Plant Spacing Effects on Stem Secondary Growth Dynamics in Tobacco (Nicotiana tabacum)

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Research Article

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Abstract

Plant spacing usually refers to distances between plants within and between rows in the field. Different spacing of crop planting would generally influence the size, plant architecture, economical productivity et al. Present research provided a time course monitoring on the tissue transmission in tobacco stem development from the plants with different spacing. The result showed cambium activity, vascular bundle thickness, lignin, cellulose, and hemicellulose content differed in the stem because of the varied plant spacing, as well as the macro nutrients deposition. Furthermore, the genes coding the homologous of key transcription factors HB8 and NST3 (NtHB8s and NtNST3s) which involved in plant secondary growth were clone in tobacco. In the time course, they also indicated diverse expression patterns among altered plant spacing treatment. Their transcriptomic activities were validated and the motifs which might bind transcription factor in their promoter regions were predicted. Promoters of NtHB8s and NtNST3s gene were rich in light response elements, as a result, light might be the main environmental factor for plant spacing to regulate stem secondary growth.

1. Introduction

Stem is a central part of plant that supports body, connects various body parts and transports important substances[1, 2]. The stem development in crops can influence several agronomic traits, that is, traits that are related to the cultivation and production of the crop. These traits include plant stand, lodging resistance, and crop yield. Therefore, stem development is an important factor in crop agronomy. Adequate stem development ensures proper plant stand, lodging resistance, and crop yield. The growth of stem can be divided into two stages: primary growth and secondary growth. Primary growth is responsible for elongation at the tips of plant axes, while secondary growth contributes to the thickening of plant axes. Primary growth gradually transitions to secondary growth[3].

Secondary growth resulted in thickening, at this stage, asymmetric divisions of cambium gave rise to secondary phloem outwards and secondary xylem inwards [4, 5]. Arabidopsis always used as model plant to research stem development [1, 4, 6–9]. There also have research to compare major regulators of stem secondary growth between poplar and Arabidopsis[10]. Phloem and xylem, which divided from cambium are the main components of vascular bundles in plant[11]. To achieve their roles as conduits of water and nutrients, as well as support their whole body[12]. Cell wall in plant vascular tissues are always highly specialized modifications[2].

The processes of cell differentiation in xylem and phloem tissues and secondary cell wall formation have been intensively studied, many regulatory genes have been identified and characterized[13]. For instance, ATHB-8 in Arabidopsis, a member of HD-ZIPIII family, regulate by auxin positively, an early marker of the procambial cells and of the cambium during vascular regeneration after wounding[14, 15]. The overexpression of ATHB-8 increase the number of xylem cells in the vascular bundle of the stem[16]. Another Arabidopsis gene AtNST3 play a crucial role in the formation of secondary walls in woody tissues. Overexpression of AtNST3 induced ectopic secondary wall thickenings in various aboveground tissues[17, 18]. In poplar, PtrHB7 and PtrHB8 are close homologues of Arabidopsis ATHB-8[19, 20], suppression of PtrHB7/PtrHB8 expression impedes secondary xylem differentiation[19]. PtrWND1A/ PtrWND1B, which homologues of Arabidopsis AtNST3, relate to secondary cell wall formation in poplar[21, 22].

Crop spacing can directly affect the stem development of the crop. The distance between the plants affects the availability of light, water, and nutrients that each plant receives, which can affect stem growth. Moreover, crop yield, plant morphological structure and disease resistance are closely related to plant spacing[23–27]. The effect of plant spacing on anatomical structure, chemical composition and nutrient transport of plant stem has not been studied in any detail to date. We describe here the development law of secondary growth of tobacco stem, as well as the effects of plant spacing on the growth and development, anatomical structure, chemical composition content and nutrient transport of tobacco stem. Furthermore, we cloned NtHB8s and NtNST3s, and identified their expression dynamic in the
different spacing treatments. The result showed that different spacing treatment could lead to variation in expression pattern of these genes. Summarily, present research provide evidence that different spacing in tobacco cultivation lead to the secondary growth variation; and the light could be the key element influencing the plant via NtHB8s and NtNST3s transcription factors.

