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Review

Molecular Regulation of Flowering Time in Grasses

Fiorella D. B. Nuñez [1] and Toshihiko Yamada [2,*]

[1] Graduate School of Environmental Science, Hokkaido University, Kita 10 Nishi 5, Sapporo, Hokkaido 060-0810, Japan; fiorepkm@gmail.com
[2] Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan
* Correspondence: yamada@fsc.hokudai.ac.jp; Tel.: +81-11-706-3644

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Abstract: Flowering time is a key target trait for extending the vegetative phase to increase biomass in bioenergy crops such as perennial C_4 grasses. Molecular genetic studies allow the identification of genes involved in the control of flowering in different species. Some regulatory factors of the *Arabidopsis* pathway are conserved in other plant species such as grasses. However, differences in the function of particular genes confer specific responses to flowering. One of the major pathways is photoperiod regulation, based on the interaction of the circadian clock and environmental light signals. Depending on their requirements for day-length plants can be classified as long-day (LD), short-day (SD), and day-neutral. The *CONSTANS* (*CO*) and *Heading Date 1* (*Hd1*), orthologos genes, are central regulators in the flowering of *Arabidopsis* and rice, LD and SD plants, respectively. Additionally, *Early heading date 1* (*Ehd1*) induces the expression of *Heading date 3a* (*Hd3a*), conferring SD promotion and controls *Rice Flowering Locus T 1* (*RFT1*) in LD conditions, independently of *Hd1*. Nevertheless, the mechanisms promoting flowering in perennial bioenergy crops are poorly understood. Recent progress on the regulatory network of important gramineous crops and components involved in flowering control will be discussed.

Keywords: flowering; *Arabidopsis*; grasses; photoperiod; circadian clock

1. Introduction

Global climate change and energy security issues have promoted interest in the production and increased availability of alternative energy sources. Lignocellulosic biomass is a promising feedstock source for biorefineries producing biofuel, which can mitigate greenhouse gas emissions [1–3] and reduce dependency on fossil oil [4,5]. Perennial C_4 bioenergy crops such as switchgrass (*Panicum virgatum* L.) and *Miscanthus* spp. provide good targets as non-edible plant species [6–8] having advantages with regard to land utilization and the avoidance of conflict with food security [9–11] to provide efficient production systems at low cost.

One of the most important traits in the plant life cycle is the timing of flowering, the floral transition between the vegetative and reproductive phases of plant development [12,13]. Consideration of flowering time is an important strategy in the cultivation of grain crops in northern latitudes. Early flowering is useful in regions where growing seasons are short to enhance grain yield stability by avoiding drought or adverse temperatures [14–16]. Flowering time is also a major determinant of biomass yield in perennial C_4 bioenergy crops, because delayed flowering time allows an extended period of vegetative growth and produces more biomass. Thus, earlier flowering will produce lower yields than late flowering in terms of feedstock production [17]. However, biomass potential also depends on environmental conditions. In switchgrass, lowland ecotypes that originate from southern areas flower later in high latitude areas, but the yield advantages of these southern switchgrasses are often not realized at northern latitudes due to high winter mortality [18].

Natural variation in flowering time is related to latitude in several plant species. Migration of plants into different latitudes often require the adoption of different signals to induce flowering and promote adaptive responses to diverse growing seasons [19]. Factors such as photoperiod and temperature that vary over large geographical scales are involved [20]. Plants possess an internal biological clock providing circadian rhythms that respond to fluctuations in day-length and thus anticipate upcoming seasonal changes [21] to regulate flowering. Depending on their requirements for day-length (light period in a 24-h cycle) to promote flowering, plants can be classified as long-day (LD) plants when photoperiod exceeds a critical day-length, short-day (SD) plants when photoperiod is shorter than a critical day-length and day-neutral plants when flowering occurs irrespective of day-length [22–24]. Winter annuals (e.g., wheat (*Triticum aestivum* L.)), biennials (e.g., sugar beet (*Beta vulgaris* L.)) and numerous perennials (e.g., orchardgrass (*Dactylis glomerata* L.)) are obligatory LD plants. These plants, however, flower only after vernalization during a cold period [23]. The molecular basis of flowering time regulation has been extensively studied using classical Quantitative Trait Loci (QTL) approaches in model plant species such as Arabidopsis thaliana [25–27], a LD plant, and rice (*Oryza sativa* L.), a SD plant [28]. Grasses have multiple pathways to control flowering time but only some of them are conserved in *Arabidopsis thaliana* (L.) Heynh. [29]. These studies have been crucial in establishing the multiple pathways that control flowering, of which the photoperiod pathway is of major importance [30]. The use of model species has played a major role in understanding the molecular mechanisms involved in flowering time to help in the genetic improvement of crop development. Nevertheless, little is known about the mechanisms promoting flowering in perennial C_4 bioenergy crops. In this review, we discuss recent progress concerning the regulatory network and components involved with flowering control in different species including C_4 grasses such as sorghum (*Sorghum bicolor* (L.) Moench), switchgrass and *Miscanthus* spp. To understand how plants initiate flowering is a crucial step in developing selection criteria in breeding programs of grasses used as bioenergy crops.

