Auxins Regulations of Branched Spike Development and Expression of *TFL*, a *LEAFY*-Like Gene in Branched Spike Wheat (*Triticum aestivum*)

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Abstract

Branched spike wheat is a hexaploid germplasm with branched rachis on its main rachises, and the crucial period for branched rachises occurrence and development is just after the two ridges stage of shoot apex. Natural [indole-3-acetic acid (IAA), indole-3butyric acid (IBA)] and synthetic [(1-naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D)] auxins were applied at this period to investigate the spike traits, seedling growth and photosynthesis related characters and expression of a putative homologue of the *LEAFY* in branched spike wheat. The four types of experienced auxins induced similar effects on these foresaid characters, although the impact extents were different among the auxins treatments. More branched rachis, spikelets, fertile florets and longer branched rachis were obtained in plants with IAA and IBA at 0.1 mM or NAA and 2,4-D at 1.0mM than those plants with no auxin treated. Auxin treatments also increased fresh and dry mass, photosynthetic pigment and parameters. *TFL*, a *LEAFY*-like gene was cloned in branched spike wheat and *TFL* mRNA expression was quantified using real-time reverse transcriptase-PCR. Application of the auxins accelerated the rise in *TFL* expression during the periods of branched rachises occurrence and extension. The data supports the hypothesis that auxins play a central role in the regulation branched spike development and *TFL* might correlate with the development of branched rachises in branched spike wheat.

Keywords: auxins, branched rachis, *LEAFY*, photosynthetic parameters, *TFL*, *Triticum aestivum*

1. Introduction

Branched spike wheat is a special hexaploid wheat germplasm which has branches (branched rachises) on its main rachis (inflorescence axis) and bears an overabundance of spikelets and grains on a spike. The grain number in a branched spike can reach 70-130 while that in a single normal spike generally is 35-70. Branched spike wheat has great application potentials in wheat production areas with high speed of grain filling or long term of grain filling period.

A number of studies have been conducted on field performances of the branched spike wheat. The supernumerary spikelet character in this type of spike was affected by light, temperature, and nutrients conditions (Koric, 1975; Peanell & Halloran, 1983), and environmental factors might play a minor effect on the character expression during spike differentiation stage at the same eco-region (Sun et al., 2000).

Traditionally, plant hormones and synthetic plant growth regulators are used as valuable research tools to elucidated physiological responses of plant or probe biochemical control mechanisms. In many bioassays, it has been shown that auxins play an important role in plant growth and development (Cooke et al., 2002). Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are naturally-occurring, plant hormone of the auxin class. IAA is the most common in the auxins, and has been the subject of extensive studies by plant physiologists (Simen & Petrášek, 2011). IBA is a plant hormone in the auxin family and is an ingredient in many commercial horticultural plant rooting products (Ludwig-Müller, 2000). 1-naphthaleneacetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) are synthetic plant hormone in the auxin family and also used for plant tissue culture. NAA is a rooting agent and used for the vegetative propagation of plants from stem and leaf cutting (Naduvilpurakkal et al., 2014). 2,4-D is one of the most widely used herbicides in the world. 2,4-D and as a synthetic auxin it is often used in laboratories for plant research and as a supplement in plant cell culture media such as MS medium (Andrew & Reade, 2010).

Our previous studies indicated that as the young spike developed, endogenous IAA contents in branched spike wheat were much higher than those in normal spike lines. Meanwhile, the relative expression of *TaIAR3*, an auxin synthesis related gene, in branched spike wheat line was also significantly higher than those in normal spike lines. Higher IAA content might involve in the formation and growth of branched rachis in branched spike wheat (Data published lately).

LEAFY, or *FLORICOULA* (*FLO*)/*LEAFY* (*LFY*) genes were characterized initially as floral meristem genes in *Antirrhinum* and *Arabidopsis* (Coen et al., 1990; Weigel et al., 1992), which act as genetic switches during the choice of floral versus shoot fates (Yanofsky, 1995). Expression of *FLO/LFY* has been observed in young vegetative shoot apices and leaf primordia and might involve in the inflorescence architecture which distinct controlling the initiation of floral meristems in many plant species, including *Oryza sativa* (Kyozuka et al., 1998) and *Z. mays* (Bomblies et al., 2003).

