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Effect of Nitrate Concentration on the Growth, Bolting and Related Gene Expression in Flowering Chinese Cabbage

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Abstract: Nitrogen concentration affects growth and bolting of plants, but its regulation mechanism is still unclear. In this work, three nitrate concentration treatments (5%, 100%, 200%) in nutrient solution were conducted to explore the internal relationship between nitrogen and bolting in flowering Chinese cabbage. The results showed that the bolting and flowering time was earlier under the treatment with low nitrate and, the lower the nitrate concentration, the earlier the bolting and flowering. Low-nitrate treatment reduced the content of nitrate, soluble protein, free amino acid and total nitrogen, and increased the C/N ratio. The C/N ratio was significantly negatively correlated with plant height, stem thickness and biomass, while it was significantly positively correlated with flowering rate. Thus, it was indicated that nitrogen may affect bolting and flowering by regulating the C/N ratio of flowering Chinese cabbage plants. The expression of flowering-related genes (*SOC1*, *LFY*) was increased significantly under low nitrate treatment. In addition, the pith cell area at the stem tip was significantly reduced under low nitrate treatment, resulting in a significant decrease in stem thickness. The expression of cyclin- and expansin-related genes (*CYCD3-3*, *CYCB1-1* and *EXPA8*) was significantly reduced, which indicated that nitrogen may regulate the stem development of flowering Chinese cabbage by regulating the expression of cyclin- and expansin-related genes.

Keywords: nitrogen; flowering Chinese cabbage; bolting; flowering; cell structure

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1. Introduction

The complete life cycle of plants includes two stages of vegetative growth and reproductive growth. Bolting and flowering marks the shift from vegetative growth to reproductive growth in plants, which is essential for plant reproduction [1,2]. In Arabidopsis (*Arabidopsis thaliana* L.), six important regulatory pathways determine flowering, namely the photoperiod, vernalization, autonomous, age, ambient temperature and gibberellin (GA) pathways [3–5]. FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (*SOC1*) and LEAFY (*LFY*) are the main integrators of multiple flowering pathways [6–8]. In addition to the external environment and endogenous hormones inducing flower bud differentiation, it is also very important to provide a certain amount of nutrients for plant flowering.

Nitrogen (N) is one of the essential elements in the growth and development of crops. The amount of nitrogen directly affects cell division and growth, and plant nutrient growth is often inhibited by nitrogen deficiency [9,10]. Nitrate is one of the main forms of nitrogen uptake by plants, and is involved in regulating many aspects of plant development, such as seed germination, root and leaf growth, root structure, flowering time, branch branching and plant senescence and yield [11,12]. In Arabidopsis, nitrate availability controls the flowering transition; lower nitrate concentration promotes early flowering while higher nitrate concentration delays flowering time [13–15]. Lin et al. concluded that different nitrate availability leads to a U-shaped flowering curve, and optimal nitrate concentration

promotes flowering higher or lower than the optimal concentration of delayed flowering in Arabidopsis [16].

Flowering Chinese cabbage (*Brassica campestris* L. ssp. *chinesis* var. *utilis* Tsenet Lee.) is a subspecies of Chinese cabbage originally from Southern China that is now planted throughout the country [17]. The major food product of flowering Chinese cabbage is the stalk, the development of which is directly related to plant quality and yield [17], but the molecular mechanisms underlying this development are not well understood. The main pathways controlling flowering in B. campestris may be autonomous, distinct from vernalization-associated pathways, and dependent on FRIGIDA (FRI) [18]. However, our previous research showed that low temperature treatment at 15 °C could promote early bolting of flowering Chinese cabbage, which differed in bolting times [19].

In this study, the effect of different nitrate concentrations on the stem development of flowering Chinese cabbage was conducted to explore the internal relationship and regulatory mechanism between nitrogen and the bolting of flowering Chinese cabbage, so as to provide a theoretical basis for research on the bolting mechanism of flowering Chinese cabbage.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The experiment was carried out in a greenhouse of the College of Horticulture, South China Agricultural University, with flowering Chinese cabbage "Youqing 33". The seeds flowering Chinese cabbage were sterilized with 1% sodium hypochlorite for 10 min, washed with deionized water 3 to 4 times and then directly sown in perlite. After the cotyledons were flattened, seedlings were watered with a 1/4 dose of Hoagland nutrient solution and, four days later, seedlings were watered with a 1/2 dose of Hoagland nutrient solution. Seedlings with three leaves and one core were transplanted into hydroponic containers (25 L in volume, 12 seedlings per container). As a repeat, pure water was added to the original volume every three days.