2. Conservation and Divergence in Flowering Pathways

2.1. Arabidopsis and Rice

In the last decades, studies on the model plant *Arabidopsis* have revealed that molecular mechanisms discovered in that species are evolutionally conserved in other species [15]. Genetic approaches in *Arabidopsis* have identified three genes that control flowering: *GIGANTEA (GI)—CONSTANS (CO)—FLOWERING LOCUS (FT)* [15,28]. Loss of function mutations in each gene for flowering control delay flowering under LD conditions but no effect is produced under SD [31]. *GI* is a key regulator of the photoperiodic pathway and in the evening promotes *CO* transcription under LD conditions [32]. The most extensively gene studied in *Arabidopsis* flowering is *CO* that confers LD responses. *CO* encodes a B-box zinc finger transcription factor and CCT domain genes that promote flowering under LD conditions and activate the expression of *FT* [33–35], a major component of the florigen that induces flower differentiation [29]. Its inactivation causes flowering delay, while its over-expression induces early flowering. *CO* and *FT* are expressed in the phloem and act there to promote flowering [31]. This signalling pathway is conserved in rice: *OsGI-Hd1-Hd3a* [28], mediated by LD responses. *OsGIGANTEA (OsGI)* acts an activator of *Heading date1 (Hd1)*, an ortholog of *CO*, and controls flowering time by modulating rhythmic flowering under SD [36]. *Hd1* encodes a zinc finger type transcriptional activator with the conserved CCT *(CO*, *CO-like*, *TIMING OF CAB EXPRESSION1* (*TOC1)*) domain [15,37]. In contrast to *Arabidopsis CO*, *Hd1* promotes *Heading date 3a (Hd3a)* expression in SD but expression is modified in LD conditions [38,39] where *Hd1* function is converted into a repressor. Rice involves at least two flowering pathways that control the expression of the florigens: *Hd1* that is conserved in rice and *Arabidopsis*, and *Early heading date 1*(*Ehd1*), without an ortholog in *Arabidopsis* [39,40] (Figure 1). Moreover, *Grain Number, Plant Height, and Heading Date7* (*Ghd7*) is unique in grasses [16]. *Ehd1* is a B-type response regulator that induces the expression of *Hd3a* in rice,

conferring SD promotion of flowering in the absence of a functional allele of *Hd1*. It also controls *Rice Flowering Locus T (RFT)* gene in LD conditions independently of *Hd1* [40]. In LD conditions *Hd1* acts as a flowering repressor inhibiting *Hd3a* expression but promotes its expression and subsequent flowering in *SD* [41]. *Ghd7* is a small protein with a CCT-domain that represses *Ehd1* expression and downstream *Hd3a/RFT1* expression in LD conditions to delay flowering [42–45]. Recent studies demonstrated that the interaction between *Ghd7* and *Hd1* can play a critical role in repressing *Ehd1*. Under SD conditions *Hd1* activates the expression of *Ehd1* at night but not in the day while under LD conditions *Hd1* represses its expression in the morning. Indeed, *Hd1* repressor activity requires a proper *Ghd7* function under LD conditions to repress *Ehd1* in the morning. In contrast, *Ghd7* can repress the expression of *Ehd1*, *Hd3a* and *RFT1* by itself under all photoperiod conditions [15].

