *4*, *6*, *7*) and the three-dwarf lines (*bmr12*, *19*) the difference in flowering time is apparent, which indicates that the effect is not limited to a spe-

Table 1: Comparison of mean, standard deviation and median flowering time (in days after planting) *of bmr* mutants and their corresponding wild-type controls in the two locations.

Location	Rep	Genotype	*n*	Mean	SD	Median
A	1	*bmr2*	22	99.1	4.51	99
		N2	56	88.5	2.70	88
	2	*bmr2*	38	98.1	3.37	98
		N2	66	92.0	5.42	90
	1	*bmr3*	56	86.6	1.42	87
		N3	86	86.5	1.09	87
	2	*bmr3*	59	87.4	1.82	87
		N3	85	86.2	1.17	86
	1	*bmr4*	48	92.9	2.95	94
		N4	65	87.0	1.87	87
	2	*bmr4*	41	91.1	4.17	89
		N4	44	87.9	2.01	87
	1	*bmr6*	60	94.0	4.72	94
		N6	54	88.9	2.15	88
	2	*bmr6*	56	93.5	4.16	94
		N6	53	91.6	2.54	92
	1	*bmr7*	48	94.6	3.62	95
		N7	51	97.8	4.14	97
	2	*bmr7*	45	93.3	3.12	94
		N7	41	99.3	3.68	99
	1	*bmr12*	49	98.9	4.34	99
		N12	65	93.5	4.55	94
	2	*bmr12*	56	101.6	2.29	103
		N12	55	93.9	3.88	94
	1	*bmr18*	44	95.9	2.34	96
		N18	57	95.0	4.11	95
	2	*bmr18*	27	97.2	3.31	97
		N18	49	98.6	4.29	99
	1	*bmr19*	32	93.0	3.46	94
		N19	75	87.6	1.42	87
	2	*bmr19*	42	95.6	4.9	95
		N19	52	87.6	1.87	87
B		bmr6	44	92.6	2.78	92
		N6	49	90.4	3.92	90
		bmr12	17	95.7	3.08	96
		N12	17	88.8	3.15	88
		bmr18	29	92.9	2.93	93
		N18	22	94.5	2.11	94

cific genetic background. It is also interesting to note that the allelic mutants *bmr12* and *bmr18* behave differently, even though they are both in the three-dwarf background. This could be the result of variation in the penetrance of the mutation.

Plant height and development

Given the significant differences in the flowering time between most *bmr* lines and their wild-type counterparts, the question arises what the origin of these differences is. One possibility is that the *bmr* lines develop at a slower or faster growth rate and reach the point at which they transition from the vegetative to the reproductive state at a different time. Differences in growth rate may be apparent all the time or only during select stages such as germination or the formation of floral structures, and they may or may not result in a difference in height at maturity. Alternatively, the *bmr* lines may develop at a normal rate, but delay the time at which they flower at the level of the flower itself. In that case the flower may be present, but may start blooming earlier or later than the wild-type flowers. It is possible that different mutations result in different developmental effects.

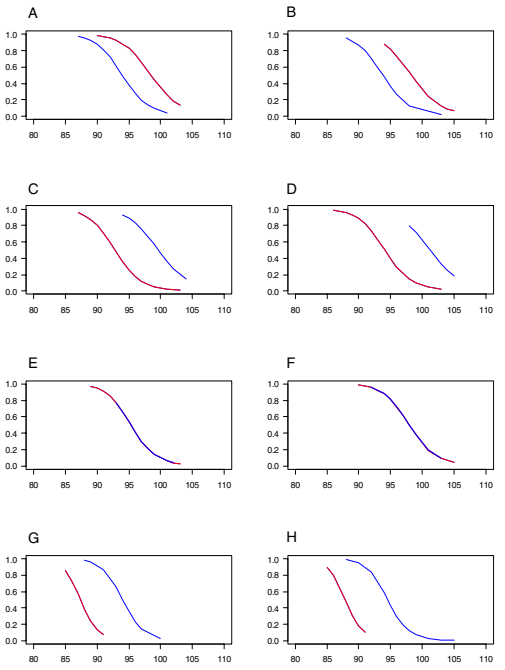

Figure 2
Modeling of flowering dynamics using a log-logistic survival model with data from both replicates at location A. The horizontal axis displays the flowering time (in days after planting), the vertical axis displays the predicted probability of not flowering (a value of 1.0 indicates none of the plants are flowering). In each graph the *bmr* mutant is represented by a blue line and the corresponding N-line by a red line. **A, B** *bmr7-N7* **C, D** *bmr12-N12* **E, F** *bmr18-N18* **G, H** *bmr19-N19*. For each pair of mutant and wild type the panel on the left represents replicate 1, and the panel on the right represents replicate 2.