2. Results

2.1. Effect of plant spacing on vascular bundle of tobacco stem

From edge to the core, the stem was composed of epidermis, cortex, vascular bundle and pith (in the center), the cell type of cortex and pith was parenchyma cell. The vascular bundle of tobacco stem which contained phloem, cambial, xylem was bicollateral vascular bundle, i.e. with phloem on both sides of xylem. (Fig. 2A). The phloem fibers visible clearly in mature vascular tissue (Fig. 2B, F). According to the anatomical structure of tobacco stem cross-section, the cell number of xylem were more than that in phloem. It could be seen that cambium cells divide one phloem cell outward and several xylem cells inward. Cambial cells were composed of stem cells and arranged orderly, new phloem cells (phloem mother cell) differentiate on the peripheral side of the cambium and new xylem cells (xylem mother cell) differentiate on the internal side. It could identify the true initialed of phloem mother cell and xylem mother cell on cell morphology (Fig. 2C). Xylem of tobacco stem was mainly composed of primary xylem (Fig. 2E) and secondary xylem (Fig. 2D), and it contented vascular ray, xylem fiber and xylem vessel.

The development stage of tobacco stems treated with different plant spacing was similar on the marking day. On the 4th day after marking, the vascular bundles of tobacco stems under plant spacing of 30 and 40 cm were in the transition stage (Fig. 3A). Typical secondary structures of vascular bundles in tobacco stems under plant spacing of 50 and 60 cm were basically formed. It could be seen from Fig. 3B that the thickness of primary xylem under plant spacing of 50 and 60 cm were similar, but the thickness of secondary xylem under plant spacing of 60 cm was significantly greater than that of 50 cm. At 12th and 40th day after marking, the xylem thickness under plant spacing of 60 cm treatment was significantly greater than that of other treatments (Fig. 3C, D).

With the development of stem, the phloem thickness of each treatment increased gradually. 40 days after marking, the phloem thickness of 60 cm plant spacing was significantly thicker than 30 cm (Fig. 3E). With the stem thickening, the cambium thickness of each treatment was showing a trend of increases at first and then decreases. The cambium thickness of 30 cm plant spacing reached the peak at 4 days after marking. The cambium thickness of 40–60 cm plant spacing reached the peak at 12 days after marking. The cell number of cambium of 60 cm plant spacing decreased slightly 40 days after marking. (Fig. 3F). 4 days after marking, the xylem thickness of 60 cm plant spacing treatment was significantly greater than that of 30 cm treatment, and the xylem thickness of 40 cm and 50 cm plant spacing treatment was significantly greater than that of 30 cm treatment 12 days after marking. 40 days after marking, there was no significant difference in xylem thickness under the different plant spacing. However, the xylem thickness of 30–50 cm plant spacing was significantly less than that of 60 cm treatment (Fig. 3G). 4–12 days after marking, the maximum vessel diameter of 60 cm treatment was significantly larger than 30 cm plant spacing, and there was no significant difference in the maximum vessel diameter of every treatment 40 days after marking (Fig. 3H). The results showed that plant spacing could affect vascular bundle thickness and then stem girth by regulating cambium cell activity.

2.2. Effect of plant spacing on secondary cell wall formation

Except for the secondary cell wall thickness of xylem vessels of 50 and 60 cm plant spacing was significantly greater than that of 30 cm on the day of marking, there was no significant difference in the secondary cell wall thickness of xylem vessels in other periods (Fig. 4A). The secondary cell wall thickness of wood fiber of 30 cm plant spacing was
significantly greater than that of 50 cm on the 12th day after marking, and there was no significant difference in the thickness of secondary cell wall of every treatment on the 40th day after marking (Fig. 4B). On the 12th day after marking, the secondary cell wall thickness of phloem fibers in 60 cm plant spacing were significantly greater than those in other treatments. On the 40th day after marking, the secondary cell wall thickness of phloem fibers in 30 cm and 40 cm plant spacing was significantly greater than that of 50 and 60 cm treatments (Fig. 4C). The thickness of the secondary cell wall of the perimedullary phloem fibers in 30 cm and 50 cm plant spacing was significantly greater than that in the 40 cm and 60 cm treatments on the 12th day after marking, and the thickness of the secondary cell wall of the perimedullary phloem fibers in 60 cm plant spacing on the 40th day after marking was significantly greater than that in other treatments (Fig. 4D). 40 days after marking, the lignin content of 60 cm plant spacing was significantly higher than that of 30 cm, and the lignin content of 30 cm and 60 cm plant spacing was significantly higher than 40 cm and 50 cm. The cellulose content of 60 cm plant spacing was significantly higher than the other treatments, and there was no significant difference between the hemicellulose content among the treatments. (Fig. 4E).

