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Awn microstructural observation revealing multifunction of awn-inhibitor Gene *B1* in near-isogenic lines with different awn length

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Abstract. Awn is one of wheat morphological characteristics and acts as a highly effective organ for photosynthesis in wheat. Variation in awn length is controlled primarily by three major genes, most commonly the dominant awn suppressor *Tipped1* (*B1*). So far, the function of *B1* is not well understood. In this paper, we identified a pair of near-isogenic lines (NILs) containing different awn inhibition gene *B1* alleles and observed microstructures and ultra-microstructure of their awns. The typical awns differences between the NILs represented by the cross-sectional area and chloroplasts number. Long awn line had a larger cross-sectional area, and more cells in various parts of tissues, especially the cells containing more and larger chloroplasts, which could attribute to a strong cytological basis for photosynthesis. The results may suggest that the gene has pleiotropic effects in the control development of awn tissue structure and grain yield.

Keywords: Common wheat, NILs-*B1*, awn length, SEM, anatomy.

INTRODUCTION

Wheat awn is a long and stiff filamentous prolongation of the lemma, which plays an important role in seed dispersal, burial, transpiration and photosynthesis (Grundbacher, 1963; Elbaum et al., 2007). It is one of the morphological characteristics of wheat and other species, such as barley (*Hordeum vulgare*), rye (*Secale cereal* L.), oat (*Avena sativa* L.), rice (*Oryza sativa* L.) and sorghum (*Sorghum bicolor* (L.) Moench). Awn has prickly barbs on its surface, which aid the seed dispersal by attaching to the animal fur (Yuo et al. 2012). In wild wheat, each spikelet has a pair of long awns, which can be guided to fall an appropriate germination site with the balance provided by awn. Moreover, the awns would bend as they dry and straighten in a damp environment, these movement driven by the daily humidity cycle propels the seeds into the ground (Elbaum et al. 2007). Awn is an important

organ of gramineous crop panicle, which ensures wheat adapt to some special environment such as warm growing regions (Motzo & Giunta, 2002). Therefore, it has important biological significance, but its function and genetic research are not well understood.

Genetic analysis has shown that the awnless trait dominated over the awned ones. Three dominant inhibitor alleles, *Hooded* (*Hd*), *Tipped 1* (*B1*), and *Tipped 2* (*B2*), were subject to simple Mendelian inheritance, which has been identified in common wheat (Sourdille et al. 2004; Mackay et al. 2014). *Hd* was located on the short arm of chromosome 4A and resulted in short and broad awns that are curved inward into a hood shape. Previous studies have shown that the effect of the wheat *B1* gene on the length of awn is the strongest, results in very short or absent awns at the base and the middle of the ear (Yoshioka et al. 2017; DeWitt et al. 2020; Huang et al. 2020; Wang et al. 2020). Recent fine mapping located *B1* to a region on the long arm of chromosome 5A containing only two predicted genes, including C2H2zinc finger transcriptional repressor TraesCS5A02G542800 (DeWitt et al. 2020). Another group also reported that *TraesCS5A02G542800* encoding a C2H2 zinc finger protein putatively functions as a transcriptional repressor predominantly responsible for awn inhibition in wheat (Huang et al. 2020; Zhang et al. 2020) identified 4 SNPs in the promoter region of *TraesCS5A02G542800* and proved the *TraesCS5A02G542800* promoter as the control gene of *B1* awn length inhibition site, which was named as *ALI-1* (Wang et al. 2020). All in all, there is a consensus that the *B1* gene is located in the candidate gene or promoter, but its function and regulation mechanism is still uncharacterized.

In the study, line SN051-1(long awn) and line SN051-2(short awn) are NILs with the only difference of awn-length (Du et al. 2010). Genetic analysis indicated that awn from the NILs was controlled by awn-inhibitor gene *B1*. We used optical microscope and Scanning electron microscopy (SEM) to observe the cross-sections, epidermal tissues, and anatomic features of awns and revealed their differences among NILs-*B1*. At the same time, we investigated the photosynthetic rate of the ear and thousand-grain weight of the NILs. The investigation on NILs-*B1* is expected to lay a foundation for the study on the function, genetic mechanism of the awn in common wheat.