2.2. Treatments

Treatments of eight nitrate concentrations (5%, 10%, 20%, 40%, 80%, 100%, 150%, 200%) were performed in the experiment (Table 1). NO_3^- was supplied by KNO_3 and $Ca(NO_3)_2$. KCl or $CaCl_2$ were used to maintain the same amount of total K^+ and Ca^{2+} in different treatments. The concentration of mineral element in different strength nutrient solution concentration is shown in Table S2. The nitrate level in 1/2 dose of Hoagland nutrient solution is defined as 100% (7.5 mM), and the amount of other large and medium trace elements is Hoagland nutrient solution formula. We measured the EC value and pH value of the nutrient solution every three days after planting, and we counted the flowering time and flowering rate of the flowering Chinese cabbage every day after flowering.

Table 1. Nutrient solution with different nitrate concentrations (Unit: mM).

Nitrate Levels	5%	10%	20%	40%	80%	100%	150%	200%
KNO_3	0.125	0.25	0.5	1	2	2.5	3.75	5
$Ca(NO_3)_2{\cdot}4H_2O$	0.125	0.25	0.5	1	2	2.5	3.75	5
KH_2PO_4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
$MgSO_4{\cdot}7H_2O$	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
KCl	2.375	2.25	2	1.5	0.5	-	-	-
$CaCl_2$	2.375	2.25	2	1.5	0.5	-	-	-

2.3. Growth Measurements

By comparing the flowering rate of 8 treatments with different nitrate concentrations after 24 days of treatment, three treatments of 5% N, 100% N and 200% N were selected for the following experiments. On the 24th day after treatment, the fully developed flowering

Chinese cabbages were harvested, and plant height, stem diameter and fresh and dry weight (determined after 1 h at 105 °C and 48 h at 75° in a drying oven.) were measured (4 biological replicates per treatment, 12 plants per replicate). Plant height (cm) was measured with a ruler, stem thickness (mm) was measured with a vernier caliper using the cross method and dry and fresh weight were measured by electronic balance.

2.4. Phytochemical Measurements

Nitrate content was measured colorimetrically [20]. Fresh frozen tissue (2.0 g) was soaked in a tube with 10 mL distilled water and placed in boiling water for 30 min. The extract was filtered into a 25 mL volumetric flask and distilled water was added. Then, 9.5 mL NaOH (8%) and 0.4 mL salicylic and sulfuric acid (5%) were added into 0.1 mL extracting solution. The absorbance of mixture was detected at 410 nm by a UV spectrophotometer.

Soluble protein content was determined by Coomassie blue staining [21]. Fresh frozen tissue (0.5 g) was ground up by a mortar and pestle with liquid nitrogen, and then added into 5 mL distilled water. After centrifuging at 10,000 rpm for 10 min, 0.5 mL of supernatant was diluted in the same volume of distilled water and added into 4 mL Coomassie brilliant blue G-250 solution, then examined by a UV-spectrophotometer at 595 nm.

Free amino acids content was examined by a Ninhydrin reaction [22]. Fresh frozen sample (1.0 g) was heated in a water bath for 30 min at 80 °C with 10 mL deionized water. After centrifuging at 13,000$\times$ *g* for 10 min. The 0.2 mL supernatant was mixed with 19 mL NaOH (4 mol·LL1) and 0.8 mL salicylic acid (5% (*w*/*v*)). The absorbance of the mixture was determined at 410 nm by a UV spectrophotometer.

Soluble sugar content was performed by Anthrone colorimetry [23]. Fresh frozen samples (0.5 g) were heated for 30 min in a boiling water bath with 10 mL distilled water. Then, 0.1 mL supernatant was mixed with 1.9 mL distilled water, 5 mL vitriol and 0.5 mL anthrone ethyl acetate. After shaking and cooling to ambient temperature, the solution was measured by the UV-spectrophotometer at 630 nm.

Determination of total nitrogen and total carbon was conducted according to the method suggested by Lu et al. [24]. Frozen sample (0.1 g) was heated with 1 ml distilled water and 5 mL digestive liquid at 250 °C for 10 min, and cooled for 5 min. Next, 0.5 mL H_2O_2 was added and heated at 370 °C for 30 min. After cooling, the volume was set at constant to 50 mL for the determination of total N content. Frozen sample (20 mg) and 10 mL $K_2Cr_2O_7$ solution were heated at 180 °C and boiled for 5 min. After cooling, the lotion was collected and 2 drops of indicator were added. The solution was reddish brown. We added the $FeSO_4$ standard solution until the solution turned blue-green. The amount of $FeSO_4$ standard solution was recorded to analyze total C content.

2.5. Cell Structure Observation

During the harvest period, we cut the stem tips (5 mm) of different treatments of Chinese cabbage and put them into a sample bottle containing FAA fixative (70% alcohol: acetic acid: formaldehyde = 90:5:5; *v*:*v*:*v*). Then, we vacuum pumped for 20 min, placed them in a refrigerator at 4 °C for 2 days, replaced the FAA with 70% alcohol, stored them in a refrigerator at 4 °C and used them for paraffin sections to observe the cell structure. Refer to Chen et al. (2021) for paraffin section method [25].

