

## PHYSIOLOGICAL AND BIOCHEMICAL PROPERTIES ANALYSIS OF LATE-BOLTING TRANSGENIC CHINESE CABBAGE (*BRASSICA RAPA* L. SSP. *PEKINENSIS*)

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### ABSTRACT

*LFY* gene plays important roles in determining plant flowering mainly by controlling the timing of phase transition. Constitutive under-expression of *LFY* gene in *Arabidopsis* resulted in the formation of late flowering and highly branching phenotype. In this paper, we investigated some physiological and biochemical properties of the transformants that were down-regulated *LFY* gene expression to delay bolting and flowering of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) by RNAi approach. The results showed that transgenic plants had more leaves, but a shorter height compared to control. Meanwhile, it was greatly reduced whatever the expression of *LFY* gene and the content of soluble sugar, sucrose, starch, IAA, GA, ABA, or activities of peroxidase,  $\alpha$ -amylase and esterase, although soluble protein content and zeatin were increased. All results indicated that the variation of nutrient metabolism and endogenous hormones were the root of late-bolting and flowering in transformants. They affected different signal transduction pathways and induced late-bolting and flowering.

**Keywords:** Chinese cabbage; *LFY* gene; RNA interference; physiological biochemistry properties.

### INTRODUCTION

In higher plants, flowering transition represents a crucial transition from the vegetative stage to the reproductive stage in life cycle. This process is controlled by both endogenous and environmental factors. In *Arabidopsis* and rice, four pathways, including photoperiod pathway, vernalization pathway, autonomous pathway, and GA pathway were involved in flowering control (Levy and Dean 1998; Blazquez *et al.* 1998; Ross and O'Nelli 2001; Weigel and Meyerowitz 1993). The different pathways promote and activate the expression of genes, then cause the floral transition. Genetic and molecular approaches had identified some of the key genes regulating flowering induction and development in species. Two genes, *LEAFY* (*LFY*) and *APETALA1* (*API*), are necessary to determine flower meristem identity. Constitutive expression of *LFY* promoted flower initiation in transgenic tobacco, hybrid aspen and induced flowers precociously during the vegetative phase (Weigel and Nilsson 1995). At the same time, some late-flower mutants blossomed early by 35S::*LFY* in *Arabidopsis* (Putterill *et al.* 1995; Ross and O'Nelli 2001). Moreover, over-expression of *LFY* specifically caused early flowering under long day conditions compared with non-transgenic plants. It indicated *LFY* played roles in promoting a direct effect of *LFY* on meristem emergence (Zhang *et al.* 2013, Liu *et al.* 2014). A variant of *LEAFY* reveals its capacity to stimulate meristem development by direct induction of REGULATOR OF AXILLARY

MERISTEMS1 (*RAX1*) (Chahtane *et al.* 2013). However, an inverted repeat Pt*LFY* fragment (Pt*LFY*-IR) effectively blocked flowering of transgenic tobacco plants (An *et al.* 2011). Therefore, down-regulation in expression of the *LFY* gene may delay the onset of flowering in plants. *LFY* and *API* were mutual transcriptional activators (Liljegren *et al.* 1999). *LFY* executes its meristem identity role in part by activating *API* expression directly (Parcy *et al.* 1998; Wagner *et al.* 1999). *API* was triggered by some related protein complex, such as FT, FD and 14-3-3 (Abe *et al.* 2005; Wigge *et al.* 2005; Taoka *et al.* 2011). *FT* was also able to activate *LFY* expression through the transcription factor SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (*SOC1*) (Lee *et al.* 2008; Yoo *et al.* 2005).

It has been reported that many factors, such as carbohydrate, hormone, protein and isozymes, affect flowering. High carbon-nitrogen ratio is one of the major factors which affect many plants process from vegetative to reproductive phase. Gibberellins (GAs) play a central role in the control of flower initiation (Yamaguchi *et al.* 2014). Its effect on *LFY* is mediated through a GA-response site in the *LFY* promoter with similarities to a GA-myb binding site (Blazquez and Weigel 2000). During bolting and flowering time, the activities of peroxidase, esterase and  $\alpha$ -amylase are also increased, and have some new bands appeared (Lay-Yee *et al.* 1987).

