Effects of Gibberellic Acid and Auxin on Expression of Genes Involved in Flixweed Flowering

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Abstract

Flixweed is an annual or biennial herbaceous plant. The seeds of this plant are small, slightly elongated, and typically come in two colors that form inside the pod. A lack of synchronized care for the sorrel pods on the main stem leads to grain loss and a reduction in economic yield. The flowering process and the genes involved play a crucial role in coordinating the formation, growth, and maturation of Flixweed pods. The aim of this study was to investigate the expression patterns of genes involved in the flowering process of Flixweed—namely, LFY, TFL, AG, FLC, AP1, and MYB24—under different concentrations of gibberellin and auxin treatments. Thus, increasing or decreasing the expression of these genes can impact the rate of Flixweed grain loss. The results of the analysis of variance showed that the effects of auxin and gibberellin foliar application levels on the relative expression of the LFY, TFL, AG, FLC, AP1, and MYB24 genes were significant at the 1% probability level. Based on a comparison of the results, the mean relative expression levels of the LFY, TFL, AG, and MYB24 genes were influenced by treatments with gibberellic acid and auxin. The highest relative expression of FLC was observed with the application of 60 mg/L auxin. The lowest relative expression levels of LFY (2.9466), TFL (5.6466), AG (4.3066), and MYB24 (-1.6867) were observed with the application of 30 mg/L auxin. The lowest relative expression of FLC and AP1 genes was achieved with the foliar application of 15 mg/L auxin and 60 mg/L gibberellin, respectively.

Keywords: Real Time PCR, Gene expression, Plant growth regulators, Flowering process, Crop physiology

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Introduction

Flixweed (*Sisymbrium sophia*), a member of the Brassicaceae family, is an important species used in the treatment of asthma, fevers, bronchitis, oedema, and dysentery. Plants belonging to the Brassicaceae family, including Flixweed, typically disperse their seeds via dispersal agents like wind. Although pod opening is a useful mechanism for dispersing seeds in nature, it is one of the major problems in Flixweed cultivation. Determining the right time to harvest is very difficult because the ripening of seeds on a flowering branch does not happen simultaneously. In other words, newly formed flowers are observed in the upper third of

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a flowering branch, ripe pods ready for harvest in the middle third, and split pods in the lower third, respectively.

Flowering in plants occurs only during certain seasons of the year, regulated by several networks that interpret environmental signals such as day length and temperature fluctuations (Kim and Sung, 2013). Flower development results from the expression of a set of genes and is considered an important biological phenomenon. One class of genes involved in this process is the flowering time genes that affect the flowering time and can control the morphology of flowers and inflorescences (Bernier and Périlleux, 2005).

Inflorescence and flower formation and reproductive transfer in plants involve several continuous stages. Generally, several gene groups control the flowering pathway in plants. Flowering stimulus genes are the first group in the flowering pathway. The second group is floral meristem identity genes, and the third group is flower organ identity genes (Ingrouille, 2009).

Both Flixweed and Arabidopsis belong to the Brassicaceae (mustard family). Fortunately, comprehensive studies have been conducted on the genetic, molecular, and physiological processes of Arabidopsis, which can help in better understanding the molecular processes of Flixweed. Studies on Arabidopsis have shown that the flowering process is carried out under the ABCDE model, and by a set of transcription factors, mostly MADS-box genes. Class A genes specify sepal identity in the outer domain of the floral meristem; Class B genes, in combination with Class A genes, regulate petal identity in the second whorl; Class C genes, together with Class B genes, determine male reproductive organ identity, and C-function genes alone regulate the identity of female organs in the fourth whorl. In addition, Class D genes direct the specification of ovule development. The expression of Class E genes is extremely important for the identity of all flower organs (Bowman et al., 2012; Ingrouille, 2009; Irish, 2017; Shiri et al., 2020; 2018; Su et al., 2013).

The first step in the flowering process is the differentiation of flower meristem cells, which is regulated in Arabidopsis by genes such as LFY and AP1 (Gregis et al., 2009; Parcy et al., 1998). In contrast, late flowering is controlled by increasing the TFL1 gene expression (Hanano and Goto, 2011). Increased expression of LFY and AP1 suppresses TFL1 gene expression in flower meristem tissue, whereas increased TFL1 gene expression suppresses LFY and AP1 gene expression in inflorescence meristem cells (Hanano and Goto, 2011).