2.6. Analysis of Gene Expression

After treatment for 20 days, total RNA was isolated from leaves and stems of different nitrate treatment by the Total RNA Extraction Kit (Promega, Shanghai, China), according to the manufacturer's instructions. All the samples were used for the real-time quantitative PCR (qRT-PCR) (3 biological replicates per treatment, 3 technical replicates per biological replicate). The primers used in this study are listed in Table S1.

2.7. Statistical Analysis

All assays were performed in 3 biological replicates. Variance analysis of different nitrate treatment was performed by using SPSS17.0 software to determine the significance at $p < 0.05$ level. The tables and figures were made by Excel 2013 and SigmaPlot 11.0, respectively.

3. Results

3.1. Flowering Time and Flowering Rate

The flowering rate of flowering Chinese cabbage was affected by different nitrate concentrations (Figure 1a). Compared to the control (100% N), the flowering rate significantly improved after 24 days of treatment with low nitrate and, with the decrease in nitrate concentration, the flowering rate increased gradually and reached its highest at 5% N. The flowering rate of the treatment with high nitrate is also higher than that of the control, but the effect was not significant (Figure 1b). In order to further explore the effect of low nitrate and high nitrate concentrations on the bolting and flowering of flowering Chinese cabbage, combined with the previous phenotype and flowering rate, we selected two extreme concentrations (5% N and 200% N) for further analysis. Further dynamic analysis was conducted on flowering time and flowering rate under three different nitrate treatments of 5% N, 100% N and 200% N (Figure 1c). The flowering rate of 5% N treatment was the highest after 18 days of treatment. With the extension of the treatment time, the flowering rate increased the most under 5% N treatment. After 24 days of treatment, the flowering rate of the 5% N treatment reached approximately 97%, while the flowering rates of the control and 200% N treatments only reached about 56% and 64%, respectively. These results indicated that low nitrate treatment could significantly improve the flowering time and flowering rate of flowering Chinese cabbage.

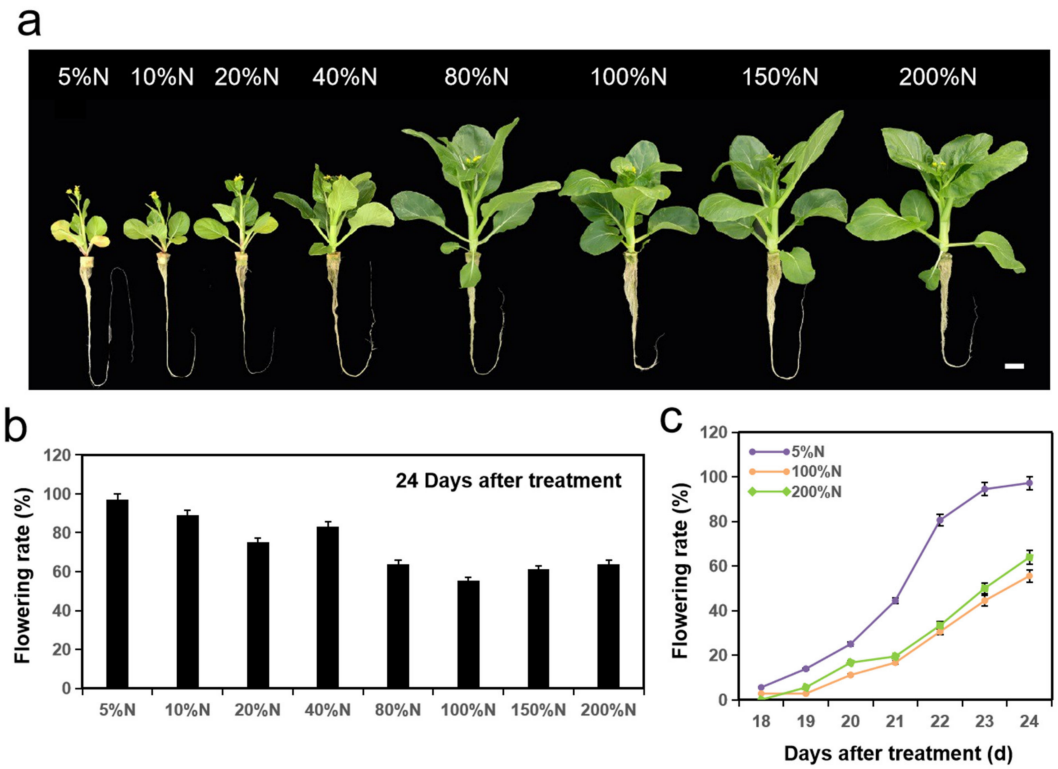

Figure 1. The effects of different nitrate concentrations on flowering time and flowering rate of flowering Chinese cabbage: (**a**) plant morphology of flowering Chinese cabbage treated with different nitrate concentrations for 24 days. Bar = 2 cm; (**b**) flowering rate of flowering Chinese cabbage treated with different nitrate concentrations for 24 days; (**c**) analysis of flowering time and flowering rate of flowering Chinese cabbage under 5% N, 100% N and 200% N treatments. Error bars indicate standard errors.