The objective of the present study was to compare the effect of natural and synthetic auxins on the branched spike traits, growth and photosynthesis related characters, and the expression pattern of *TFL* in the shoot apex of plant was also determined in responses to auxins. The obtained results may be important for elucidation of the plant hormone role in branched spike development in wheat.

2. Materials and Methods

2.1 Plant Materials, Growth and Treatments

Fen 33, a released branched spike wheat cultivar in HuangHuan wheat zone in China, was grown in Agronomy Experimental Station of Shandong Agriculture University, Taian City, Shandong Province, China, from 2012 to 2013 growing season. The experiment plot was about 8.0 m^2 and the wheat plants were thinly sowed at 6 cm plant spacing and 22 cm row spacing on 8th October 2012. Cultivation management was similar with common practice at local place.

Sowed wheat lines turned green at mid February in 2013. The young shoots were tripped out and observed under microscopy to determine the development stages of shoot apexes. Auxins dissolved in 50% ethanol were applied at experimental concentrations, 0.1 mM IBA, 0.1 mM IAA, 1.0 mM NAA and 1.0 mM 2,4-D (Sigma-Aldrich Co., USA), respectively. An equal amount of 50% ethanol was added to the control. Auxins treatments were performed three times on 3, 6 and 9 days after the two ridges stage of shoot apex. Plants were foliar sprayed until considerable run-off on the leaf surface occurred at early morning. The experiments were carried out with complete random block design with four replicates.

2.2 Determination of Pike Characters

After wheat maturation at June, twenty spikes of main shoots in each replicate plot were randomly taken out to investigate the number and the length of longest branched rachis, the number of spikelet and fertile floret. Numbers of branched rachis and spikelet were counted. The length of branched rachies was measured by using a meter scale. Fertile floret was represented as the grain number in a spike which was counted at the same time.

2.3 Determination of Growth Traits and Photosynthesis Related Characters

After 30 d of first auxins treatment, the plants were removed from the field along with the soils and were dipped in a bucket filled with water. The plants were moved smoothly to remove the adhering soils particles were weighted for fresh mass, and then placed in an oven run at 80 $^{\circ}$C for 24 h. These dried plants were weighed to record the plant dry mass.

Chlorophylls a, b and carotenoids contents (mg g^{-1} FW) were detected according to the methods given by Arnon (1949). Clean leaf materials were homogenized with 80% acetone and centrifuged; the optical density of the acetone extract was measured at 663, 645 and 470 nm using a UV-160A UV Visible Recording Spectrometer, Shimadzu, Japan.

The measured photosynthetic parameters included photosynthesis rate (Photo), intercellular concentration of carbon oxide (Ci), stomatal conductance (Cond) and transpiration rate (Tr). These parameters were detested simultaneously in an open-type leaf pathway by LI-6400 portable photosynthetic apparatus (LI-COR Company, USA). The detesting condition: T = 20±1 ^{o}C, air carbon concentration = 380±5 µl (Lu and Gao 2003).

2.4 Isolation of LFY/FLO Homologue TFL in Branched Spike Wheat

The strategy of direct sequencing of a reverse transcription PCR product was used to obtain the putative homologue of *LFY*/*FLO*, using degenerate primers and cDNA from shoot apical meristems of branched spike wheat plants expected to express a *LFY/FLO* homologue. Total RNA was isolated from young shoot apex tissues using TRIzol reagent (Invitrogen) and RT-PCR was performed using the degenerate forward primer 5′CGC(G)GAGCTC(G)GACGACATGA3′ and reverse primer 5′GCACTGCTCGTAG(C)AGA(G)TGGA3′ to obtain the middle region of *TFL*. The following thermocycling conditions were employed: initial denaturation at 94 ^{o}C for 3 min; 30 cycles of 94 ^{o}C for 30 s, 59 ^{o}C for 45 s, and 72 ^{o}C for 1 min; final extension at 72 ^{o}C for 10 min. The amplified products were separated on a 1.5% agarose gel, visualised and photographed.