MATERIALS AND METHODS

Plant material and growth conditions

A pair of wheat lines with different awn lengths, SN051-1 (long awn, *b1b1*) and SN051-2 (short awn, *B1B1*), were used in the current study. They were developed from the F_8 progeny of Octoploid *Triticale* Jinsong49 and Octoploid *Tritittrigia* Xiaoyan7430, and then were self-crossed for 5 generations (Du et al. 2010). The morphological, genetic and molecular marker analyses showed that they differed in the wheat awn inhibition gene *B1*: line SN051-2 contained the dominant allele *B1*, and SN051-1 possessed the recessive allele *b1*. The hybrid was obtained from the cross of SN051-1 × SN051-2, F_2 population and two back-crosses BC_1F_1 populations including SN051-1/SN051-2//SN051-1) and (SN051-2/SN051-1//SN051-1) BC_1 were developed to study genetic of awn length. All the materials, including SN051-1, SN051-2, their F_1, F_2, and BC_1F_1 hybrid, were grown in two replications as 1 m rows spaced 30 cm apart at Tai'an, Shandong Agricultural University. At heading stage, presence or absence and awn length of the materials were studied. Thousand-grain weight of the lines were investigated at the harvest stage.

SSR Polymorphic Analysis

Total DNA was extracted by the SDS-phenol method (Liu Cheng et al. 2006). The primers used included Xgwm, Xgdm, WMC, BARC, CFA, CFD, STS-MAG, EST-KSUM, EST-CWES, and EST-DUPW in 1690 pairs. The sequence and location of the primers used can be found at http://wheat.pw.usda.gov, and the primers used were synthesized by Shanghai bioengineering co. LTD. The PCR system was 15μl, including 10×Buffer 1.5μl, 25 mmol/L $MgCl_2$ 1.2μl, 2.5 mmol/L dNTP 0.9μl, 25 ng/ 1l primer 3μl, 5U/μL *Taq* enzyme 0.12μl, deionized water 5.28μl, 80 ng/1μl genomic DNA 3μl. Amplification program reference Hao method (Hao et al. 2008), 94 °C modified 4 min, then 15 cycles touch down PCR process sequence, 94 °C modified 45s per cycle, 65 °Crenaturation 50 s diminishing per cycle (1 °C) and 72 °C 55s extension, the final 30 cycles of ordinary PCR process sequence is 94 °C modified 40s, 40s, 50 °C renaturation 72 °C extends 40s, then, it was extended at 72 °C for 5min, and stored at 10 °C after amplification. The amplified products were electrophoresis with 6% nondenatured polyacrylamide gel and stained with silver nitrate.

Microstructure observation of awn and its cross-section

Ten days after the anthesis, awns were collected from spikes of 2 or 3 plants and immediately fixed in 2.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer (pH 7.0) overnight at room temperature, post-fixed with 1% (w/v) osmium tetroxide in phos-

phate buffer at 4 °C, and then embedded in Epon812 (Shell Chemical, Houston, TX, USA) following a standard dehydration procedure. Transverse sections (about 1 cm from the base of the awn, 2.5 μm thick, were cut with an LKB-V microtome, and then stained in 1% (w/v) toluidine blue in 1% (w/v) disodium tetraborate, and observed under an optical microscope (Olympus BX-51, Japan) with automatic camera.

Determination of photosynthetic rate in ear

The photosynthetic rate of flag leaf was determined by LI-6400 portable photosynthesis systems, the panicle photosynthetic rate was measured by GXH-305 infrared CO_2 gas analyzer and a special assimilation chamber on May 25. Each treatment was repeated three times.

RESULT

Genetic analysis of awn length phenotype

SN051-1 and SN051-2 showed no significant difference on plant morphology, but only on awn length, F_1 hybrids presented short awn (Figure 1), F_2 individuals separated into two groups (short awn phenotype, long awn phenotype) which fitting a 3:1 segregation ratio, BC_1 individuals separated into two groups (short awn phenotype, long awn phenotype) which generally fitting a 1:1 segregation ratio, which were consistent with Mendelian segregation of a single gene (Table 1). This result indicated a single genetic locus, represented here as *B1*, which was associated with the dominant short awn phenotype that could be used as a phenotypic marker.