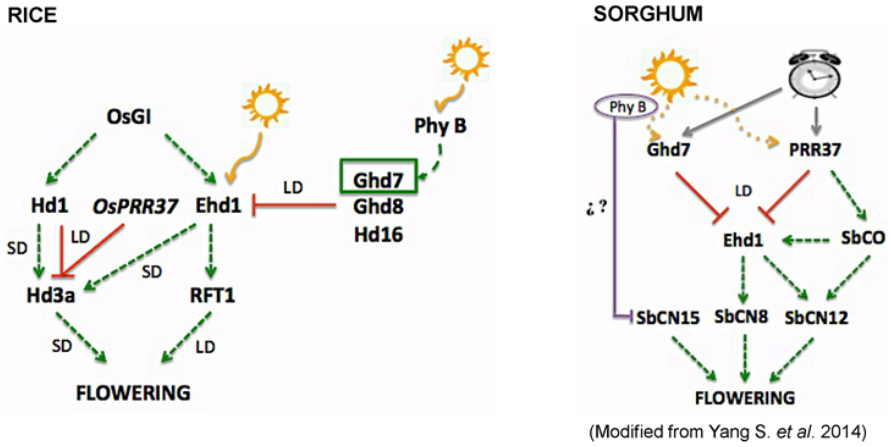

Figure 1. A simplified model of flowering time under short-day (SD) and long-day (LD) conditions in rice and sorghum. A dashed green arrow indicates transcriptional activation and a solid red line indicates transcriptional repression.

2.2. C_4 Grasses

Previous studies identified two floral activators in sorghum, a SD plant: *SbEhd1* and *SbCO*. *SbCO* is a homolog of the floral activator *CO* in *Arabidopsis* and an ortholog of *Hd1* in rice. It promotes early flowering in both LD and SD conditions, and increases the expression of *SbEhd1*, *SbCN8*, *SbCN12* and *SbCN15* [16]. Genetic analyses and expression studies in sorghum reveal that *SbCO* shares a conserved CCT-domain with *TOC1*, *PSEUDORESPONSE REGULATOR PROTEIN 37* (*PRR37*), *Ghd7* and *HEME ACTIVATOR PROTEINS (HAP)*. *SbCO* also increases expression of *SbEhd1*, a promoter of *Hd3a* in rice. In comparison with rice, *Ehd1* regulates positively the expression of *RFT1* to promote flowering; however, no ortholog of *RFT1* is present in the sorghum genome. *SbPRR37* (Ma_1) and *Ghd7* (Ma_6) inhibit flowering, reducing the expression of *SbEhd1* and *SbCN8/12* (florigens) under LD conditions, but not in SD (Figure 1). The ability of *SbPRR37* to inhibit their expression could be due to inhibition of *SbEhd1* or *SbCO*, activators of *SbCN8* and *SbCN12* expression [14,16]. The *PhyB* regulation of *SbCN15* expression may modify flowering time in a photoperiod-insensitive manner [46] (Figure 1). In switchgrass, the flowering time regulatory network is similarly to maize (*Zea mays* L.) and is regulated by both photoperiod-dependent and autonomous pathways. Some conserved flowering genes such as *FT-like gene* (*ZCN8* in maize) and *INDETERMINATE 1 (ID1)* have also been identified in the maize genome. The study of genes involved in flowering of switchgrass is relatively new. Hence, the functions of *FT-like* gene in switchgrass germplasm have not been clarified yet but may contribute to delayed flowering time as in maize [18]. Thus, the switchgrass *FT* homolog may have similar functions to the maize *FT* gene and is down-regulated by the expression of *LONG VEGETATIVE PHASE ONE (AtLOV1)* in switchgrass [18]. Overexpression of *AtLOV1* causes delayed flowering time in switchgrass but does not enhance cold tolerance as in *Arabidopsis* [18]. Sorghum is closely related

to *Miscanthus* spp., a promising candidate C_4 bioenergy crop in temperate climates. The *CO/Hd1* sequence in *Miscanthus sinensis* Andersson was identified as *MsiHd1* with two types of diverged loci, *MsiHd1a* and *MsiHd1b*. The *MsiHd1* gene encodes two conserved B-box zinc finger domains and a CCT domain. Two to five different alleles of *MsiHd1* were found in *Miscanthus* accessions from mainland Asia and from Japan, suggesting that *MsiHd1* consists of at least three loci in the *Miscanthus* genome with small differences in the number of functional alleles [38]. From preliminary data we identified at least three alleles suggesting that *MsiEhd1* has two loci in the *Miscanthus* genome, *MsiEhd1*a and *MsiEhd1b* in comparison to sorghum, rice and maize, which have only one gene. We also detected two loci in *MsiGhd7*. The current diploid *M. sinensis* evolved from genome duplication of its progenitor that was very close to a sorghum ancestor [46]. Gene duplication is a key mechanism in evolution because it can provide genes with new functions.