2.3. Effect of plant spacing on NtHB8s and NtNST3s genes expression

Different plant spacing lead to the variation in light transmittance in the plant cultivation. The light transmittance between plant and between row were measured. The result is demonstrated in Fig. 5A, the light transmittance increased along with plant spacing, but there is not difference between the plant spacing of 50 and 60 cm.

The full length of NtHB8sy, NtHB8to, NtNST3sy and NtNST3to had cloned and inserted into pBD-GAL4 vector. It could be seen from Fig. 5B that the yeast transformant constructed with pBD-GAL4-NtHB8sy, pBD-GAL4-NtHB8to, pBD-GAL4-NtNST3sy and pBD-GAL4-NtNST3to turned blue in the presence of X-α-Gal and grown normally on the SD medium (SD/-Leu-Trp-His-Ade), whereas the negative control did not. NtHB8sy and NtHB8to classed into the same group, and homologous to AtHB8. NtNST3sy and NtNST3to classed into the same group, and homologous to AtNST3 (Fig. 5C). The expression patterns of NtHB8sy, NtHB8to, NtNST3sy and NtNST3to on the day of marking were identical, gene expression levels of 40 cm plant spacing was significantly higher than that of 30 cm and 50 cm, 30 cm and 50 cm were significantly higher than that of 60 cm. Four days after marking, the gene expression levels of NtHB8sy and NtNST3to of 40 cm treatment was significantly higher, and the expression of NtHB8to and NtNST3sy of 30 cm treatment were significantly higher than the other treatments. The gene expression levels of NtHB8sy, NtHB8to and NtNST3sy of 60 cm treatment were significantly higher, and the gene expression level of NtNST3to of 40 cm treatment was significantly higher than the other treatments in 12 days after marking. The regularity of each gene expression level was that the earlier of each gene expression reached the peak in the smaller of the plant spacing (Fig. 5D).

2.4. cis-acting element analysis of key genes

The 3 kb upstream regions of NtHB8 and NtNST3 were analyzed in the PlantCARE database to find useful information about the regulatory mechanism (Table 1). For promoters studied, all of them contained light response elements, hormone responsive elements, low-temperature elements, MYB and MYC elements. Abundant light response elements indicated that NtHB8 and NtNST3 were relate to light responsive. NtHB8 and NtNST3 might relate to hormone and stress responsive, as well as regulated by MYB and MYC transcription factors. However, the elements of allelic gene promoters were not identical, such as only NtHB8sy had anaerobic elements, only NtHB8to had SA elements, only NtNST3sy had ABA elements. The number of the same elements in allelic gene promoters were not identical too.
Table 1
The distribution of main cis-acting elements in the 3 kb upstream promoter regions of key genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Light</th>
<th>Circadian</th>
<th>Auxin</th>
<th>ABA</th>
<th>GA</th>
<th>MeJA</th>
<th>SA</th>
<th>Anaerobic</th>
<th>Low-temperature</th>
<th>MYB</th>
<th>MYC</th>
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<tr>
<td>NtHB8syP</td>
<td>18</td>
<td>1</td>
<td>1</td>
<td>2</td>
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<td>1</td>
<td>14</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NtHB8toP</td>
<td>19</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>/</td>
<td>2</td>
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<td>8</td>
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<tr>
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<td>/</td>
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<td>2</td>
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<td>NtNST3toP</td>
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<td>1</td>
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<td>7</td>
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</table>

2.5. Effect of plant spacing on nutrient content

Total accumulation of nitrogen, phosphorus and potassium in leaves and stems of tobacco of 60 cm plant spacing were significantly higher than the other treatments, and there was no significant difference among the treatments (Fig. 6A). The accumulation of nitrogen, phosphorus and potassium in leaves of each treatment were higher than that in stems of corresponding parts. The nitrogen content in leaves of 60 cm plant spacing was significantly higher than that of the other treatments, and there was no significant difference among these treatments. Nitrogen content in stems of 40, 50 and 60 cm plant spacing was significantly higher than that of 30 cm plant spacing (Fig. 6B). The phosphorus content in leaves of 60 cm plant spacing was significantly higher than that of 30 and 40 cm plant spacing. The accumulation of phosphorus in stems of 60 cm plant spacing was significantly greater than the 30 and 40 cm plant spacing (Fig. 6C). The accumulation of potassium in leaves of 60 cm plant spacing was significantly higher than these treatments, and lower than that of 40 cm plant spacing (Fig. 6D).