Genetic background and difference analysis based on DNA level on the parents

The F_2 population was analyzed with polymorphic SSR primer Xgwm291, and a band of 140 bp was amplified in SN051-1 and most F_2 long-awn plants, and a band of 110 bp was amplified in SN051-2 and most F_2 short-awn plants (Figure 2). The marker has been located at the end of the long arm of 5A chromosome in wheat, and the genetic distance between the marker and the *B1* of awn length suppressor gene at the end of 5A chromosome is 2.0~4.5cm (Paillard et al. 2003; Somers et al. 2004). According to the results of the molecular marker and the characterization between near-isogenic gene line, SN051-1 and SN051-2 are the near-isogenic gene lines of awn-length inhibiting gene *B1*. This is consistent with the study of the *B1* gene by DeWitt (2019) and Wang DZ (2020).

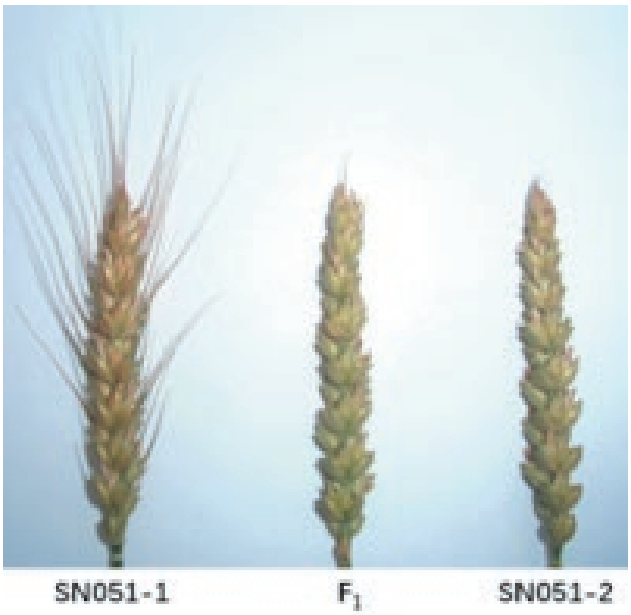

Figure 1. Awn performance of SN051-1, F_1, and SN051-2. SN051-1: long awn phenotype; SN051-2: short awn phenotype; F_1: short awn phenotype.

In a total of 1690 pairs of SSR and 209 sequence-tagged sites (STS), primers were selected for genomic DNA polymorphism analysis of SN051-1 and SN051-2. A total of 64 SSR primer pairs and 7 STS primers were

Table 1. Segregation for long awn or short awn in F_2 populations derived from F_1 of SN051-1 × SN051-2.

Cross combination and generation	Plant number	Observed		Expected		χ^2	Segregation ratio
		As	Ad	As	Ad		
(SN051-1/SN051-2) F_2	536	401	135	402	134	0.00995	3:1
(SN051-2/SN051-1) F_2	456	347	109	342	114	0.292	3:1
(SN051-1/SN051-2//SN051-1) BC_1	142	75	67	71	71	0.45	1:1
(SN051-2/SN051-1//SN051-1) BC_1	128	73	55	64	64	2.531	1:1

Ad: awned; As: awnless.

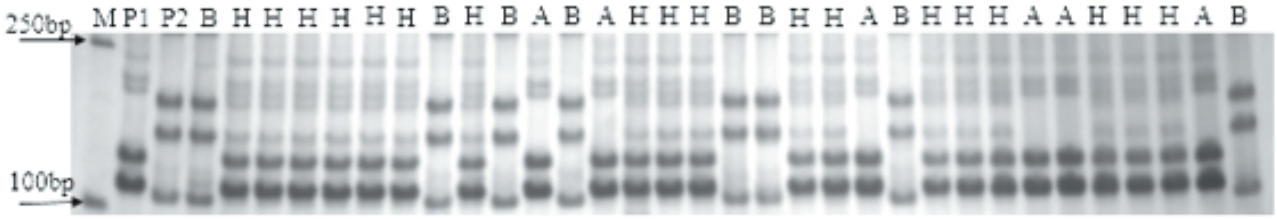

Figure 2. Amplification results with primer Xgwm291 in parents and the F_2 population. M: DL2000 marker; P1: SN051-2; P2: SN051-1; B: F_2 individuals with long awn in; H: F_2 individuals with a short awn.