The Chinese cabbage is a representative of the family *Brassica*, and it is also an important vegetable crop that is cultivated extensively in China, Japan and Korea. It is used throughout the year in China. However,

Premature-bolting is a persistent problem in Chinese cabbage production in spring (Elers 1984). Previous attempts of transferring the late-flowering trait from late-bolting varieties into commercially important genotypes by conventional breeding produced a hybrid of poor quality (Mero and Honma 1985). In this study, after we had obtained of late-bolting by RNA interference (Xia *et al.* 2007), some physiological characteristics, which were related to flowering induction from transgenic Chinese cabbage were analysis. It provided the proof of late-bolting and flowering in transgenic Chinese cabbage.

## MATERIALS AND METHODS

**Breeding transgenic plants:** Cotyledons each with one to two millimeter petioles were excised from 4-5-day-old seedlings of 1039 and then pre-cultured for three days for 10-15 min with *Agrobacterium tumefaciens* strain LBA4404 infected, then co-cultured for two-to three days and inoculated into the regeneration medium containing Kanamycin (Kan) (10mg/L) to obtain Kan resistant (KanR) shoot. When KanR seedlings grew to 2-3cm high, they were transferred into root induced medium. Formation of roots was induced for about 20 d, and leaf tissue of the transformants was identified by RT-PCR, southern blot (Xia *et al.* 2007), then the transgenic plants were transplanted into pots in greenhouse for the seeds.

**RT-PCR analysis:** Total RNA were isolated from transformants. For RT-PCR analysis, the first strand cDNA was generated from Oligo-dT primers (Takara bio-chemicals), and then used as a template for PCR reaction with *LFY* specific primers. Nucleotide pairs of primers used to amplify *LFY* cDNAs were as follows: Forward- 5'-CTAAAATGCGACACTACG-3'; Reverse- 5'-GCTAATACCGCCAATA-3'. Beta-*actin* primers: Forward-5'-GCTCAGTCCAAGAGAGGTATTC-3'; Reverse-5'- CGAAGGATAGCATGAGGAAGAG-3' as control.

**Sugar constituent and isozymes extraction and assaying:** The shoot meristems (1g) were dissected and crushed with 15ml 80% alcohol at 75°C for 10 min, then centrifuged at 12000rpm/min for 15 min. Following the supernatant washed in 5ml80% alcohol, and filtered by 0.45  $\mu$  m Millipore filter. Fractions were collected and dried for capillary electrophoresis.

As for isozymes extraction, samples (100 mg) were dissected from shoot meristem, the shoot meristems were each pooled into three replicate samples, crushed and incubated at 25°C for one hour within the buffer (40 mM sodium-phosphate buffer at pH: 7.0 containing 1%

(v/v) Triton X-100 and 0.5 M KCl). Sucrose (25 mM) was added and the homogenate was centrifuged at 5500 g at 4°C for 10 min. The supernatant was stored at 25°C prepared for peroxide, -amylase and esterase according to Waghorn *et al.* (2003).

**Capillary electrophoresis assay for sugar constituent:** Before filtering, sugar, sucrose and starch obtained by Sigma were quantified using as an internal standard. Samples were injected in negative poles with 10mmol/L sodium benzoate and 0.5 mmol/LCTAB separating buffer, and run at 20KV voltage, 3.44kpa pressure for two second by using UV (220nm) absorption detection.

**Hormone extraction and purification:** Tissue was homogenized and the extracts held at 4°C for 24 h. Before filtering, endogenous GA3, auxin (IAA), abscisic acid (ABA) and zeatin (ZT) were quantified using as an internal standard. In the metabolism and quantification experiments, aliquots were purified by Step-Pak C18 cartridges, and then dried for HPLC (Ross *et al.* 1995)

**High-performance liquid chromatography:** Samples were subjected to HPLC as a free acid. The solvent program ran from 20% to 75% methanol in 0.4% acetic acid over 25min with a linear gradient followed by isocratic 75% elution. The flow rate was two ml/min and 1-min fractions were collected. Fractions were pooled according to the reaction time of a trituated tracer, dried and methylated. Then taken up in 1 ml of distilled water, and partitioned into the diethylene, which was then removed under nitrogen. Internal metabolites were subjected to HPLC-radio counting as methyl esters. The solvent program ran from 30% to 60% methanol in distilled water for 35 min using an exponential program, followed by isocratic (60%) elution. The flow rate was 1.6 ml/min and 1-min fractions were collected and assayed for radioactivity as before (Ross *et al.* 1995).