In a first attempt to examine these different possibilities the height at half-bloom was measured for each individual plant in location B. Table 2 displays the height at half-bloom of the lines *bmr6, bmr12* and *bmr18* and their wild-type counterparts. A comparison of the height at half-bloom reveals no significant variation between *bmr12* and N12 and between *bmr18* and N18, but the *bmr6* plants are somewhat shorter than the N6 plants ($P = 0.0017$). There is a slight height difference between the *bmr12* and *bmr18* plants and their respective normal counterparts, but it is not statistically significant at the $\alpha = 0.05$ level. It may be worth examining a larger number of plants to explore this possible variation in height. The height data indicate that there may be an effect from the *bmr* mutations on plant growth, but it is likely that this effect is not universal, but rather dependent on the specific mutation.

Discussion

The results from this study indicate that changes in cell wall biosynthesis affect flowering dynamics in sorghum. Most of the *bmr* mutants we investigated flowered later than their wild-type counterparts, but the *bmr7* mutant flowered earlier. In the case of N18 and *bmr18* and N3 and *bmr3* there is no significant difference. Differences across replications were consistent in direction, but varied somewhat in the estimate of difference between the wild type and *bmr* mutant. These changes in flowering time are similar to what was observed for the maize *bm* mutants [12]. If the correlation between flowering dynamics and cell wall biosynthesis were spurious, mutations affecting cell wall composition in evolutionarily related species with different flower morphology would not show association with flowering dynamics. Therefore, the verification of this association in sorghum, when it has been demonstrated in maize, provides evidence for an evolutionarily conserved mechanism that links cell wall biogenesis with flowering time. This opens the possibility that a similar mechanism is present in other plant species, including the model plant Arabidopsis.

Taken together, the maize and sorghum data agree well with the model of trade-offs between current survival and future reproduction. The fact that the change in flowering time is not in one direction suggests that the different mutations have different effects on the energy balance. Additional experiments are necessary to obtain further details on the relationship between cell wall composition and flowering time. In the case of *bmr6* we have evidence that not only flowering time, but also height is affected as a result of the mutation. It may be interesting to explore variation in height in other mutant/wild-type combinations. Furthermore, determining during what developmental stage the difference between a particular *bmr* mutant and its wild-type counterpart becomes apparent may reveal whether or not there is a direct effect on the floral transition. It will be necessary to complete allelism tests before a relationship between the (bio)chemical effect of the various *bmr* mutations and the flowering time can be defined with confidence.

One hypothesis that can be tested is the involvement of phenylpropanoid intermediates as signaling molecules in plant development. Mutations in lignin biosynthesis may affect the effective concentration of such molecules, which could include dehydrodiconiferyl alcohol glucosides (DCGs). DCGs are lignan-glucosides with cytokinin-like activity [28,29]. An alternative hypothesis is that as a result of the *bmr* mutations the physico-chemical characteristics of the vasculature have been altered in such a way

Table 2: Comparison of mean, standard deviation and median height (in cm) of *bmr* mutants and their corresponding wild-type controls in location B. The *P*-values were calculated based on linear regression and were based on sample size *n*.

Genotype	*n*	mean	SD	median	*P*-value
N6	49	114	9.6	114	
bmr6	44	108	7.4	108	0.0017
N12	17	139	24.5	127	
bmr12	17	139	19.3	143	0.914
N18	22	117	7.8	117	
bmr18	29	114	6.5	116	0.128

that it affects the transport of signaling compounds. Colasanti and Sundaresan [11] proposed a role for the vasculature in the control of transport of signaling molecules between leaves and the shoot-apical meristem. Indeed, a recent study on the nitrogen content of phloem and xylem exudates during the transition to flowering in Arabidopsis and *Sinapis alba* showed changes in the transport of amino acids through the vasculature [30].