amplified difference between the two materials, accounting for the proportion of polymorphism primers of 3.79% and 1.38%, respectively, which reflected the similar genetic background and the mine genetic difference between the two lines. Other than SSR and STS markers, we also used Specific-locus amplified fragment sequencing (SLAF-seq) method to verify the single nucleotide polymorphisms (SNP) difference between SN051-1 and SN051-2. The results showed that only 3,679 tags among 216,192 tags produced were accounted for about 1.7% of polymorphic percentage, which also illustrated the mine difference. Therefore the combination of the phenotype characteristics and DNA genetic analysis, SN051-1 and SN051-2, can be considered as near iso-genic lines.

Awn primordial development of the NILs

Besides the awn length trait, we further investigated other awn related traits on the parental pair lines. Awns emerge from the lemma of young spikelets at an early developmental stage. Awn development process in a spikelet (Figure 3) showed SN051-1, and SN051-2 appeared awn primordial without differences on pistil and stamen differentiation stage (Figure 3A). while further develop to anther connective formation stage (Figure 3B), awn primordial of SN051-1 elongated and grew longer, while the awn length of SN501-2 stopped elongating. On the tetrad formation stage, the awn length difference became more obvious, with the characteristics of SN051-1 having long awn and SN051-2 short awn. Scanning electron microscopy (SEM) observation indicated the two NILs significant differences in awn development.

Awn external surface and cross-section features of NILs

Awn external surface contained several stomata and hairs in both SN051-1 and SN051-2, while the number of stomata in a unit area of SN051-2 awns had more stomata and SN051-1 awns had more hairs (Figure 4A). The cross-section of awn contains vascular bundles, parenchyma, and sclerenchyma in NILs (Figure 4B), while SN051-1 awns displayed more areas for each tissue, more complete cell structure and more regular cell arrangement. Moreover, the difference showed by morpho-anatomical structure using the semi-thin section was more obvious (Figure 4C). The above results suggest that SN051-1 has a more obvious xerophytic structure.

Chloroplast in awn cell of NILs

Observation of the ultrastructure of chloroplast in awn cell by transmission electron microscopy (TEM) showed that chloroplast arranged along the cell wall and

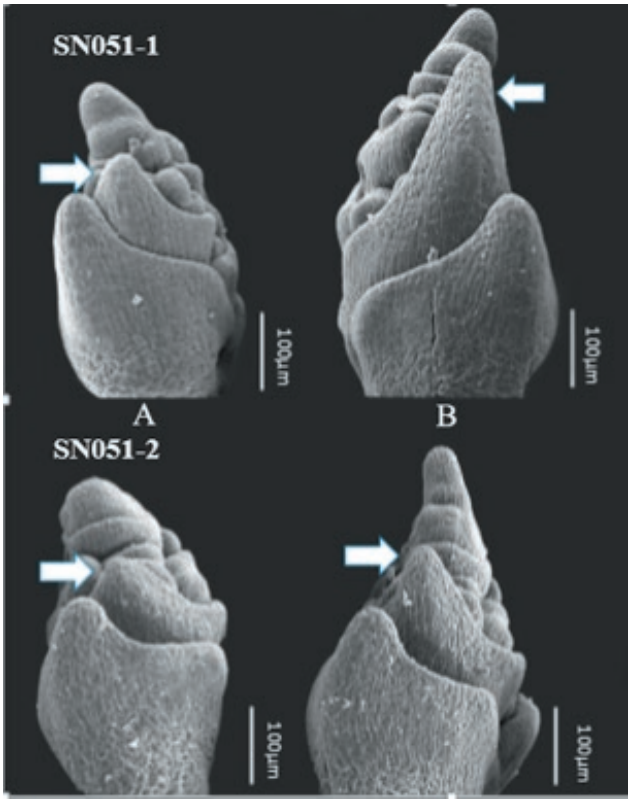

Figure 3. SEM images of SN051-1 and SN051-2 spikelets at different development stages. Arrows point to awn primordia; A pistil and stamen differentiation stage; B anther connective formation stage.