## RESULTS

**Down-regulation of *LFY* gene expression in transgenic Chinese cabbage plants:** Results of RT-PCR revealed that the expression of *LFY* in transgenic plant buds were lower compared to control plants (Fig.1). Twelve out of the 22 transgenic lines carrying 35S:anti-sense *LFY* bolted and flowered significantly 7-10 days later than their accustomed time (Table.1). In addition to the difference in bolting and flowering time, between transgenic plants and plants of the control, the transformants had lower height, more leaves, larger leaf area and lateral branches (Table 1 and Fig.2).

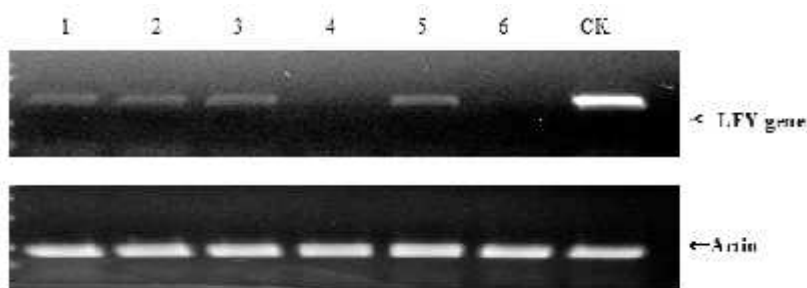


Figure 1. Effect of down-regulated *LFY* by RNAi-mediated on *LFY* gene expression in transgenic Chinese cabbage. 1-6: transformants; CK: Control. Up panel represented the expression of *LFY* gene; Low panel represented the expression of actin as control.

Table 1 Morphologic observation of transgenic and control plants

	Day to flowering (d) <sup>a</sup>	Height(cm) <sup>a</sup>	Numbers of leaves during flowering <sup>a</sup>	Leaf areas during flowering (cm <sup>2</sup> ) <sup>a</sup>	Numbers of lateral branches during flowering
Transgenic plants	93±5	31.68±3.28	12.08±4.12	48.26±3.01	2
Control plants	86±2	43.22±6.54	9.25±2.67	45.75±1.24	0

<sup>a</sup>the value was means from three time's measure of 12 plants



Figure 2. Altered growth of Chinese cabbage with anti-sense *LFY* gene fragments. Transgenic plants (left) compared to control plants (right).

**Content of carbohydrate in transgenic Chinese cabbage plants:** The metabolism of carbohydrate is related to flowering, but its mechanism is not clear. For example, there are at least five *Arabidopsis* mutants, *adg1*, *cam1*, *gi*, *pgm*, and *sex1*, which are altered in starch synthesis, accumulation, or mobilization and which flower late under some conditions (Eimert *et al.* 1995). Relevant studied also showed that high content of carbohydrate in plants can induce bud differentiation early. The results of our experiment showed that content of soluble sugar, sucrose and starch in transformants decreased 32.8%, 36.9% and 44.6% than the control, respectively (Fig.3). There was a significant difference between transformants and control ( $p < 0.01$ ).