3. Discussion

3.1. Anatomic structure and gene expression pattern analysis of the transition from primary to secondary stem development

Secondary growth usually occurs in gymnosperms and dicotyledons[28], poplar and Arabidopsis had proven to be an excellent model for studying secondary growth[9]. In cross section, the stem of poplar is similar to the hypocotyl of Arabidopsis, such as both plants have vessel elements, sieve-tube elements, companion cells and parenchyma cells and fibers[29]. However rays, radially organized files of parenchyma cells in the secondary vascular tissues of hybrid aspen, were not unequivocally identified in Arabidopsis[30]. Although Busse and Evert observed rays in the secondary xylem of Arabidopsis hypocotyls (under proper growth conditions), they are obviously not the common feature of secondary growth elongation in Arabidopsis[31]. In addition, secondary growth only to be produced at the basal region of the Arabidopsis shoot[4], and the regulatory network in poplar is more complex[32]. From the anatomical structure of tobacco stem cross-section, the cortex, phloem, cambial, xylem, perimedullary phloem and pith. The vascular ray, xylem fiber and xylem vessel in xylem of tobacco are similar to that in poplar (Fig. 2). However, there also have some difference between tobacco and poplar, such as pith in tobacco is obvious (Figure S1) and tobacco stem has perimedullary phloem.

The secondary structure was formed completely 8 days after marking. The maximum increase of xylem thickness, the maximum xylem vessel diameter and the size of pith cells occurred from 8 to 12 days after marking. The increase of stem girth was mainly due to the increase of cell number and cell diameter. Cells of vascular bundle were derived from cambium, the cell number of cambium in active cambium was more than that in dormant cambium[9, 33]. The number of cambium cells increased from 4 to 12 days after marking, which indicated that the cambium was in an active state at this stage[34]. The cambium divided cells differentiate into xylem, resulting in vascular bundle thickening, which leads to the thickening of stem girth. 12 days after marking, the number of cambium cells began to decline, which indicated that...
the ability of cambium division was gradual decline, which was also the main reason for the decrease of stem girth growth (Fig. 3, Figure S2).

Development of xylem and phloem involves including cell division, cell expansion, formation of secondary cell walls (involving cellulose, hemicellulose, and lignin synthesis), and programmed cell death[35]. During the secondary growth of poplar, HB and NAC gene family members that show strong secondary growth-associated upregulation[36]. Tingting Lu et al. have reported the anatomical structure of xylem and secondary cell wall when tobacco was used as transgenic receptor material of poplar gene[37]. To date, there is no systematic report on the secondary growth of tobacco stems. On the other hand, there was neither report about the key genes associated with secondary growth for instance ATHB-8 homologous gene in tobacco. In present research, NtHB8sy, NtHB8to, NtNST3sy and NtNST3to homologous with corresponding genes in Arabidopsis and poplar were cloned, and we also proved that all of them had transcriptional activation activity, which indicated that they were function genes. As the secondary growth of tobacco, the expression level of NtHB8sy, NtHB8to, NtNST3sy and NtNST3to was strongly upregulated, which were in affirmation with the earlier findings and supporting their role in vascular differentiation (Fig. 5, Figure S3).

3.2. Morphological structure, chemical composition and gene expression pattern response of tobacco to plant spacing

The development stage of tobacco stem with different plant spacing treatments was basically the same on the day of marking, which because the size of tobacco was small and their development of were not limited. With the thickening of stem, the thickness of cambium under every treatment tended to increase first and then decrease. The thickness of cambium under 30 cm plant spacing treatment reached the peak at 4th day after marking, and the thickness of cambium of other treatments reached the peak at 12h day after marking (Fig. 4F). It can be seen that the decrease of plant spacing could impact on the activity of cambium, the smaller the plant spacing, the earlier the activity of cambium decreases. The effects of plant spacing on cambium activity and vascular bundle development were consistent. The greater the plant spacing, the higher the cambium activity, and the greater the thickness of xylem and phloem. These findings are found to be similar to maize[38].