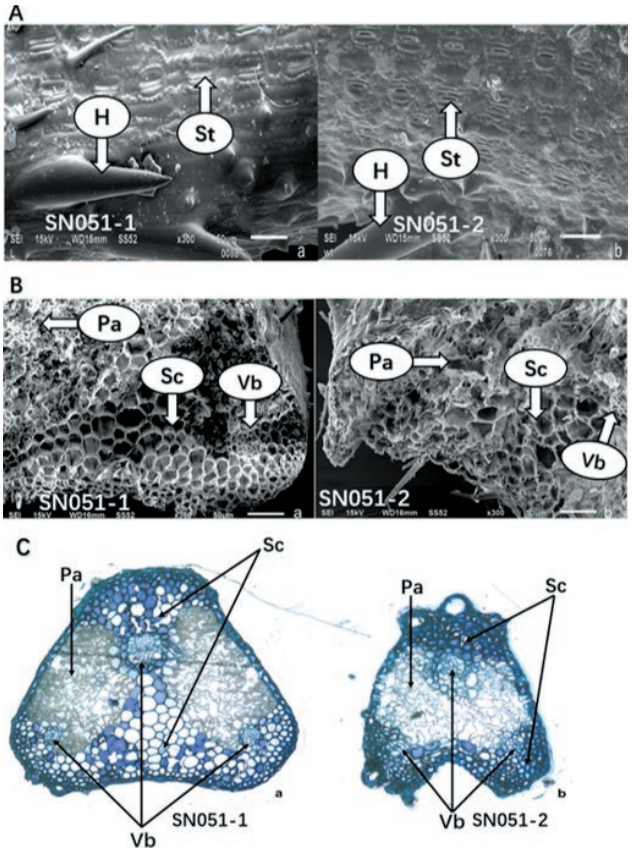

Figure 4. Awn external surface and cross-section of NILs. A. SEM images of the awn epidermis. St: stomata; H: hair. a. SEM images of the epidermis of SN051-1 awn, b. SEM images of the epidermis of the SN051-2 awn. B SEM images of the morpho-anatomical structure of awns. Vb: vascular bundle; Sc: sclerenchyma; Pa: parenchyma an SEM image of the morpho-anatomical structure of SN051-1 awn b SEM images of the morpho-anatomical structure of the SN051-2 awn. C. Morpho-anatomical structure of awns by semi-thin sections. Vb: vascular bundle; Sc: sclerenchyma; Pa: parenchyma.