3. QTLs Analysis

To understand the complex genetic network of flowering in perennial ryegrass (*Lolium perenne* L.), a C_3 forage grass, a number of genes have been identified through QTL mapping, using different plant material and genetic maps and by sequence homology with *Arabidopsis*, rice and maize [47]. The genomic and phenotypic variations associated with perennial ryegrass *LpFT3*, an ortholog of *FT*, were assessed in a diverse collection of nine European germplasm populations, identifying a total of 7 haplotypes. The results indicated a significant association between allelic variation in the *LpFT3* gene and flowering time. Haplotype C was associated with early flowering and the A and B haplotypes with late flowering. The variations were identified in the predicted sequence and in non-coding regions, mainly within the 5′ region of the coding sequence which is strongly conserved [48]. Comparative analysis established close proximity between genetic markers related to the *DGL1*, *Ph1* and *OsPIPK1* ortholoci and the corresponding perennial ryegrass QTLs. This suggests that *DGL1* and *Ph1* ortholoci may provide candidate genes for the herbage yield-related QTLs on linkage group 3 (LG3). The physical location of the *OsPIPK1* gene (a heading date locus) was located at the 28.2 Mb position of rice chromosome 3, close to the predicted *CDO795* ortholocus (23.1 Mb). Further studies have suggested that the *CDO795*-linked heading date QTL was equivalent to a rice heading date QTL, *dth3.3* (Gramene QTL Acc. ID AQFE011). As a consequence, the perennial ryegrass *OsPIPK1* ortholocus may be related to the heading date QTLs on LG4 [49]. In addition, the major QTL in the F_2/WSC and ILGI perennial ryegrass populations was identified on LG7, which is associated with the position of the genes *Hd3a* and *Hd1*, two heading date genes of rice on chromosome 6. However, analysis of the ILGI population grown in Japan identified a QTL on LG4, but not the QTL reported on LG7 [50,51]. This result emphasizes the importance of adaptation in plants to the broad range of agro-environmental conditions in which they grow. In sorghum, three significant QTL associated with flowering time, *PHYB (Ma3), PHYC (Ma5)* [52] and *SbGHD7 (Ma6)* [14,52] were identified, through analysis of flowering variation in LD using an F_2 population, which explained *~50% of the* phenotypic variance for flowering time [52]. Recessive *ma3R* alleles from 58 M populations associated with *Ma3* QTL produced early flowering time phenotypes; however, dominant alleles of Sb*Ghd7 (Ma6)* and Sb*PRR37* act in an additive manner to delay floral initiation for ~175 days until day-lengths decrease below 12.3 h [14,52]. Sorghum accessions exhibit significant variations in flowering time in response to day-length. One QTL controlling photoperiod sensitivity was detected on chromosome 1 under SD, and one QTL controlling photoperiod insensitivity expression was detected on chromosome 4 under 12 h and natural photoperiod conditions, from the SSR markers *Xtxp61* and *Xtxp51* respectively [53]. Under LD, *a cross between tropical and temperate sorghums* (Sorghum *propinquum* (Kunth) Hitchc.×*S. bicolor* (L.) Moench), revealed one QTL *FlrAvgD1* located in chromosome 6 in a 10 kb interval, which accounted for 85.7% of the variation in flowering time. This interval contains a single annotated gene, *Sb06g012260*, which is a member of the *FT* family of transcription factors. *Sb06g012260* is unique to panicoids and suppresses flowering, although it is quite distant evolutionarily from other *FT* family members that are floral suppressors [54]. In *M. sinensis* five putative flowering QTLs were detected using the Multiple

QTL model (MQM) approach for plants grown in the years 2000 and 2001 [55]. Only QTL F12 was detected in both years on LG1, F11 and F13 were only detected in the first year while F14 and F15 were detected in the second year. So these QTLs may depend on interactions between genotype and environment. A genome-wide association study may be identified association with gene network in flowering time.