3.2. Growth and Biomass

The growth indicators (plant height, stem diameter, number of leaves, fresh weight and dry weight) of flowering Chinese cabbage were determined after 24 days of treatment. Compared with the control, the growth indicators under 5% N treatment showed a significant downward trend (Table 2). The growth indicators under 200% N treatment were also increased, except for the root fresh weight and dry weight, but the effect was not significant (Table 2). The trend of growth and biomass changes were consistent with the phenotype of Chinese cabbage in Figure 1a.

Table 2. The effects of different nitrate concentrations on growth and biomass of flowering Chinese cabbage.

Treatments	Plant Height (cm)	Stem Diameter (mm)	Leaf Number	Fresh Weight (g)		Dry Weight (g)	
				Shoot	Root	Shoot	Root
5% N	16.02 ± 0.62 ^{d}	7.25 ± 0.08 ^{g}	5.67 ± 0.19 ^{d}	8.30 ± 0.04 ^{h}	2.70 ± 0.02 ^{h}	0.89 ± 0.00 ^{g}	0.58 ± 0.00 ^{g}
100% N	21.61 ± 1.35 ^{b}	18.33 ± 0.10 ^{c}	9.00 ± 0.19 ^{b}	69.43 ± 0.53 ^{d}	8.63 ± 0.02 ^{b}	3.88 ± 0.02 ^{c}	0.92 ± 0.00 ^{b}
200% N	22.33 ± 0.25 ^{b}	19.15 ± 0.07 ^{b}	10.00 ± 0.19 ^{a}	77.91 ± 0.25 ^{b}	7.41 ± 0.14 ^{d}	4.56 ± 0.03 ^{b}	0.88 ± 0.01 ^{cd}

Data are mean ± standard error. Different letters in the same row indicate significant differences ($p < 0.05$). Significant differences among the treatments were determined by SPSS 17.0 for ANOVA.

3.3. The Contents of Nitrate, Soluble Proteins, Free Amino Acids and Total Nitrogen

After 24 days of treatment, the nitrogenous compounds content of product organs in flowering Chinese cabbage was analyzed, and the results showed that, compared with the control, the content of nitrate, soluble protein, free amino acid and total nitrogen were significantly reduced under 5% N treatment (Figure 2). The nitrate content increased significantly under 200% N treatment, while no significant distinctions were observed in the content of soluble protein, free amino acid and total nitrogen (Figure 2). This suggests that high nitrate concentration increased the nitrate content in product organs of flowering Chinese cabbage, but there was no increase in nitrogen assimilation products and nitrogen accumulation.

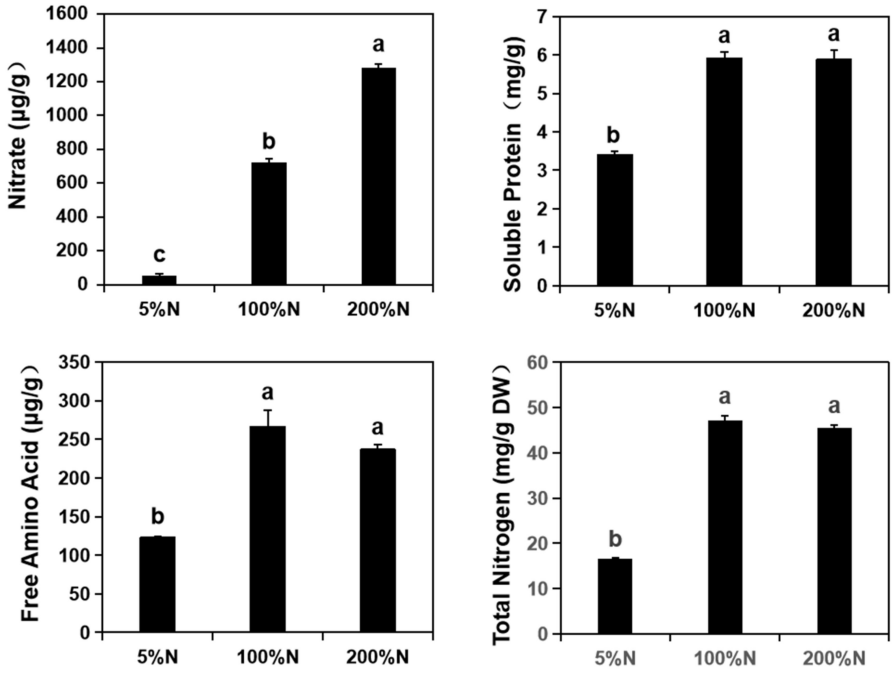

Figure 2. The contents of nitrate, soluble proteins, free amino acids and total nitrogen of flowering Chinese cabbage, as affected by different nitrate concentrations. Different letters indicate significant differences ($p < 0.05$). Significant differences among the treatments were determined by SPSS 17.0 for ANOVA.