The present study also confirms the role that the maize *brown midrib* genes play in flowering dynamics. The maize *bm* mutants were isolated more than fifty years ago and the wild-type progenitors of the original mutants are no longer available. Therefore, studies with the maize *bm* mutants need to be done with near-isogenic lines. While comparisons between a wild type and a mutant that has been subjected to six or seven backcrosses are generally considered acceptable, there is still a small risk of linkage drag, creating the possibility that genes linked to the *brown midrib* genes are affecting the development. In the case of sorghum, however, the *bmr* mutants can be compared to a line derived from a wild-type sibling, which minimizes the source of genetic variation. Given that the sorghum *bmr* mutants show different flowering dynamics, we can attribute the variation in flowering dynamics in maize more confidently to the *bm* mutations.

Now that it has been shown that the *bmr* mutations of sorghum also affect the flowering time, the experimental population that can be used to determine the underlying effect has become significantly larger. Furthermore, the incorporation specific *bmr* mutations in sorghum breeding programs may be of use for the manipulation of flowering time.

Materials and Methods

Approximately 2,000 seeds were planted in a randomized complete block design at the Agronomy Research Center in West Lafayette, IN (location A) in the Spring of 2000 in the following manner: two replicates of approximately 60 plants each for the following entries N2, *bmr2*, N3, *bmr3*, N4, *bmr4*, N6, *bmr6*, N7, *bmr7*, N12, *bmr12*, N18, *bmr18*, N19, and *bmr19*. The *bmr* mutations were introduced via chemical mutagenesis with diethyl sulfate-ethylsulfonate (DES) [21]. Briefly, an M_3 population, obtained from selfing M_1 and M_2 plants (M_1 refers to plants grown from the mutagenized seeds) was screened for *bmr* mutants. The *bmr* mutants were identified among segregating head rows. The corresponding wild-type N-lines were derived from wild-type siblings in the same head row. Both the N and *bmr* plants were advanced to the M_5 generation via selfing, to result in near-isogenic lines. The genetic similarity between these lines is far greater than can be achieved through recurrent backcrosssing, because there is essentially no linkage drag.

The mutants *bmr2*, *bmr3*, *bmr4*, *bmr6* and *bmr7* were derived from the three-dwarf parent line 954114. The mutants *bmr12*, *bmr18*, and *bmr19* were derived from the two-dwarf parent line 954104, and tend to be taller than the mutants derived from line 954114. Note that the different numbers of the *bmr* mutants do not necessarily designate separate loci. Preliminary allelism tests indicated there are at least three different *bmr* loci [25]. The *bmr* mutants that were part of this study did not show any major abnormalities in their growth and development.

In addition, two 4×4 Latin squares with 24 plants per entry were planted at the Throckmorton Agricultural Center near West Lafayette, IN (location B) in the Spring of 2000. The first Latin square contained the entries N6, *bmr6*, N12 and *bmr12;* the second Latin square contained N6, *bmr6*, N18 and *bmr18*.

Flowering time was defined as the time to half-bloom (extrusion of mature anthers having progressed halfway down the head) and was recorded as days after planting (DAP) for each individual plant by daily survey in both locations. The height at half-bloom (in cm) of each plant in location B was measured.

All data analyses were performed using the statistical software package SAS (SAS Institute, Cary, NC). Height comparisons between genotypes were made based on linear regression. Basic statistics on the flowering time including the mean, median and standard deviation for each genotype were calculated. In some cases data were skewed, as is common in time-to-event data. Differences in flowering time between genotypes were assessed with two different statistical modeling techniques: linear regression and survival analysis. A log-transformation was performed before the linear regression when the data were not normally distributed.

Survival analysis covers a broad class of models that deal with the analysis of time-dependent data. In brief, analysis of survival data involves two interrelated functions: the hazard function h(t), which given survival until time t, gives the instantaneous potential of an individual to undergo the event of interest, and the survival function S(t) which gives the probability of survival longer than time t[31]. The hazard and survival functions vary depending on the specific model. Models can be parametric or non-parametric. Non-parametric survival models assume no baseline hazard. This can result in a loss of precision and in some cases a loss of power. In contrast, the parametric models specify a baseline survival function, which can have serious consequences if mis-specified. For a more detailed description of survival analysis, see [31,32]. Survival models can also be implemented in SAS [33]. The application of survival analysis to developmental traits was discussed by Vermerris and McIntyre [12].

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