The vascular system fulfills two main functions, long distance transport and mechanical support. Xylem cells, with thick secondary cell walls rich in lignin, cellulose and hemicellulose, play important role in providing support to the plant, transport water, nutrients and minerals from the root to shoot[39]. The lignin content of 30 and 60 cm plant spacing at 40th day after marking was significantly higher than that of other treatments, but the reasons for the higher lignin content of these two treatments might be different (Fig. 4E). The thickness of xylem at 60 cm plant spacing and the thickness of secondary cell wall of phloem fiber were significantly greater than that in other treatments. The lignin content in 60 cm plant spacing treatment was significantly greater than that in other treatments, which might be caused by the large number of lignified cells and the large thickness of secondary cell wall of phloem fiber. The high lignin content of 30 cm plant spacing might be caused by too small plant spacing, which limits the development of tobacco plants and promoted the lignification of tobacco plants. The gene expression patterns of NtHB8sy, NtHB8to, NtNST3sy and NtNST3to at 12th day after marking was also a sufficient proof of above inference (Fig. 5D).

The light might be playing an important role in procedure depicted above. As the demonstration in the result part, the transmittance was influenced largely by plant spacing. Simultaneously, the 3kp upstream regions of these genes are abundant with the motifs banding light responding transcription factors. These results implied the expression pattern of these genes could be determined by the different light conditions due to the plant spacing variation.

On the other hand, plant spacing could also lead to the other subjective condition for individual crop, for instance delivery water, essential mineral nutrients, sugars and amino acids; and transportation of those nutrients to the various plant
organs was the essential function performed by the vascular system[40]. Up to date, the effect of plant spacing on the chemistry and physiology of plants is extremely understudied[41]. In this study, the accumulation of nitrogen, phosphorus and potassium in leaves and stems increased with the increase of plant spacing. The accumulation of nitrogen, phosphorus and potassium in stems and leaves of 60 cm plant spacing treatment was significantly greater than that of 30 cm plant spacing. Close planting might reduce the nutrients in the soil corresponding to single plant, and then affect the aboveground nutrient accumulation. It could be seen that the increase of plant spacing is helpful to the transportation and accumulation of nutrients from soil to plant. Interestingly, with the increase of plant spacing, the potassium accumulation in stems decreased significantly, while the potassium accumulation in leaves increased significantly. The sum of potassium accumulation in stems and leaves increased significantly with the increase of plant spacing. Therefore, the increase of plant spacing contributed to the accumulation of potassium and significantly promoted the transport of potassium to leaves (Fig. 6).

Close planting will lead to shading of leaves among plants and affect the photosynthetic photo flux density of plants. Close planting will also lead to less nutrients absorbed by a single plant, which will affect the stem phenotype[25, 42, 43]. Studies have shown that shading has a greater impact on plants than nutrient depletion[44, 45]. Cambial cell divisions was controlled by photoperiod[34]. Light could promote xylem fiber-like cellular differentiation and regulate synthesis of main chemical components of secondary cell wall[46, 47]. Abundant light response elements in the promoter of NtHB8 and NtNST3 indicated that these genes relate to "plant spacing-light-vascular bundle development" relationship.

4. Materials and Methods

4.1. Plant material and growth conditions

Seeds of Nicotiana. tabacum (ZC208) were sown on seedling substrate and grown in greenhouse. After seedling stage (Fig. 1), tobacco seedlings were moved to field. Four different plant spacing treatments were set, which were 30, 40, 50, 60 cm. And the row space of different treatments was the same, which were 120 cm. This experiment was conducted by employing randomized block design, and each treatment had three repeats. Compound fertilizer (m(N):m(P₂O₅):m(K₂O) = 1: 1: 2.5) was applied to field, which the total nitrogen application rate was 67.5 kg·ha⁻¹.

At rosette stage (Fig. 1), when the stem pitch of nodes between the 4th and 5th leaf positions (counted from shoot to root) reached to 1 cm, the node between the 4th and 5th leaf positions was marked with plastic rope. The stem circumference of the marked nodes was measured every 4 days. Samples used for stem girth, stem anatomic structure, physiological index and gene expression analysis were sampled 0, 4, 8, 12, 16, 20, 40 days after marking.

4.2. Measurements of stem girth and microscopy

Due to the stem of tobacco was irregular circle, soft ruler was used to measure stem girth. Samples for cross section observation were stocked in FAA (70% ethanol, 5% formaldehyde, 5% acetic acid), and were sectioned using vibration slicer (VT1000 S, Leica) to a thickness of 25 µm. Sections were stained with Toluidine blue (TBO, T3260, Sigma) and observed under the light microscope (DMC2900, Leica)[48, 49]. Anatomical structure of tobacco stem cross-section was indicated in Fig. 2.