3.4. The C/N Ratio

At the five periods of 0 d, 5 d, 10 d, 15 d and 20 d after treatment, total nitrogen and total carbon contents of product organs in flowering Chinese cabbage were determined (Figure S1). An analysis of the C/N ratio was conducted. The C/N ratio of 5% N treatment gradually increased with the extension of treatment time, and was significantly higher than the control and the 200% N treatment (Figure 3). Throughout the treatment process, no significant differences were observed in the C/N ratio between the 200% N treatment and the control (Figure 3).

The correlation analysis was performed between the C/N ratio and plant height, stem diameter, flowering rate, number of leaves and biomass. The C/N ratio was negatively correlated with plant height (r = 0.870), stem diameter (r = 0.965), number of leaves (r = 0.948) and biomass (r > 0.982), and positively correlated with flowering rate (Figure 3).

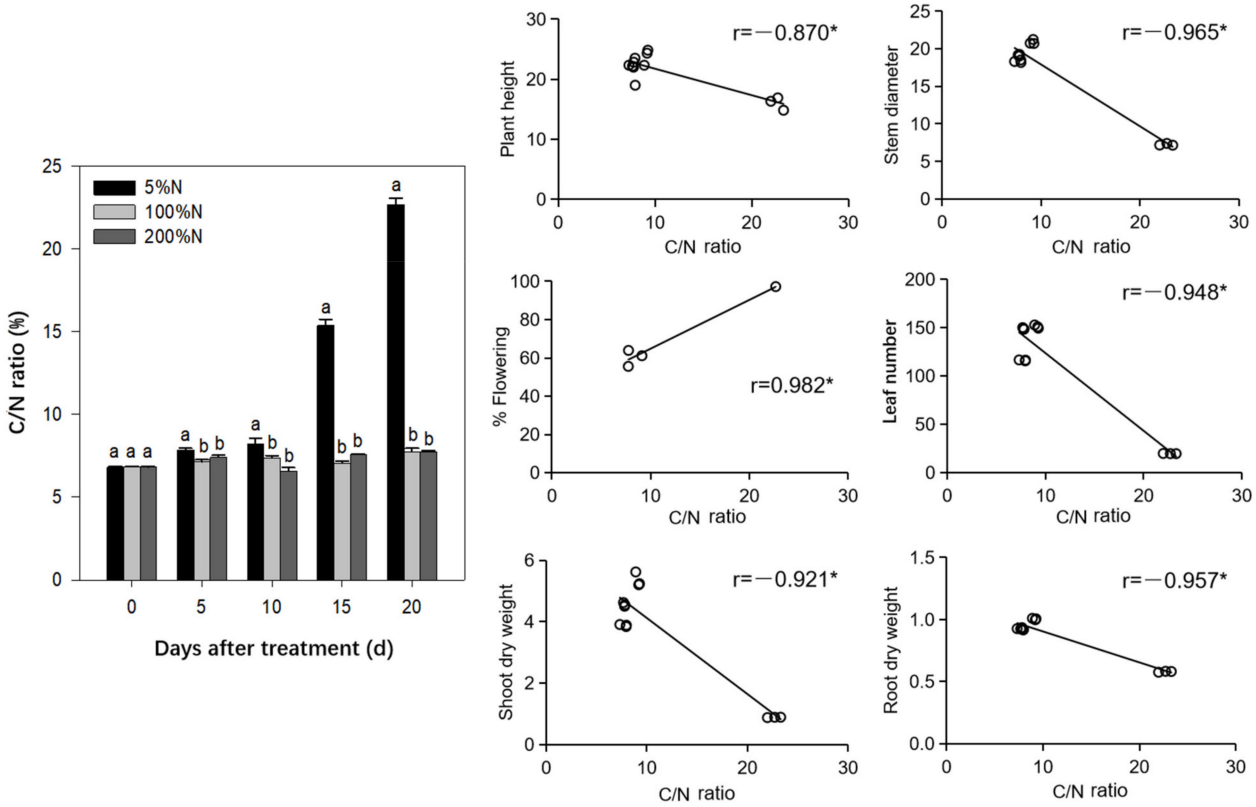

Figure 3. The C/N ratio of flowering Chinese cabbage, as affected by different nitrate concentrations. Different letters indicate significant differences ($p < 0.05$). * represented the significant difference ($p < 0.05$). Significant differences among the treatments were determined by SPSS 17.0 for ANOVA.

3.5. Expression Analysis of BcSOC1 and BcLFY in Flowering Chinese Cabbage

After 20 days of treatment, the expressions of flowering-related genes *SOC1* and *LFY* were examined in the leaves and stem tips of flowering Chinese cabbage using real-time PCR. The results revealed that the transcript levels of *BcSOC1* and *BcLFY* were significantly increased under 5% N treatment compared with that of the control and the 200% N treatment, while no significant distinctions were observed between the control and the 200% N treatment (Figure 4). This indicated that low nitrate treatment could significantly improve the expressions of *BcSOC1* and *BcLFY*, which coincides with the conclusion in Figure 1.