The amplified cDNA fragment was independently cloned at least twice into pGM-T Easy vector (Promega) and sequenced. After sequencing, the sequence was submitted to GenBank to identify similar genes. To obtain the full length cDNA sequence of *TFL*, a set of specific primers were designed for the 5′ and 3′ RACEs (Rapid Amplification of cDNA Ends) by using the SMARTTM RACE cDNA Amplification Kit (Clontech, Palo Alto, CA). These primers were:

5′Race: PF: 5′TTTCTTGGCCTTTCTCGCCA3′;

3′Race: PR: 5′AAGAAGAACGGGCTGGACTA3′;

The amplified sequences were cloned and sequenced. The full length cDNA containing ORF for *TFL* was obtained by PCR with forward primer (5′ATGGATCCATGGATCGCCACGACGCCT3′) and reverse primer (5′TTCTAGCCTGTTGCCCGGAGGATCC3′). The primers contained the generated BamHI recognition site (GGATCC) to facilitate the cloning of the cDNAs.

2.5 Phylogenetic Analysis

Phylogenetic comparisons of amino acid sequences of different *LFY/FLO* homologues were obtained from GenBank (http://www.ncbi.nlm.nih.gov/) and aligned with ClustalW mega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic trees based on the complete sequences were generated using MEGA4 and constructed by the neighbor joining (NJ) method. Bootstrap values were derived from 1000 replicate runs.

2.6 Real-Time RT-PCR

For experiments on apices total RNA was extracted from the apical part of the plants. The samples were taken before the beginning of the auxins treatment and then every 10 d until the 30th d after the beginning of the auxins first treatment. At first sampling the apical part of the plants included apex and a little of non-removable young shoots, and afterwards, only included the young spike. RNA was isolated TRIzol from Invitrogen following manufacturer's protocol. Each RNA sample was treated with RNase-Free. DNase (Promega) following manufacturer's protocol in an effort to remove any residual genomic DNA (gDNA). DNase treated RNA was subjected to reverse transcriptase reactions using oligo-dT primer and PrimeScriptTM Reverse Transcriptase (Takara) according to manufacturer's protocol. The gene-specific primers used in RT-PCR were 5′AGAACGACTGCGACGACGA3′ and 5′CCGCATCTTGGGCTTGTTGA3′. As a control, the cDNA sequence of the actin gene was amplified by using the two primers 5′CACGGCATCGTAAGCAACTG3′ and 5′TCCTTCGTAAATGGGCACGGT3′. The following thermocycling conditions were employed: initial denaturation at 94 ^{o}C for 3 min; 30 cycles of 94 ^{o}C for 30 s, 57 ^{o}C for 45 s, and 72 ^{o}C for 1 min; final extension at 72 ^{o}C for 7 min. The amplified products were separated on a 1.5% agarose gel, visualised and photographed. The obtained CT values were analyzed by averaging three independently calculated normalized expression values for each sample. PCR products were sequenced to ensure amplification products for the purpose of gene fragment. Expression values are given as the mean of the normalized expression values of the triplicates, calculated according to $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

2.7 Statistical Analysis

Branched spike wheat with auxins treatments were arranged in a completely randomized design with four treatments. Analysis of variance was performed using the SPSS software package. Analysis of variance (ANOVA) was performed on the data to determine the least significant difference (LSD) among treatment at P < 0.05 and Duncan's multiple range tests were applied for comparing the means.

3. Results

The two ridges stage of shoot apex in branched spike wheat in the field ended at about February 20th, in the spring of 2013. The shoot apex entered the glume differentiation stage and the stage lasted approximately 11 days. Afterwards, the floret differentiation stage and stamen and pistil differentiation stage lasted about 12 days and 7 days respectively. The plants entered anther connective stage at about March 22rd. There were about 30 days from the end of two ridges stage to pistil and stamen differentiation stage in the spike lines.