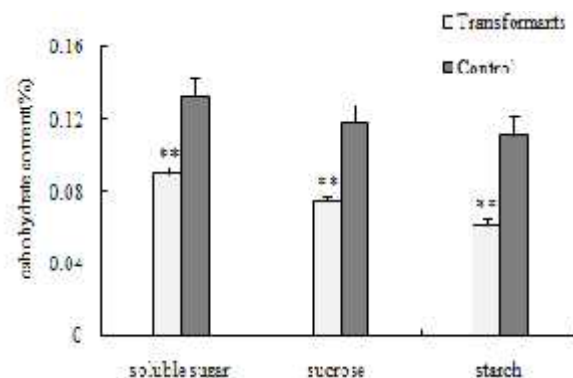
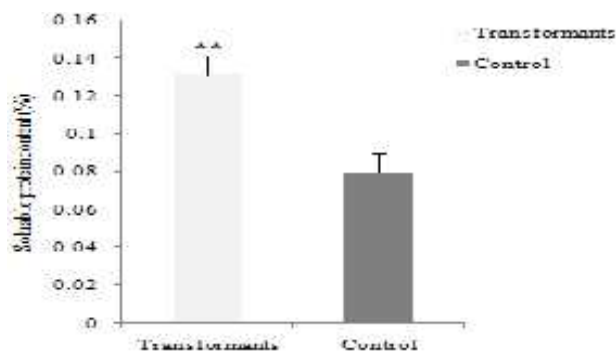
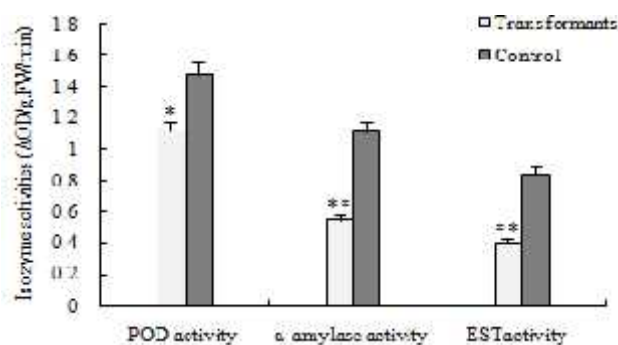


Figure 3. Content of carbohydrate in transgenic and control plants. All data were represented as the means  $\pm$  SEM of three independent experiments. \*\*  $p < 0.01$  as compared with Control.

**Analysis of soluble protein and isozymes in transgenic Chinese cabbage plants:** Isozymes is the direct product of gene, they play important role in plant transition. The increase of protein is necessary for bud differentiation. Compared to control, the content of soluble protein increased 39.1% (Fig.4), while the specific activity of peroxidase,  $\alpha$ -amylase and esterase decreased 23.7%, 50.4% and 51.5%, respectively (Fig.5). There was significant difference for the activity of  $\alpha$ -amylase and esterase between transformants and control groups ( $p < 0.01$ ) except peroxidase, which showed the remarkable difference ( $p < 0.05$ ). The lower content of them might induce the delay from vegetative to generative.



**Fig.4. Soluble protein content in transgenic and control plants. All data were represented as the means  $\pm$  SEM of three independent experiments. \*\* $p < 0.01$  as compared with Control.**



**Fig.5. Isozyme activities in transgenic and control plants. All data were represented as the means  $\pm$  SEM of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$  as compared with Control.**

#### Hormone assay in transgenic plants.

The development of plants is also controlled by the content of endogenous hormone. For example, Abscisic acid (ABA) regulates many agronomical important aspects of plant development, including the inhibition of the phase transitions from embryonic to germinative growth and from vegetative to reproductive growth. In the current experiment, the level of GA, IAA, and ABA in shoot meristem of transgenic Chinese cabbage reduced 3.7 fold, 4.7 fold and 40 fold, respectively, but the ZT level increased 3.2 fold than that of non-transgenic plant (Table 2).

**Table 2. Contents of the endogenous hormones in the transgenic Chinese cabbage (~g/g.FW)**

Hormones	Content in transformants	Content in the control	Fold <sup>a</sup>
GA	715.5	269.2	-3.76
IAA	50.8	240.8	-4.74
ABA	26.8	1096.0	-40.90
ZT	18.3	5.7	+3.23

<sup>a</sup> : “-” means a decreasing fold while “+” means an increasing fold compared with non-transformants.