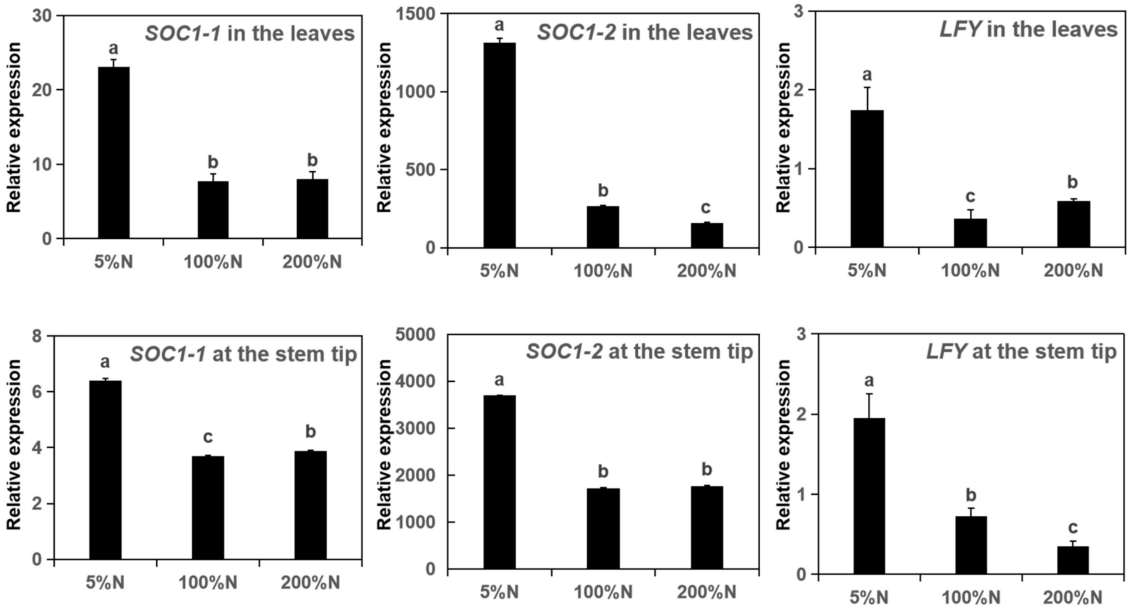

Figure 4. The expressions of *BcSOC1-1*, *BcSOC1-2* and *BcLFY*, as affected by different nitrate concentrations. Different letters indicate significant differences ($p < 0.05$). Significant differences among the treatments were determined by SPSS 17.0 for ANOVA.

3.6. Analysis of Cell Structure and Related Gene Expression in Flowering Chinese Cabbage

Paraffin sections were performed on the stem tip and the longest internode segment (between the 4th and 5th true leaves) of flowering Chinese cabbage at harvest time. In the cross-cut state, whether the stem tip or the longest intersegment, the area of the pith cells was significantly reduced, and the number of cell layers increased under the 5% N treatment, while the area of the pith cells increased significantly and the number of cell layers decreased under the 200% N treatment (Figure 5). Cortical parenchyma cells showed short columnar in longitudinal section, and the length of cortical parenchyma cells is significantly reduced under the 5% N treatment compared with the control, while the cortex was thin-walled under the 200% N treatment and the length of cortical parenchyma cells increased significantly (Figure 5). The changes of cell structure were consistent with those of plant height and stem diameter in Table 2 under different nitrate concentrations, which indicated that the expansion and elongation of flowering Chinese cabbage stalk are inseparable from the expansion and elongation of pith cells.

After 5 days of treatment, we examined the expressions of cyclin-related genes (*CYCD3-3*, *CYCB1-1*) and expansin-related genes (*EXPA8*) of the stem tip of flowering Chinese cabbage using real-time PCR. The results revealed that the transcript levels of *CYCD3-3*, *CYCB1-1* and *EXPA8* were significantly reduced under the 5% N treatment compared with that of the control and the 200% N treatment (Figure 5), which was consistent with the suppression of the horizontal growth of flowering Chinese cabbage stalks under low nitrate conditions (Figure 5).