3.1 Effect of Auxins on Branched Spike Characters

Auxins altered notably the characters of branched rachis. The experiment showed that IAA and IBA at 0.1 mM, and PAA and NAA at 1.0 mM were effective in inducing inerratic branched rachis (Figure 1). IBA at 0.1 mM induced the highest increase in the number of branched rachis by 82.6%, 0.1 mM IAA by 78.3%, 1.0 mM NAA by 46.7% and 1.0 mM 2,4-D by 57.6% in comparison with the plants with no auxin treatment. The highest length of branched rachis (19.7mm) was observed in plants treated with IBA. The length of the longest branched rachis were 18.9 mm, 18.4 mm and 14.5 for IAA, NAA and 2,4-D, respectively. For the number of spikelet in a whole spike, IBA and IAA reached 74.9 and 73.6, respectively. The number of spikelet in whole spike treated with 1.0 mM NAA and 2,4-D reached 57.2 and 53.4, respectively. The fertile floret per spike in wheat treated with 0.1 mM IBA, 0.1mM IAA, 1.0 mM NAA and 1.0 mM 2, 4-D reached 90.6, 87.6, 84.9 and 75.4 respectively.
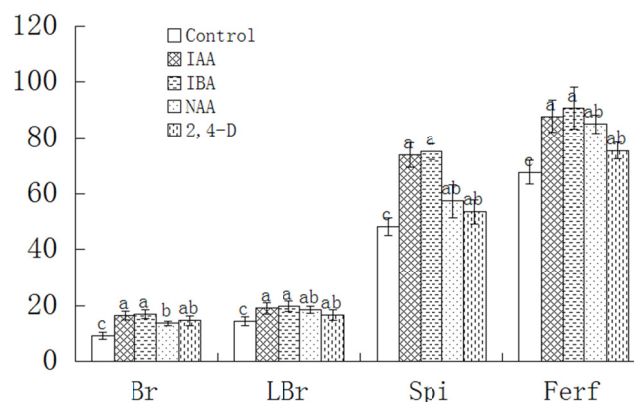

Figure 1. The effect of auxins (IAA, IBA, NAA, 2,4-D) on the number of branched rachies (Br), the length of the longest branched rachies (LBr) (mm), spikelet number (Spi) and fertile floret number (Ferf) in a branched wheat inflorescence after harvest

Note. Data are the means of four independent experiments ± SD. Treatment with at least one letter the same are not significantly different according to Duncan's test.

3.2 Growth Traits and Pigment Contents

Branched wheat treated with IBA at 0.1 mM was characterized by the highest (23.0%) increase in plant fresh after 30 d of first auxin treatment (Table 1). Other auxins showed weaker biological activity in fresh weight with a 14.5% increase in fresh weight level measured in response to 0.1 mM IAA, 4.9% and a 3.3% in the case of 1.0 mM NAA and 1.0 mM 2,4-D, respectively, in comparison with the control. The dry mass of wheat treated with auxins increased by 2.9-24.2% than the control, and the increase order in these auxins was IBA > IAA > NAA > 2,4-D.

Table 1. The effect of auxins (IAA, IBA, NAA, 2,4-D)on growth traits, pigment contents, and photosynthesis related characters in branched spike wheat after 30 days of first treatment.Data are the means of four independent experiments ± SD

	Control	IAA	IBA	NAA	2,4-D
Plant fresh (g)	10.98±1.03	12.57±1.36	13.5±0.98	11.52±0.087	11.34±0.79
Dry mass (g)	2.07±0.31	2.43±0.36	2.57±0.298	2.31±0.234	2.13±0.198
Chloro a (mg·g^{-1} fresh weight)	1.365±0.045	1.47±0.069	1.489±0.036	1.419±0.047	1.398±0.033
Chloro b (mg·g^{-1} fresh weight)	0.357±0.039	0.472±0.052	0.532±0.047	0.387±0.034	0.361±0.059
Carotenoids (mg·g^{-1} fresh weight)	0.462±0.015	0.483±0.021	0.503±0.014	0.493±0.028	0.481±0.015
Photosynthesis rate (μmol CO_2 m^{-2}·s^{-1})	32.7±4.26	34.21±3.69	37.06±2.87	38.29±4.35	34.2±3.96
Intercellular concentration of carbon oxide (μmol CO^2·mol^{-1})	240.6±28.9	298.6±23.6	327.5±28.7	272.3±15.6	268.1±32.1
Stamatal conductance (mmol H_2O m^{-2}·s^{-1})	0.32±0.049	0.35±0.053	0.39±0.035	0.37±0.028	0.34±0.041
Transpiration rate (mmol H_2O m^{-2}·s^{-1})	8.7±0.93	9.31±1.32	9.52±1.21	9.28±1.42	8.86±1.09

IBA applied at 0.1 mM had the most stimulatory effect on chlorophyll a, chlorophyll b and carotenoid accumulation after 30 d of first treatment. Other auxins were characterized by lower stimulatory effects on photosynthetic pigment levels in branched spike wheat. IAA at a concentration of 0.1 mM stimulated chlorophyll a accumulation by 7.69%, chlorophyll b by 32.2% and carotenoid by 4.54% after 30 d of first auxin treatment. The 2.41-3.95% increase in chlorophyll a level and 1.12-8.40% increase in chlorophyll b were noted in response to 1.0 mM NAA and l.0 m 2,4-D. These auxins also stimulated the carotenoid content by 4.11-8.87% after 30 d of first treatment.