The main role of flower meristem differentiation genes is to activate a small group of genes whose task is to differentiate flower organs. The AG gene is the key gene in Class C and plays an essential role in the differentiation of flower organs (Shiri et al. 2018, Shiri et al. 2020). Two genes, MYB21 and MYB24, play a key role in the growth of petals, stamens, and female organs, and mutants of these two genes produce incomplete flowers with indehiscent anthers (Cheng et al., 2009; Song et al., 2020). The FLC gene is a flowering inhibitory transcription factor in Arabidopsis (Michaels and Amasino, 2001; Shu et al., 2016). The key role of this gene is observed in the plant's cooling needs to begin the flowering process. Before the cold requirement is met, the FLC gene prevents the terminal meristem from turning into a reproductive structure (Deng et al., 2011). After a cold winter, in the spring, FLC gene expression is suppressed, and plants are able to flower.

Currently, studies about regulating flowering by plant growth regulators (PGRs) focus primarily on gibberellins (GAs), indole-3-acetic acid (IAA), indole butyric acid (IBA), abscisic acid (ABA), and cytokinins (CKs), which play different roles in the process of plant development. GA is one of the key plant growth regulators that plays an important role in the growth and development of plants.

Due to the role of genes involved in the developmental process of flower organs, it is possible that these genes undergo expression changes under the influence of gibberellin and auxin PGRs. Despite the importance of flowering in the process of pod and fruit formation of

Table 1
Names and sequences of designed primers

Gene Nam	Gene ID	Direction	Sequence 5'-3'	Product Length
AG	XM_024149777.1	FW	GCTGAAGTCGCACTCATCGT	176
		RV	TGACGCAATTTGGCCGATTC	
LFY	XM_024151678.1	FW	AGACGCCGTCATTTGCTACT	104
		RV	GGCTCCTCTGATAACCCTGT	
AP1	XM_006390994.2	FW	TATTGCACCTGAGTCGGACG	135
		RV	AGCTCCTTAGGGCTCATTGC	
TFL1	XM_006398779.2	FW	AGGGTTGAGATCCATGGTGG	184
		RV	TTGGCCTTGGCAGCTCATAA	
MYB24	XM_006405442.2	FW	AGCCACGTCTTGAATGCTCA	123
		RV	GATCGAATCCGACGAAGGCA	
FLC	XM_006399442.2	FW	GCGACTTGAACCGTACCTGA	140
		RV	CGTTGCGTCGTTTGGAGAAG	
Actin 7	XM_006399401.2	FW	TTCCCAAGTATTGTTGGTCGTC	168
		RV	CCAGATCTTCTCCATGTCATCC	

Table 2
Mixture of reaction components designed for cDNA synthesis using reversible primers

Reagent	Volume (µl)
Total RNA (100 ng)	3
Specific Primer (10 µM)	0.5
dNTP (10 mM)	0.5
RTase reaction buffer (10X)	1
DTT (0/1 mM)	1
HYPER Script TM Reverse Trans criptase 200u/ µl	0.5
Zym ALLTM RNase inhibitor	0.5
Nuclease free water	3

Flixweed, and the key role of plant growth regulators, especially gibberellin in the flowering process, the expression pattern of genes involved in this process under the influence of PGRs treatment in Flixweed has not been studied. The present study aims to investigate the expression pattern of genes involved in the flowering process of Flixweed (LFY, TFL, AG, FLC, AP1, and MYB24) under different PGR concentrations, while identifying the optimal concentration of each, to clarify the role of auxin and gibberellin PGRs in such expression modifications.

Materials and Methods

This study was conducted to investigate the effect of GA (Gibberellic Acid) and IBA (Indole-3-butyric Acid) solutions on the expression of genes involved in the flowering process of Flixweed (LFY, TFL, AG, FLC, AP1, and MYB24) in a completely randomized design with three replications at the research farm of Zabol University (located in the Chah-Nimeh district). One week before flowering and during flowering, foliar applications were carried out with GA and IBA at concentrations of 15, 30, 60, and 120 mg/L, respectively, based on the recommended concentrations (Shiri et al. 2020). Simultaneously, the control plants were sprayed with distilled water to ensure the same environmental conditions for both the control and treated plants. One week after the last foliar application, flowering stems from both treated and control plants were sampled. The obtained samples were transferred to the laboratory after being immediately placed in liquid nitrogen (-196°C) and stored at -80°C.