4. Circadian Clock and Photoperiod Response

The circadian clock plays an important role in seasonal flowering time regulation of angiosperms; photoperiodic time measurement is based on the interaction between the endogenous circadian clock and environmental light signals in *Arabidopsis* [56,57]. The plant circadian system consists of biochemical timing mechanisms that temporarily modulate the function of several signalling pathways to measures changes in day-length and promote suitable timing of flowering to maximize reproductive success [2,3,6,7,9,10,13–15,21,24,26,28,29,31,36,38,41,42,46,48,49,56–66]. The photoperiod response on flowering time varies among grasses. Barley (*Hordeum vulgare* L.) and wheat are LD plants, while rice and sorghum are SD plants [52]. Flowering is regulated through the *CO* and *FT* genes [66]. *GI* plays an important role in regulating the circadian clock and flowering, promoting *CO* gene expression and light response. The rice ortholog of *GI*, *OsGI*, is a positive regulator of *Hd1* expression under both SDs and LDs [24]. Mutation in *OsGI* reduced photoperiod sensitivity in rice [36] and affected the expression of *LATE ELONGATED HYPOCOTYL (LHY)* and several *PSEUDO RESPONSE REGULATOR (PRR)* genes. However, *PRR37* expression was not affected in the *osgi* mutant, suggesting independent control of heading date by these factors [66]. *Hd1* is predominantly regulated by the circadian clock through *OsGIGANTEA (OsGI)* [64] and possesses two contrasting functions in the regulation of the rice ortholog of *Arabidopsis FT* gene, *Hd3a*. The bi-functionally mechanism of *Hd1* involves the action of the red-light photoreceptor *phytochrome B (phyB)*, a primary cause of long-day suppression of flowering in rice [39,60]. Over-expression of *Hd1* causes a delay in flowering under SD conditions and a single extension of day-length decreases *Hd3a* expression consistently with the duration of daylight [44,59]. The repression of flowering by *Hd1* under LD conditions is enhanced by the kinase activity of *Heading date 6 (Hd6)*, a gene encoding the α subunit of protein kinase CK2 (CK2α) [58]. *Hd6* is a QTL involved in photoperiod response in rice. To induce delayed flowering under LD conditions, *Hd6* requires the presence of functional *Hd1* alleles and plays a critical role in *Hd1* activity. Despite this, *Hd6* regulation is not mediated by changes in the circadian clock [37,65]. The rice genome contains two important genes for photoperiodic regulation: *Ehd1* and *Ghd7*, specific to grass species such as rice, maize and sorghum but absent in the *Arabidopsis* genome [44]. The expression of *Hd3a* is also regulated by *Ehd1* conferring SD promotion of flowering and controlling *FT-like* gene expression independently of the *Hd1/CO* photoperiodic flowering pathway [30,40] (Figure 1). *Hd1* and *Ehd1* expression are controlled by the circadian clock, although *Ehd1* is also regulated by both blue and red light. In sorghum, *SbCO* expression is not altered significantly in response to day-length. However, *Ghd7*, a floral repressor regulated by the circadian clock and light, represses the expression of *SbEhd1* and *SbCN8* [14]. *Ehd1* expression in rice is strongly repressed by *Ghd7* in LD conditions but in SD conditions *Ghd7* rarely affects flowering time [36].

5. Photoreceptors Involved in Flowering Time

Plants use the phytochrome system to regulate time of flowering and adjust growth based on the duration of dark and light periods (photoperiodism), while the spectrum of the light also affects flowering. Plants use many photoreceptors to detect the intensity and quality of light, including PHYTOCHROMES (PHY), which absorb the red and far-red region of the visible spectrum, and the CRYPTOCHROMES (CRY) [63]. *Arabidopsis* contains five PHYs (A-E), where accumulation of *CO* in LD is due to stabilization mediated by *phytochrome A* (*PHYA*), *cryptochromes* (*CRY1/2*) and *SUPPRESSOR OF PHYA-105 (SPA1)* [61]. However, it has been shown that PHYB signals delay flowering by destabilizing CO protein during the morning and have an inhibitory effect on *FT*