3.3 Photosynthesis Rate, Intercellular Concentration of Carbon Oxide, Stamatal Conductance and Transpiration Rate Determination

Wheat in the presence of 0.1 mM IBA had 13.3%, 36.1%, 21.9% and 9.42% more photosynthesis rate, intercellular concentration of carbon oxide, stamatal conductance and transpiration rate, respectively, than the control after 30 d of first treatment (Table 1). A significant increase in the photosynthetic parameters (4.62% in the case of photosynthesis rate, 24.1% in the case of intercellular concentration of carbon oxide, 9.36% in the case of stamatal conductance and 7.01% in the case of transpiration rate) was also observed with 0.1 mM IAA application. In addition, exposure of branched wheat to 1.0 mM NAA or 2,4-D caused a weaker, but statistically significant increase in the photosynthesis rate (4.58-17.1%), intercellular concentration of carbon oxide (11.4-13.7%), stamatal conductance (6.25-15.6%)and transpiration rate (1.83-6.67%) after 30 d of first treatment.

3.4 Expression of TFL during Spike Development

The effect of auxins on the expression of the *TFL* gene was examined (Genbank accession number: KP408434). The coding region of *TFL* is 1,185 bp, and encodes a putative protein of 394 amino acids, which is 59.2% identical to *WFL* (GenBank accession No: AB231888), a *LFY/FLO* homologous gene from the species of common wheat (Shitsukawa et al., 2006). Comparison of an amino acid sequence alignment containing *TFL* and other *LFY/FLO* proteins showed the presence of several conserved regions. The putative amino acid sequence of *TFL* showed 64.9% homology with *LtLFY* of *Lolium temulentum* (AF321273), 59.9% with *RFL* of *Oryza Sativa* (AB005620), 54.7% with *LEAFY*-like of *B. distachyon* (XM_003580387); 52.9% with *zfl1* of *Zea mays* (AY179881), 53.4% with *LEAFY*-like of *S. italic* (XM_004976618), 52.9% with *LEAFY* like of *O. brachyantha* (XM_006652684), 50.7% with *LEAFY*-like of *S. bicolor* (XM_002446991) (Figures 2 and 3).
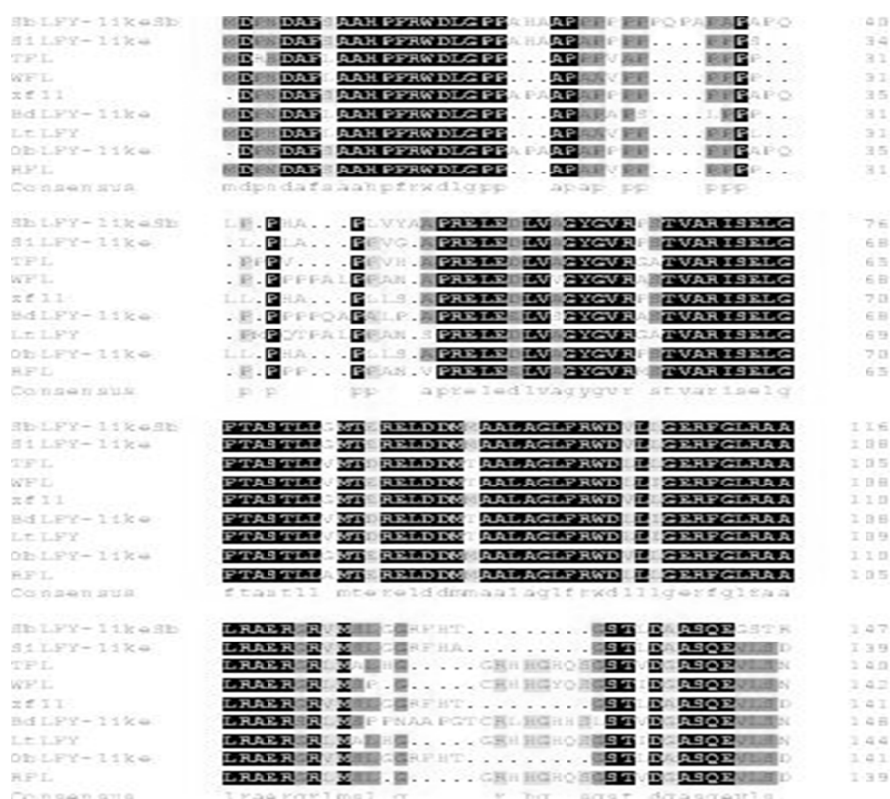
Figure 2. Comparison of amino acid sequences of *TFL* with representative *FLO/LFY*-like proteins