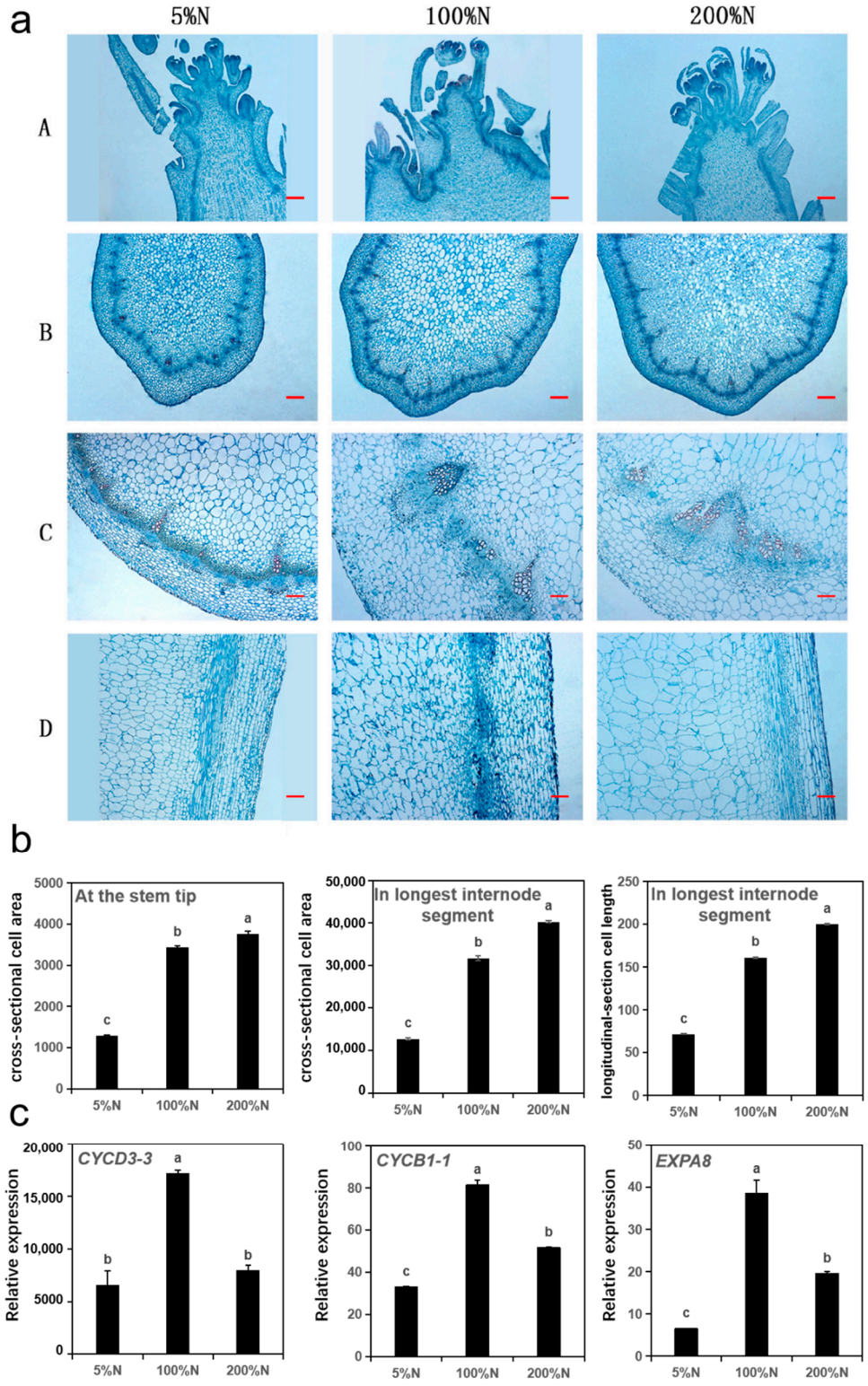

Figure 5. The pith cell structure and related genes expression, as affected by different nitrate concentrations (**a**). Scanning electron microscopy images of stem tip longitudinal section (**A**); stem tip cross-sections (**B**); longest internode segment cross-sections (**C**); and longest internode segment longitudinal section (**D**). Bar = 200 μm. (**b**) The cross-sectional area and longitudinal-section length of pith cells in the stem; (**c**) the expressions analysis of cyclin- and expansin-related genes *CYCD3-3*, *CYCB1-1* and *EXPA8* at the stem tip. Different letters indicate significant differences ($p < 0.05$). Significant differences among the treatments were determined by SPSS 17.0 for ANOVA.

4. Discussion

Nitrogen, as a component of protein, has significant effects on chlorophyll, leaf area, photosynthetic rate, photorespiration and light energy utilization rate of plants, thus affecting the final yield of plants [10,26]. Nitrate is one of the main forms of nitrogen utilization in plants. In this experiment, NO_3^- was supplied by KNO_3 and $Ca(NO_3)_2$. KCl or $CaCl_2$ were used to maintain the same amount of total K^+ and Ca^{2+} in different treatments. Thus, only the amount Cl^- of nutrient solution was different among the treatments. Our previous results showed that Cl^- had no obvious effect of the absorption of NO_3^- [27]. Therefore, the change of different Cl^- content does not affect the comparison between different nitrate treatments. This study showed that low-nitrate treatment significantly reduced plant height, stem thickness, leaf number and biomass, and restricted plant growth, while high-nitrate treatment improved these growth indicators. Previous studies have shown that low nitrate supply promotes early flowering of Arabidopsis [11,12,16,28]. In this study, flowering Chinese cabbage flowers grew under low-nitrate treatment, and the flowering rate of was the highest compared with the control and high-nitrate treatment, at the same time. The flowering time and flowering rate were improved under high nitrate treatment compared with the control, but the effect was not significant (Figure 1).