Design of Primers

Table 3
Materials required for Real Time PCR

PCR components	concentration	(µl value)
Eva Green qPCR Mix-Rox	x 5	4
Input Primer	Pmol/ul10	5/0
output primer	Pmol/ul10	5/0
cDNA	-	1
Double-sterilized distilled water	-	14
final Volume		20

Table 4
Real-time polymerase chain reaction temperature program

Step	Temperature & Time
Initialization	95°C for 15 min
Denaturation	95°C for 92 seconds
Annealing	57.5 °C for 45 seconds
Extension	72 °C for 92 seconds
Final extension	33 °C - 52 °C (1 °C after 5 seconds)

By referring to the NCBI database, the nucleotide sequences of the desired genes were obtained, and then the nucleotide sequences of the selected genes were used to design PCR-specific primers using CLC Main Workbench software, considering desirable properties for PCR (Table 1). Specific primers for LFY, AP1, FLC, MYB21, TFL1, and AG, along with the Actin7 gene, were used as an internal control or housekeeping gene.

RNA Extraction

Total RNA was extracted from Flixweed flowering stems based on the Japelaghi protocol (Japelaghi et al., 2011). Agarose gel electrophoresis was used to determine the quality of the extracted RNA, and the concentration of total RNA was determined using a spectrophotometer. The concentration of total RNA extracted for all samples examined by the spectrophotometer ranged from 1.81 to 1.91 ng/µl. cDNA synthesis was performed using a kit purchased from REXu in South Korea following the manufacturer's instructions (Table 2). During the experiments, to prevent errors and for greater convenience and speed of operation, the master mix solution was prepared separately for each pair of primers (Table 3). The total volume of the reaction mixture was 20 µl per sample, consisting of 1 µl of cDNA and 19 µl of primer solution. The tubes were then placed in a thermocycler, and the temperature cycle program was set according to Table 4.

All PCR reactions were performed in duplicate. Data analysis from the real-time PCR device was performed using SAS version 9.1 software. The expression level of each gene was calculated using the 2^(-ΔΔCt) formula (Livak and Schmittgen, 2001). The formula for ΔΔCt is:

ΔΔCt = (Δ test sample) Ct - (control sample) Ct = (target gene) Ct - (housekeeping gene) Ct

For each sample, three replicates for the specific gene and three replicates for the internal control gene were considered. Analysis of the results begins with the start of the reaction, where the amplification curve is drawn by the device, and the Ct method is used to analyze the data. In this method, the difference between the Cts of the internal control gene and the target gene is calculated. This difference is applied to the Ct of the target gene and the control gene, and the data are normalized, which are the same as the Ct values.

To normalize the data, the Ct of the studied gene in each sample was subtracted from the Ct of the internal control gene of the same sample. Since the normalized data for each sample is obtained by subtracting the Ct of the target gene from the Ct of the internal control gene, we minimized the normalized data by calculating ΔCt:

ΔCT = Ct (Internal control gene) - Ct (target gene)

ΔCt is based on a logarithm with a base of 2 (because, in real-time PCR, each cycle results in a doubling of the product), so Ct values must be subtracted rather than divided. We minimized the new parameter obtained by subtracting the ΔCt of the sample from the ΔCt of the control sample:

ΔΔ CT = ΔCt (Internal control gene) - ΔCt (target gene)

Ct, ΔCt, and ΔΔCt are logarithms based on 2, so we made them linear using the formula:

Relative Gene Expression (RGE) = 2^(-ΔΔCt)

Finally, analysis of variance (ANOVA) was performed using SAS version 9.3. The comparison of means was done using the Duncan method at 1% and 5% significance levels.

Results

The results of the analysis of variance (ANOVA) presented in Table 5 showed that the relative expression of the LFY, TFL, AG, FLC, AP1, and MYB24 genes was significantly affected by plant growth regulators (PGRs) treatment. The differences in gene expression were statistically significant at the 1% level.

The results of the mean comparison presented in Table 6 showed that the relative expression of the LFY, TFL, AG, and MYB24 genes was affected by the PGR treatments of GA (gibberellic acid) and IBA (indole-3-butyric acid). The highest expression levels of these genes were observed during the foliar application of 30 mg/L GA. In contrast, the highest expression of the AP1 and FLC genes was obtained during the foliar application of 60 mg/L IBA.