expression [60]. The phytochromes PHYA, PHYB, CRY1 and CRY2 are directly clock regulated under specific light conditions. For example, over-expression of the photoreceptor PHYA under SD conditions promotes flowering, but *phyA* mutants delay flowering in LD conditions. In contrast to *Arabidopsis*, rice and sorghum encodes three phytochromes (PHYA, PHYB and PHYC) [12,52], where *phyA* mutants of rice do not produce significant alterations in flowering time [52,63]. This is despite the high similarity between the *PHYA* locus in *Arabidopsis* rice, sorghum and maize [62] suggesting that a similar response would be expected. However, PHYA mutations in combination with PHYB or PHYC cause early flowering in rice [61]. In addition, studies have shown that PHYC plays an essential role in the acceleration of wheat flowering under LD photoperiods. Moreover, it is stable and functionally active even in the absences of other phytochromes, compared with rice and *Arabidopsis* [67]. Blue and far-red lights promote flowering in *Arabidopsis* and rice, acting through the action of PHYA, CRY1 and CRY2 photoreceptors in *Arabidopsis*, while red light delays flowering [12,33,43,67]. On the other hand, PHYB modulates the expression of genes in response to red light and is the main component of the shade-avoidance mechanism in *Arabidopsis*. *PhyB* mutants revealed that PHYB inhibits flowering under both LD and SD photoperiods, but an over-expression of PHYB in LDs results in early flowering [22]. PHYB is responsible for delayed flowering and *Hd3a* suppression in the presence of a night-break (NB) treatment and activates the *Hd1* expression in rice. The NB treatment is a short exposure to light in the middle of night and was widely used to understand the role of the circadian clock and light on flowering. In recent studies, *photoperiodic sensitivity 5* (*SE5*) and PHYB also suppress *Ehd1* expression, by suppressing *Oryza sativa CO-like4 (OsCOL4)* [39]. Rice mutants deficient in PHYB have reduced sensitivity to red light and are early flowering [43,60]. In contrast, *phyB* null mutations in wheat are connected with delayed flowering [24]. In sorghum, under LD, PHYB (Ma_3) is required for elevated expression of *SbPRR37* and *SbGHD7* during the evening to inhibit flowering (Figure 1). This response results in repression of *SbEHD1*, *SbCN12*, *SbCN8* and floral initiation. *Ghd7* represses *Ehd1* expression in response to the red light signal in the morning mediated by phytochromes. In SD conditions, PHYB may have a limited effect on the expression of these genes as peak *SbPRR37* and *SbGHD7* expression is highest in the morning and lowest during the evening compared with expression in LD. The inactivation of PHYB results in early flowering in LD [43,52].

6. Conclusions and Perspectives

Genetic analysis in *Arabidopsis* has allowed the identification of different pathways that promote flowering in response to environmental conditions and developmental regulation. The primary mechanism of the photoperiod pathway in plants is evolutionary conserved for flowering signalling. *CO* is the central regulator in promoting flowering and exhibits complex regulation. In addition day-length and the circadian clock control critical aspects of flowering. The effect of *GI* on flowering is associated with promoting expression of genes related to the circadian pathway. *Arabidopsis* is considered as a model plant to understand flower development while some grasses have defined their own responses and adaptation strategies. For example, *Ehd1* and *Ghd7* genes are unique in grasses in relation to the promotion and repression of flowering time respectively. Due to the lack of nucleotide information in *Miscanthus*, few genetic resources have been developed to clarify the relationship of the *Miscanthus* genome to its close relatives, sorghum and sugarcane (*Saccharum* spp.). Comparisons between the *Sorghum* genome and the genus *Miscanthus* reveal that whole genome duplication occurred in *Miscanthus* after its divergence from a common ancestor shared with sorghum. The base chromosome number of *Miscanthus* is approximately twice that of sorghum with nominally diploid and tetraploid species [46,68]. Analysis of natural variation in flowering in different ecotypes of grasses with economic value, such as *Miscanthus* spp. and switchgrass, is necessary to clarify the molecular network of flowering time control in these species. Through breeding programs, favorable alleles of QTLs then can then be efficiently introduced into elite cultivars to generate new varieties with high biomass productivity and beneficial adaptations to environmental changes.

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