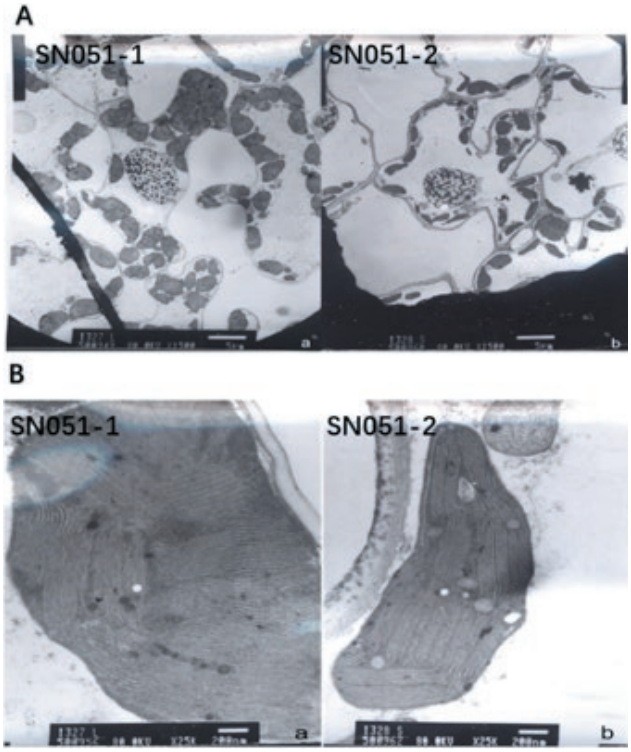

Figure 5. Ultrastructure and TEM results of chloroplast. A. Ultrastructure of chloroplast in SN051-1 awn cells b ultrastructure of chloroplast in SN051-1awn cells. The chloroplast in SN051-1 awn cells was bigger and more than that in SN051-2. B. TEM images show the ultrastructure of a single chloroplast in awns. An ultrastructure of single chloroplast in SN051-1 awn cell b ultrastructure of single chloroplast in SN051-1 awn cell. The chloroplast in SN051-1 awn cell was bigger than that in SN051-2.

are similar to those in leaves and well developed (Figure 5A). SN051-1 had more chloroplasts in each cell (Figure 5A), and the volume of the chloroplast of SN051-1 awn cell was significantly bigger than that in SN051-2 cells (Figure 5B). Besides, because the long awn has a larger surface area than the short one, it means that it has more chloroplasts, suggesting that wheat lines with long awn had a better capacity of photosynthetic than short awn plants.

Differences in photosynthetic rate and grout rate and ear in the near-isogenic lines (NILs) with the difference of awn-length

The photosynthetic rates of flag leaves and the panicle of SN051-1 and SN051-2 were measured 27 days after flowering (Figure 6). It can be seen that there was no

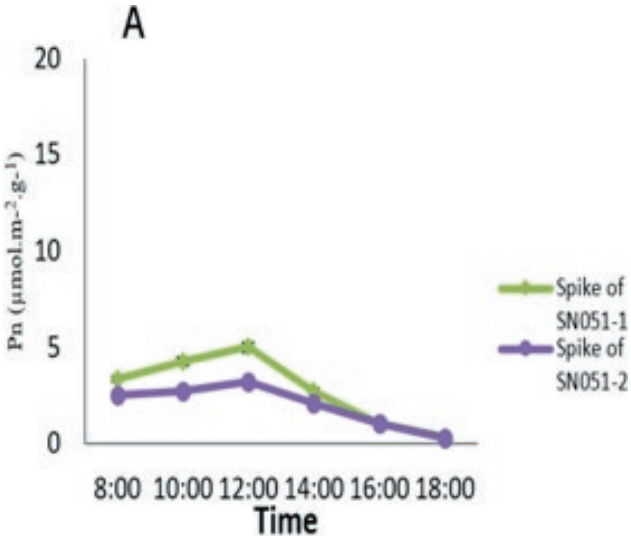

Figure 6. Curve of the daily net photosynthetic rate of flag leaf for SN051-1 and SN051-2.

Table 2. the thousand seed weight of long awn plants and short awn plants.

Materials	Awns	Average TKW	F Value
(SN051-1/SN051-2) F2	Long awn	32.396	8.637**
	Short awn	30.778	
(SN051-2/SN051-1//SN051-1) BC1	Long awn	32.709	5.146*
	Short awn	30.743	

obvious midday depression in a panicle; the panicle net photosynthetic rate of SN051-1 was significantly higher than that of SN051-2, and the difference reached a significant level. This result indicated that the presence of awn significantly increased the photosynthetic capacity of the panicle.

The comparison of thousand-grain weight per line in F_2 generation between long awn plant and short awn indicated that thousand kernels weight with long awn is higher than that without an awn (Table 2). This result suggested that awn played an important role in the accumulation of assimilates and photosynthesis in the later stage of grain filling, which resulted in the difference of thousand-grain weight between long awn plants and short awn plants.

DISCUSSION

Awn-inhibitor gene B1

In common wheat, *B1* (5AL), *B2* (4AS), and *Hd* (6BL) are known dominant suppressor genes of awn, and different combinations of them can lead to changes in awn phenotype (Sourdille et al., 2004; Mackay et al., 2014). Homozygotes of three recessive alleles *b1*, *b2,* and *hd* were awned phenotypes, the presence of one dominant allele inhibited the elongation of awn, and the plants containing two dominant inhibitory alleles were short awn phenotypes (Antonyuk et al., 2012). Previous studies have shown that the effect of the wheat *B1* gene on the length of awn is the strongest, results in very short or absent awns at the base and the middle of the ear. (Yoshioka et al., 2017; DeWitt et al., 2020; Huang et al., 2020). So far, although the *B1* gene has been cloned, the mutation site and function of the *B1* gene are still controversial in previous researches, and further experimental verification is needed, and its expression and inhibition mechanism are still a mystery.