On the other hand, the lowest relative expression levels of LFY (2.9466), TFL (5.6466), AG (4.3066), and MYB24 (-1.6867) genes were observed during the foliar application of 30 mg/L auxin. The lowest relative expression of the FLC gene was found during the foliar application of 15 mg/L auxin, while the lowest expression of the AP1 gene was observed at a concentration of 60 mg/L gibberellin.

Discussion

Research has shown that gibberellic acid (GA) can affect the expression of FT in leaves and induce flowering through a DELLA-dependent mechanism (Fukazawa et al., 2021; Zhang et al., 2023) or by

Table 5
Results of the variance analysis of the relative expression of genes involved in the flowering process of Flixweed under PGRs treatments

		Mean					
SOV	df	LFY	TFL	AG	FLC	AP1	MYB24
PGRs	7	38.3288358**	69.2795138**	39.429696**	70.1335605**	28.95265**	43.592705**
error	14	0.0022286	0.0000622	0.0000420	0.0145008	0.0259429	0.0269125
CV	-	2.665852	5.086760	7.135656	22.11226	4.128179	10.11357

Table 6
Comparison of the mean relative expression of genes involved in the flowering process of Flixweed under the PGRs treatments of gibberellic acid and auxin

PGRs	LFY	TFL	AG	FLC	AP1	MYB24
GA3-15	-0.5766 d	-2.5266 f	-1.4566 e	-1.9966d	-4.1967c	-1.2267 e
GA3-30	7.033 a	8.7133 a	5.9833 a	7.6033 a	-0.7067 a	8.3633 a
GA3-60	3.0433 c	-0.8666c	-1.1566 d	0.9233 b	-7.4367 d	-0.5267 d
GA3-120	-0.5466 d	-1.2333 d	-2.2066 f	0.3133 c	-7.0667 d	-0.3667 d
IBA-15	0.8766 e	-3.7466 g	-2.4166 g	-4.8066 f	-0.7367 a	-0.3867 d
IBA-30	-2.9466 f	-5.6466 h	-4.3066 h	-2.3466 d	-7.5067 d	-1.6867 e
IBA-60	6.08 b	5.43 b	4.52 b	7.98 a	-0.6100 a	6.5400 b
IBA-120	2.9566 c	-1.3633 e	1.7666 c	-3.3133 e	-2.9533 b	2.2667 c

enhancing the expression levels of SOC1 and LFY independently of the DELLA mechanism (Bao et al., 2020; Zhang et al., 2019). Studies have also demonstrated that in Arabidopsis, GA stimulates the normal development of flower organs by activating the AP3, PI, and AG genes (Yao et al., 2022)and the LEAFY (LFY) gene in *Lolium temulentum* (Eriksson et al., 2006). During inflorescence growth, GA promotes flowering by increasing levels of GA1 and GA4 at the apex of the branch (King et al., 2001).

Research on the molecular mechanisms and genetic control of flowering in model plants such as Arabidopsis has shown that the AP1 gene is located on chromosomes one, two, three, and five, has eight exons and seven introns in conserved regions, and is a specific transcription factor belonging to the MADS-box family (Díaz-Riquelme et al., 2009). AP1 is known as a flower meristem identity gene (Gregis et al. 2009). This gene not only controls the transition from the vegetative stage to inflorescence and flower meristems but also regulates the growth of the two outer whorls of floral organs, including sepals and petals (Pabón-Mora et al., 2012). The AP1 gene is a MADS-box protein that stimulates flowering and plays a dual role in either activating or suppressing flowering (Sridhar et al., 2006).

Expression analysis in different species has shown that AP1 is continuously expressed in the floral primordium during flower organ differentiation (Kong et al., 2015). AP1 primarily exerts an inhibitory effect during the onset of full flower development by reducing the activity of certain genes at the time of flowering, thus determining the identity of the flower meristem (Wellmer and Riechmann, 2010). Another role of AP1 in thermo-flowering is to stimulate the transcription of class B and E genes (flower organ identity genes) (Ó'Maoiléidigh et al., 2014). According to the results of this study, the relative expression of the AP1 gene during the application of PGRs (plant growth regulators) treatments showed a decreasing trend. The highest expression was observed during the application of 60 mg/L IBA, and the lowest expression occurred during the application of gibberellin treatments. A study by Goldberg-Moeller et al. (Goldberg-Moeller et al., 2013) supports these findings; the researchers found that gibberellin treatment during the flowering induction period reduced AP1 gene expression in citrus sprouts (Goldberg-Moeller et al. 2013).