Note. Identical and conserved amino acids are shaded. Sequence segments notoverlapping with the *TFL* fragment were not included. *TFL* (KP408434), branched spike wheat; *WFLa* (AB231888), *Triticum aestivum*; *RFL* (AB005620), *Oryza sativa*; *LtLFY* (AF321273), *Lolium temulentum*; *zfl1* (AY179881), *Zea mays*; *SbLFY*-likeSB (XM_002446991), *Sorghum bicolor*; *ObLFY*-like (XM_006652684), *Oryza brachyantha*; *SiLFY*-like (XM_004976618), *Setaria italica*; *BdLFY*-like (XM_003580387), *Brachypodium distachyon*.

The abundance of transcripts of *TFL* in shoot tips was determined by using Q-PCR while the branched rachis was occurring and growing (Figure 4). In branched spike wheat with no auxin treatment, as spike development, the expression of *TFL* increased slowly at first treatment, reached the highest expression level and then decreased at 30 d. Similar changing patterns appeared under four auxins treatments, but auxins increased notably the expression of *TFL*. Compared with the control at the same time, the expression of *TFL* increased by 5.8-20.5%, 4.6-25.6% and 25-67.8% at 10 d, 20 d and 30 d respectively. The highest increase was IBA at 30d, and the lowest increased was NAA at 20 d.
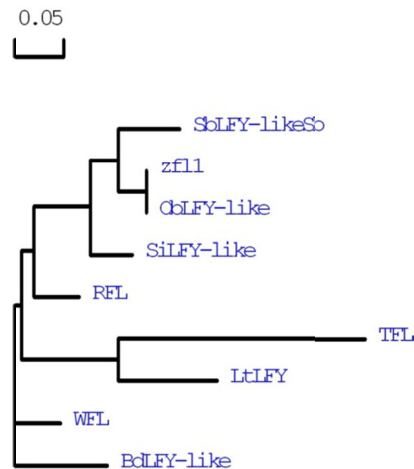
Figure 3. Neighbor-Joining phylogenetic tree of representative *LEAFY* homologues generated with 1,000 bootstrap replicates
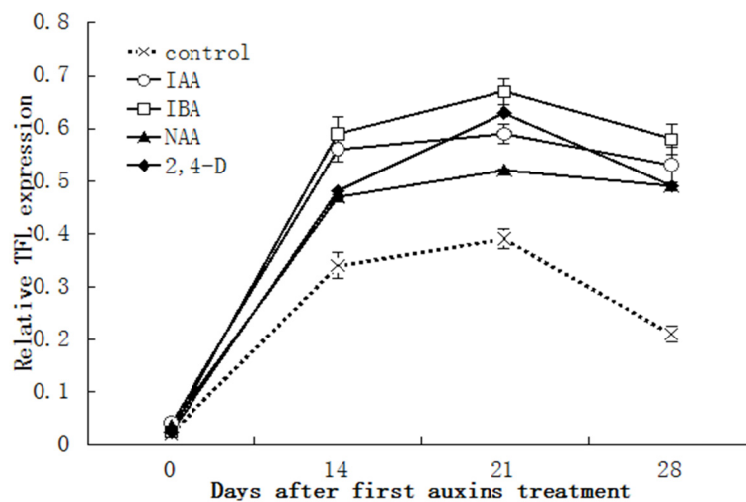

Figure 4. Relative expression of *TFL* in young spikes of branched spike wheat treated with auxins (IAA, IBA, NAA, 2,4-D)