## DISCUSSION

Physiological and genetic analysis of flowering has showed that multiple environmental and endogenous inputs influence the switching from vegetative to reproductive. Flowering of *Arabidopsis* plant is controlled by the response of flowering organs to several intrinsic and extrinsic signals which sent from leaves (Mouradov *et al.* 2002). A primary response to floral inductive signals is the transcriptional activation of the flower-meristem-identity genes *LFY*, *API* and *CAULIFLOWER (CAL)* at the shoot apex (Pidkowich *et al.* 1999), which are required to determine the floral fate of meristem and normally changes into shoot or shoot-like structures instead of the default state (Weigel and Meyerowitz 1993; Weigel *et al.* 1992). Early studies showed that a constitutive expression of a single flower-meristem-identity gene *LFY* could induce precocious flower development in Chinese cabbage as diverse as *Arabidopsis* (Weigel and Nilsson 1995). In this study, although the number of rosette leaves of the transgenic plants was not different from that of the wild-type, secondary shoots formed at lateral positions were consistently replaced by solitary flowers, and higher-order shoots were absent at much-reduced height than control plants, and the transgenic plants showed more leaves, larger leaf area and lateral branches. In *Arabidopsis*, when plants of the ecotype *Nossen* were grown in short days, flowering was delayed for 4 to 11 weeks, the number of rosette leaves produced by 35S::LFY plants were 19 instead of increased, and the total number of nodes before the terminal flower was formed from 14 to 49. Thus, the effects of constitutive *LFY* activity were weakened in short days (Weigel and Nilsson 1995). In radish, the height of 35S: anti-GI transformants was also significantly lower than that of the starting line when carrying the anti-GI gene cloned from *Arabidopsis* (Curtis *et al.* 2002). However, it is unknown whether the down-regulation of *LFY* transcript in Chinese cabbage was direct associated with a reduction of plant height and increasing of numbers of leaves and lateral branches.

Furthermore, the analysis of physiological and biochemical properties in transformants indicated that down-regulation of the expression of *LFY* gene in Chinese cabbage could also affect the metabolism of carbohydrate, protein and endogenous hormones and some isozymes activities. Compelling evidence that sucrose might be the function in long-distance signaling during floral induction comes from studies of *Sinapis alba*. It was reported that the concentration of sucrose in the phloem reaching the apex increased rapidly and transiently after induction of flowering, (Bernier *et al.* 1993). This pulse of sucrose reaching the apex appears to be derived from the mobilization of stored carbohydrates, most likely starch in the leaves and stems.

Further genetic evidence connecting carbohydrate metabolism with control of flowering is available, but the nature of this connection is unclear. In the current study, the content of soluble sugar, sucrose and starch and the activities of peroxidase,  $\alpha$ -amylase and esterase in transformants were decreased than controls, while soluble protein was on an opposite. All of the evidences indicated the conditions trend to delay a transition to flower in transformants.

The development of plants is also controlled by the content of endogenous hormones, such as GA, ABA, ZT and IAA. GAs is phytohormones that are essential for flowering, and seed development. It accumulates at the meristem prior to flowering and mutations with a severe effect on GA biosynthesis prevent flowering (Eriksson *et al.* 2006). Besides the function of GAs in changing the cellular concentration of bioactive promoting cell division and cell elongation (Hedden and Phillips 2000; Yamaguchi and Kamiya 2000), it promotes flowering either by active the expression of *SOCl* or by regulating *LEY* transcription through *MYB* transcription factors (Albani and Coupland 2010; Blazquez *et al.* 1998). Previous studies have also viewed the roles ABA, ZT and IAA in *Arabidopsis*. ABA regulates many agronomical important aspects of plant development, including the inhibition of the phase transitions from embryonic to germinative growth and from vegetative to reproductive growth (Leung and Giraudat 1998). Some evidences indicated that cytokines primarily ZT also plays a role in the transition to flowering (Bernier *et al.* 1993). In addition to ABA, GA and ZT, other hormones such as ethylene, IAA may be involved in flowering under certain circumstances and in some species (Yamaguchi and Kamiya 2000). According to the Ross's model, IAA was a messenger compound from the apical bud, activating GA biosynthesis in the elongating internodes (Ross and O'Nelli 2001) during the GAs trigger plant growth by promoting cell division and cell elongation, no significant effect on flowering time in *Arabidopsis* (Araki and Komeda 1993). The role of GAs in activation of the *LFY* promoter had been analyzed, but it was unknown whether a down-regulation of the expression of *LFY* could affect stem elongation.

In this investigation, we demonstrated that down-regulation of the expression of *LFY* gene in Chinese cabbage could delay bolting and flowering and affect the metabolism of carbohydrate, endogenous hormones and activities of some isozymes. Further studies are needed to determine whether the down-regulation of *LFY* gene has a negative effect on clock-regulated genes (such as those involved in the regulation of photosynthesis), vernalization pathway and gibberellins pathway genes. The heritage regulation of transformants will be investigated in the following work.

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