Compared with other photosynthetic organs, awn has several advantaged conditions for photosynthesis (Wang et al., 1993). The presence of awns can double the rate of net ear photosynthesis (Evans and Rawson, 1970), it contributed about 40-80% of the total spike carbon exchange rate, depending on the species (Grundbacher 1963; Das and Mukherjee 1991; Blum et al. 1985; Khaliq et al. 2008; Motzo and Giunta 2002). Statistical analysis found that spikelet number of short awn individuals was higher than that of long awn individuals, but the grain weight and bulk density of long awn individuals increased by 6.3% and 11.6% compared with that of short awn individuals. The previous results showed that the *B1* gene was significantly correlated with spikelet number, grain weight, and bulk density of wheat (DeWitt et al. 2020). However, its regulation mechanism is not clear.

The agronomic characters of SN051-1 (long awn) and SN051-2 (short awn) in this study were same, and other characters, such as anthesis date, plant height, spike length, spike number per plant and kernels per spike, were also similar, only the length of awn and grain weight were different (Du et al. 2010). The results of polymorphic primers also indicate that the genetic backgrounds of the two near-isogenic lines are highly consistent. The results of molecular markers and character analysis of awn showed that SN051-1 (long awn) and SN051-2 (short awn) were a pair of the proximal isogenic lines with awn length suppressor gene *B1* on 5AL chromosomes. SN051-1 (long awn) and SN051-2 (short awn), this excellent experimental material laid a good material foundation for the study of the role of awn and the mechanism of *B1* gene expression and inhibition.

In the present study, we applied SEM to observe long awn microstructure and found wheat long awn possessed typical xerophytes structure, which is possibly associated with high adaptation on the special region. It was reported that the proportion of awned varieties has increased over the past two decades in warm growing regions such as the southeastern U.S (Motzo & Giunta, 2002). Furthermore, the awn stomatal density of long awn Line SN051-1 with gene *b1* was less than Line SN051-2, while the awn cross-sectional area, cell volume, and volume ratio of the chloroplast of Line SN051-1 were far larger than Line SN051-2. These characteristics indicated that long awn lines SN051-1 had a stronger photosynthetic capacity, which can contribute to large grain and high grain yield in long awn wheat cultivars, particularly during the grain-filling stages. Moreover, the thousand-grain weight of the long awn NIL-*b1* SN051-1 is higher than that of short awn NIL-*B1* SN051-2. It provides cell structure evidence that awn is the major photosynthetic organ of the spike. In barley, a recent study showed that the awn preferentially expressed genes for photosynthesis, the biosynthesis of chlorophyll and

carotenoids, and reactive oxygen species scavenging, while the lemma and palea overexpressed defense-related genes compared with the awn (Abebe et al. 2009). The results suggests the lemma and palea are mainly protective organs, whereas the awn is primarily a photosynthetic organ. Therefore, molecular evidence that wheat awn is the major photosynthetic organ of the spike is still needed.

CONCLUSION

In this study, SN051-1 (long awns) and SN051-2 (short awns) derived from same cross combination showed morphological traits but awn length difference, and similar genetic background but different Xgwm291 genotype, a linkage marker with Gene *B1*, which can be regarded as near-isogenic lines (NILs) with different awn length. Besides the awn length trait, we further investigated awn cell structure and yield-related traits of the NILs. The results showed there were significant differences between awn anatomy, the photosynthetic rate, thousand kernel weight. The NIL-*b1* line, SN051-1, had long awn, which is more conducive to the cell structure of photosynthesis.

AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: FQ and XL. Performed the experiments: FQ, YZ. Manuscript preparation: FQ and XL. All authors read and approved the final manuscript.

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