4. Discussion

Our results indicate that auxins play an important role in branched spike architecture. Huang et al. (1990) have proved that adventitious branched rachis produced in some normal spike wheat when high concentration of 2,4-D was applied. This research proved that auxins might promote the occurrence or growth of branched rachis in branched spike wheat. The advantages of branched spike wheat are characterized of branched spike, and the number and length of branched rachises determines the number of spikelet, and then influences the fertile floret, or grain in a spike. Auxins significantly increased these spike parameters. In shoot branching architecture establishment, auxins can influence various stages of bud (shoot branch) development through the regulation of different genes, from a minor role in bud initiation through to a major but indirect effect on bud activity which at least involves different hormonal second messengers (Leyser, 2003). The apical dominance of auxin performed inhibition of lateral bud development, and auxin regulation involved in lateral meristem development and occurrence (Foo et al., 2005; Beveridge, 2006). Auxin also has an important role in promoting lateral root development. Before lateral root primordia breakthrough the root periderm, auxin is required to induce the expression of loading protein *LAX3*, and then triggering the activation of recombinant enzymes in the epidermal cells to promote cell separation and root formation (Swarup et al., 2008). The branches from shoots, roots and inflorescences seemed all required involvement of auxins.

Auxin plays an important role in the process of plant growth and development (Hohm et al., 2013). 0.1 mM IBA were the most effective in promoting plant growth, and photosynthetic capability. The effects of synthetic auxins NAA and 2, 4-D were significantly lower than those of IBA and IAA. A significant increment in growth, pigment content, and activities of antioxidant enzymes could be noticed in wheat young seedlings when the seeds were pretreated with IAA (Agami & Mohamed, 2013). In *Catharanthus roseus*, IBA, IAA and NAA significantly improved plant fresh and dry weights, total chlorophyll and carotenoids content, and net photosynthetic rate (Alam et al., 2012). In *Panax ginseng*, that exogenous IAA enhanced significantly net photosynthetic rate, stomatal conductance, transpiration rate at all growth stages (Li & Xu, 2014). The results in this study suggest there were no negative effects of auxins on wheat growth and photosynthesis when more fertile floret obtained in branched spike wheat by auxins application.

Evidence provided in this study supports the suggestion that *TFL* is the branched spike wheat homologue of *FLO/LFY*. A high degree of similarity was seen between the isolated cDNA molecule and fragments of known *FLO/LFY* homologues. *TFL* expressed at a low level in the shoot apex of control and auxin-treated plants at the start of the experiment, showing that *TFL* was expressed at the end of two ridges stage of wheat, at this time the shoot apex did not enter into the stage of floral organogenesis.

In no auxins and auxins treated plants, the quantities of *TFL* unregulated at the time when spike were undergoing the branched rachises differentiated and elongation. That implicates *TFL* might involved in branched rachis transition and possibly in branched rachis organogenesis.

In flower plants, *FLO/LFY* encodes a plant-specific transcriptional factor (Blazquez et al., 1997). The primary function of *FLO/LFY* homologous genes is to repress the development of vegetative organs and to promote the formation of flower meristem and then also influence blossum time (Mandel et al., 1995; Wada et al., 2002; Molinero-Rosales et al., 1999). The other function of *FLO/LFY* homologous genes is to maintain the activity of flower meristem and to activate the genes that specify the development of different flower organs (Weigel et al., 1992; Carmona et al., 2002).

Previous studied documented that *FLO/LFY* homologs might involve in inflorescence architecture. In maize, *zfl1* and *zfl2* are duplicate *FLO/LFY* homologs in maize. Transposon insertions into the two maize genes led to a disruption of floral organ identity and patterning, as well as to defects in inflorescence architecture and in the vegetative to reproductive phase transition (Bomblies & Doebley, 2005). In normal spike wheat, the expression pattern of *WFL*, a *FLO/LFY* ortholog, indicated that *WFL* is associated with spikelet formation rather than floral meristem identity (Shitsukawa et al., 2006). Auxins induced increasement of the expression of *TFL* over this time, although the extent to which *TFL* levels increased were associated with the kinds of auxins. This indicates that *TFL* may be involved in the formation and branching, and its expression might be induced by auxins.

5. Conclusions

The four types of experienced auxins induced similar effects on various physiological processes, including growth traits, photosynthetic parameters, although the impact extents were different among them. The data supports the hypothesis that auxins play a central role in the regulation branched spike characters and *TFL* might correlate with the development of branched rachises in branched spike wheat.

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