4.3. Chemical composition of secondary cell wall and nutrient content analysis

At flowering stage (Fig. 1), the stems and leaves of the marked position was sampled. Samples were heat-treated at 105°C for 30 min and dried at 65°C to a constant weight and weighted for secondary cell wall and nutrient content analysis.
The contents of lignin, cellulose and hemicellulose in the samples were determined by weighing[50, 51]. Dried the empty filter bags in oven and heat-treated at 105°C for 4 hours, then weighed them as M1. Weighed 0.5 g sample (passing 20 mesh sieve) into filter bags, weighed them as M2. The filter bags were boiled in neutral detergent (5 g/L anhydrous sodium sulfite, 30 g/L sodium dodecyl sulfate solution, 18.61 g/L disodium EDTA, 6.81 g/L sodium tetraborate, 30 g/L sodium dodecyl sulfate, 10 ml/L ethylene glycol ether) for 1 hour. Take out the filter bags, wash them with hot water, soaked them in acetone for 1–2 hours, and wash them with acetone until the liquid is colorless. Put the filter bags in the fume hood to volatilize acetone for 1–2 hours, put them into oven at 105°C for 4 hours, and weigh them as M3. Put the filter bags into acid detergent (20 g/L cetyltrimethylammonium bromide), then boil them repeatedly for 1 hour, wash them with hot water, wash them with acetone, volatilize acetone, dried the filter bags, and weigh them as M4. Put the dried filter bags into 72% sulfuric acid for 3 hours and wash them with water until free of acid. Then soaked filter bags into acetone for 1–2 hours, repeat the acetone washing, acetone volatilization and filter bag drying steps. The weight of the dried filter bags was recorded as M5. Put the filter bag into a 30 ml crucible with known weight (M6), ashed it at a temperature of 600°C ± 15°C for 2 hours and weigh them (M7). Hemicellulose content% = (M3-M4)/M1×100%; Cellulose content% = (M4-M5)/M1×100%; Lignin content% = ((M5-M1)-(M7-M6))/M1×100%.

Grinded the dried stems and leaves into powder, weighted 0.2 g of sample into the digestion tube, added 5 ml of concentrated H$_2$SO$_4$ into the same digestion tube digestion tube. Put the digestive tubes to heating digester (VELP Scientifica) with a 2-stage increase in temperature to 360°C, then cooled samples slightly, and added 2 ml of 30% H$_2$O$_2$ into every digestion tube. After 10 minutes, repeated the operation of adding hydrogen peroxide for several times until sample digested completely. The digestion samples were used for the subsequent analysis[52].

Continuous flow analyzer (AA3) was used for nitrogen and phosphorus content analysis of digested samples. The measurement wavelength was 660 nm, the injection time was 60 seconds and the flushing time was 12 seconds[52, 53].

The standard curves were made by 0, 10, 20, 30 and 40 mg/L potassium chloride. Measure the digestion samples with a flame photometer, calculate the potassium content according to the value on flame photometer and standard curve[54].

4.4. Phylogenetic Analysis and Transactivation Activity Assay of NtHB8sy, NtHB8to, NtNST3sy, NtNST3to

Multiple sequence alignments of HB8 and NST protein sequences (Supplementary file 1) at the amino acid level were performed using the ClustalW program. A neighbor-joining (NJ) phylogenetic tree was then generated based on the alignment result using MEGA 7.0 with the following parameters: Poisson model, partial deletion, and bootstrap values (1,000 replicates).

The method of transactivation activity assay of analysis was described by Na et al[55]. Simply, the PCR products of NtHB8sy, NtHB8to, NtNST3sy and NtNST3to were combined into the pBD-GAL4 vector via EcoRI/Sall sites with specific primers, and with the pBD-GAL4 vector as the negative control. The yeast strain AH109 was used to test transcriptional activation activity. The positive colonies were transferred to the SD medium lacking leucine, tryptophan, histidine, and adenine (QDO, SD/-Leu-Trp-His-Ade) supplemented with X-α-Gal plates at 30°C for 4 days.