Carbohydrates are important substances in the formation of plant organs [29–31]. Chinese cabbage with high soluble protein content and low soluble sugar content is more resistant to bolting [32]. In order to explain the rapid transition from vegetative to reproductive growth of flowering Chinese cabbage induced by low nitrate, the levels of nitrogen assimilation-related metabolites and soluble sugars in flowering Chinese cabbage were determined (Figure 2 and Figure S2). The contents of nitrate, soluble protein and free amino acid in product organs of flowering Chinese cabbage were significantly reduced, and soluble sugar content was significantly increased under low nitrate treatment, indicating that nitrogen may affect bolting and flowering by regulating the content of physiological and biochemical substances in product organs of flowering Chinese cabbage.

The C/N ratio is one of the determinants of the flower transformation process of many plants [33,34]. Klebs first proposed that the C/N ratio controls the flowering of plants, and the high C/N ratio promotes the flowering of plants, while the low C/N ratio inhibits flowering. The total nitrogen content of scallion before overwintering is lower than that of non-bolting plants, and a higher level of C/N is conducive to bolting and flowering of scallions [35]. In this study, with the growth of flowering Chinese cabbage, the C/N ratio of low nitrate treatment increased significantly, which is consistent with the conclusion that a high C/N ratio is beneficial to flower formation (Figure 3). In addition, the C/N ratio was negatively correlated with plant height, stem diameter, leaf number and biomass, and positively correlated with flowering rate. Therefore, we conclude that the C/N ratio is an important indicator that affects the growth of flowering Chinese cabbage plants, and it plays an important role in regulating the flowering of flowering Chinese cabbage (Figure 3).

SOC1 belongs to MADS-box transcription factor family which promotes flowering. Expression of *SOC1* is involved in the vernalization, gibberellin, photoperiod, age and autonomous pathway in Arabidopsis [36,37]. In this study, the expressions of *SOC1-1*, *SOC1-2*, and *LFY* were significantly upregulated under low nitrate treatment, but did not change significantly under high nitrate treatment, which is consistent with the results of early bolting and flowering of flowering Chinese cabbage under low nitrate treatment (Figure 4). This indicates that nitrogen may affect the bolting and flowering of flowering Chinese cabbage through regulating the expression of flowering genes.

The elongation of plant stems depends on the regulation of cell growth and cell cycle [38–40]. Fiber morphology, secondary cell wall structure and composition are changed after nitrogen treatment in Arabidopsis and poplar [41,42]. In this study, the cross-sectional area of the pith cells at the stem tip was significantly reduced under low nitrate treatment, while cortical parenchyma cells showed short columnar in longitudinal section, and the number of cell layers increased. This is consistent with the phenotype of flowering Chinese

cabbage with dwarf plants and thin stalks under low nitrate treatment (Figures 1a and 5a,b). In addition, The expression levels of cyclin (*CYCD3-3*, *CYCB1-1*) and expansin (*EXPA8*) were significantly downregulated at the stem tip of flowering Chinese cabbage, which was relatively consistent with the result that the horizontal growth of Chinese cabbage stalk was not obvious under low nitrate conditions (Figure 5c). It can be seen that nitrogen may influence the development of flowering Chinese cabbage stems through regulating the expression of cyclin- and expansin-related genes.

5. Conclusions

As a "life element", nitrogen is very important for the growth and development of plants. Low-nitrate treatment advances the bolting and flowering time of Chinese cabbage. It also reduces the content of nitrate, soluble protein and free amino acids in flowering Chinese cabbage. Nitrogen may affect bolting and flowering by regulating the C/N ratio of flowering Chinese cabbage plants, and it may affect the bolting and flowering of flowering Chinese cabbage through regulating the expression of flowering-related genes *SOC1-1*, *SOC1-2*, and *LFY*. Nitrogen may also regulate the development of flowering Chinese cabbage stems by regulating the expression of cyclin- and expansin-related genes *CYCD3-3*, *CYCB1-1* and *EXPA8*.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11050936/s1, Figure S1: The total nitrogen and total carbon contents of flowering Chinese cabbage as affected by different nitrate concentrations, Figure S2: Soluble sugar contents of flowering Chinese cabbage as affected by different nitrate concentrations; Table S1: The primers used in this article, Table S2: The concentration of mineral element in different strength nutrient solution concentration.

Author Contributions: S.S. conceived and designed the research. Y.W. analyzed the data and wrote the manuscript. L.C. carried out the experiments and analyzed the data. Y.H., W.S., G.S., H.L. and R.C. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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