The PEBP protein family consists of three distinct phylogenetic groups: TFL1, TSF, and FT (Melzer et al., 2013). Its expression in the early stages of flowering is suppressed by LFY and AP1 genes in flower meristem tissue, while the end of flowering is controlled by an increase in TFL1 gene expression (Hanano and Goto, 2011). In this study, during PGR treatments with gibberellin and auxin, the expression of the TFL gene showed a decreasing trend, with the highest expression observed during the application of 30 mg/L gibberellin.

LFY expression is crucial for the development of the shoot apex, lateral meristems, and the initiation of flowering in Arabidopsis, with similar effects observed in other dicotyledonous and monocotyledonous species (Dornelas and Rodriguez, 2005). In Arabidopsis, this gene is located on chromosome five, has three exons and two introns in conserved regions, and encodes a plant-specific transcription factor (Yamaguchi, 2021). LFY is expressed before other meristem identity genes, acting as a regulator of meristem identity and enhancing the activity of other essential genes involved in meristem identity (Yamaguchi, 2021). The LFY gene plays a pivotal role in the differentiation of meristematic tissue into flowers and has two key roles: coordinating the induction of flowering and activating genes in the ABCE pathway (Parcy et al. 1998). While its primary function is to control flower development, LFY also plays additional roles in some species, such as regulating stem head meristem development in tobacco, compound leaf development in legumes and tomatoes, and cluster branching in rice (Siriwardana and Lamb, 2012). The results of this study showed that the relative expression of the LFY gene increased with the use of PGRs auxin and gibberellin, aligning with findings from research in citrus (Goldberg-Moeller et al., 2013) and Arabidopsis (Yamaguchi, 2021)that confirm the increased expression of the gene when gibberellin is used.

The FLC gene is a flowering-inhibitory transcription factor in Arabidopsis (Michaels and Amasino 2001; Shu et al. 2016). This gene plays a key role in the plant's vernalization requirement to initiate flowering. Before the vernalization requirement is met, the FLC gene prevents the terminal meristem from transforming into a reproductive structure (Deng et al. 2011). After a cold winter, FLC gene expression is suppressed, allowing plants to flower in the spring. The results of this study showed that the highest expression of the FLC gene was observed during the PGR treatment of 60 mg/L auxin, which was not significantly different from the treatment with 30 mg/L gibberellin. However, expression decreased with other treatments.

The transcription factor AG is also part of the MADS-box family. The transcription factors of the MADS-box family play a crucial role in plant development by controlling the differentiation of floral organs (Smaczniak et al., 2012). AG is involved in organ differentiation during the early stages of flower formation and is the key gene of Stage C, playing a vital role in growth, development, and cell differentiation of plant organs, especially flowers (Shiri et al. 2018; Shiri et al. 2020). This gene is essential for the normal development of floral reproductive organs like stamens and carpels and for maintaining the flower meristem (Zheng et al., 2009). A study on the role of AG found that the AG gene is expressed in other plant organs besides floral organs and is involved in their development (Malabarba et al., 2017). In this study, the AG gene showed a decreasing trend under the influence of PGR treatment, with the highest expression observed during the application of 30 mg/L gibberellin (5.593333), followed by 60 mg/L auxin (4.52). Another study indicated that AG gene expression is not confined to the flowering stage and plays a role in other plant developmental processes (Mejía et al., 2007). Additionally, in an experiment using the 35S promoter of cauliflower mosaic virus, increased expression of the AG gene resulted in premature flowering and terminal flower formation in Arabidopsis (Pelayo et al., 2021).

Conclusion

The transition from vegetative to reproductive growth and flower bud morphogenesis is a complex process regulated by various factors, including external plant growth regulators. Based on the results of this study, the highest expression of the LFY, TFL, AG, and MYB24 genes was observed during the foliar application of 30 mg/L gibberellic acid. In contrast, the highest expression of the AP1 and FLC genes was found during the application of 60 mg/L auxin. Conversely, the lowest relative expression levels for LFY (2.9466), TFL (5.6466), AG (4.3066), and MYB24 (-1.6867) were recorded during the application of 30 mg/L auxin. The lowest relative expression of the FLC gene was observed during the foliar application of 15 mg/L auxin, while the lowest expression of the AP1 gene was found during the foliar application of 60 mg/L gibberellic acid.

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