4.5. Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis

The marked stems of each plant space treatment were collected and immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction. Three biological replicates were employed per sample. The method of qRT-PCR analysis was described by Na et al[55]. Due to Nicotiana. tabacum was heterotetraploid with Nicotiana. sylvestris (sy) as female parent and Nicotiana. tomentosiformis (to) as male parent, all genes are detected separately according to alleles. The qRT-PCR primers of NtHB8sy, NtHB8to, NtNST3sy, NtNST3to were shown in Supplementary file 2.
4.6. cis-Acting Regulatory Element Analysis

The genome sequences were used to retrieve the 3kb upstream regions for each gene (Supplementary file 3). cis-acting element analysis of NtHB8sy, NtHB8to, NtNST3sy and NtNST3to were carried out using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)[56].

5. Conclusions

Present research used tobacco field cultivation to investigate how plant spacing would influence the stem development. Our result showed increasing plant spacing could promote cambium cells activity; and then divide into more phloem and xylem cells; thereby stimulate the synthesis of the secondary wall and accelerate the accumulation of nutrient accumulation in stems and leaves of tobacco. Plant spacing might affect the expression of NtHB8s and NtNST3s through because of the availability of light, and then affected the morphological structure and chemical content of stem (Fig. 7).

Declarations

Ethics approval and consent to participate

All the materials and methods involved into present research were in compliance with local and national regulations.

Consent for publication

Not applicable.

Availability of data and materials

The data and materials presented in this study are available on request from the corresponding author.

Competing interests

The authors declare no competing interest.

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Author Contributions

W. Q., Y. Z. conceived and designed the experiments; N. X., L. M. performed the experiments; F. T., S. D., Y. X., S. K., Y. L. participated in data collection and analysis; N. X. wrote the manuscript; W. S., Y. L. revised the manuscript. All authors have read and approved the final manuscript.

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**Figures**
This is a figure. Schemes follow the same formatting. Tobacco development period. Tobacco seedlings transplanted to tobacco when tobacco seedlings can be transplanted. Stem nodes were marked at rosette stage (the number of leaves was 12-13). The flower stage was stage of the first central flower opening.
Figure 2

(A) Anatomical structure of tobacco stem cross-section mainly included cortex, phloem, cambial, xylem, pith. Due to the exact developmental stages were still undetermined the bars describing the zones for particular developmental zones were approximate. (B) Detailed illustration of phloem. (C) Detailed illustration of cambium. Mature phloem, phloem differentiation, cambial cell divisions, xylem differentiation, secondary cell wall formation. (D) Detailed illustration of secondary xylem. (E) Detailed illustration of primary xylem. (F) Detailed illustration of perimedullary phloem. Scale bar of (A) was 100 μm, Scale bar of (B-E) were 50 μm.
Figure 3

Cross section of vascular bundle of tobacco stem under different plant spacing treatments at 0d, 4d, 12d, 40d (A-D). Scale bar of every section was 100 μm. The key indexes of vascular bundle were shown in Figure E-F. The bars represented the standard error of the mean, the letters above each bar represent significant differences of different treatments (P<0.5).
**Figure 4**

Effect of plant spacing on secondary cell wall thickness of xylem and phloem cells (A-D). Effect of plant spacing on content of secondary cell wall main components (E). The bars represented the standard error of the mean, the letters above each bar represent significant differences of different treatments (P<0.5).
Figure 5

(A) The light transmittance between plant and between row were measured. (B) Transactivation analysis of *NtHB8sy*, *NtHB8to*, *NtNST3sy* and *NtNST3to*. Genes were combined into the GAL4 (BD) DNA binding domain in pBD-GAL4, with the gene had transcriptional activation activity as the positive control, and empty pBD-GAL4 vector as the negative control. (C) Neighbor-joining (NJ) phylogenetic relationships of *HB8s* and *NSTs*. (D) qRT-PCR analysis of *NtHB8sy*, *NtHB8to*, *NtNST3sy* and *NtNST3to* expression of tobacco stem under 30, 40, 50, 60 cm plant spacing treatments after tobacco stem marking. The bars represented the standard error of the mean, the letters above each bar represent significant differences of different treatments (P<0.5).
Figure 6

Total accumulation of nitrogen, phosphorus and potassium in marked stems and leaves (A). The accumulation of nitrogen (B), phosphorus (C) and potassium (D) in marked stems and leaves. The bars represented the standard error of the mean, the letters above each bar represent significant differences of different treatments (P<0.5).

Figure 7
The overview of the relationship of “environment-gene-structure-function”. Red upward arrows indicated increased.

**Supplementary Files**

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- SupplementaryFigures.docx
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- Supplementaryfile2.docx
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