



Exogenous application of brassinosteroids regulates tobacco leaf size and expansion via modulation of endogenous hormones content and gene expression

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Received: 8 December 2020 / Revised: 9 February 2021 / Accepted: 7 March 2021 / Published online: 27 March 2021
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Abstract Brassinosteroids (BR) play diverse roles in the regulation of plant growth and development. BR promotes plant growth by triggering cell division and expansion. However, the effect of exogenous BR application on the leaf size and expansion of tobacco is unknown. Tobacco seedlings are treated with different concentrations of exogenous 2,4-epibrassinolide (EBL) [control (CK, 0 mol L⁻¹), T1 (0.5 × 10⁻⁷ mol L⁻¹), and T2 (0.5 × 10⁻⁴ mol L⁻¹)]. The results show that T1 has 17.29% and T2 has 25.99% more leaf area than control. The epidermal cell area is increased by 24.40% and 17.13% while the number of epidermal cells is 7.06% and 21.06% higher in T1 and T2, respectively, relative to control. So the exogenous EBL application improves the leaf area by increasing cell numbers and cell area. The endogenous BR (7.5 times and 68.4 times), auxin (IAA) (4.03% and 25.29%), and gibberellin (GA₃) contents (84.42% and 91.76%) are higher in T1 and T2, respectively, in comparison with control. Additionally, *NtBR11*, *NtBIN2*, and *NtBES1* are upregulated showing that the

brassinosteroid signaling pathway is activated. Furthermore, the expression of the key biosynthesis-related genes of BR (*NtDWF4*), IAA (*NtYUCCA6*), and GA₃ (*NtGA3ox-2*) are all upregulated under EBL application. Finally, the exogenous EBL application also upregulated the expression of cell growth-related genes (*NtCYCD3;1*, *NtARGOS*, *NtGRF5*, *NtGRF8*, and *NtXTH*). The results reveal that the EBL application increases the leaf size and expansion by promoting the cell expansion and division through higher BR, IAA, and GA₃ contents along with the upregulation of cell growth-related genes. The results of the study provide a scientific basis for the effect of EBL on tobacco leaf growth at morphological, anatomical, biochemical, and molecular levels.

Keywords Brassinosteroids · Cell division and expansion · Gene expression · Hormones content · Leaf area and expansion · Tobacco leaf growth

Juan Zhang, Yan Zhang and Rayyan Khan contributed equally to this work.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12298-021-00971-x>.

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Introduction

In plants, there are diverse studies that signify the role of hormones in the regulation of various processes during plant growth and development (Kumar et al. 2014). The phytohormones play a decisive role in the plant developmental processes (Alabadí et al. 2009). Brassinosteroids (BR) are growth-promoting steroid hormones which participated in various physiological process during the plant life cycle (Cheon et al. 2013). Brassinosteroids regulate germination, root growth, shoot growth, and bring adaptation under abiotic stresses (Steber and McCourt 2001; Müssig et al. 2003; Sirhindi et al. 2009; Divi et al. 2010; Yusuf et al. 2014; Jakubowska and Janicka 2017; Soliman et al. 2020).

Many studies highlighted that BR is involved in various biological processes as the genetic analysis revealed that BR-sensitive mutants (*d2*) in rice are dwarf, short internodes, erect leaves, and shortened grain (Hong et al. 2003). Clouse et al. (1996) proposed that *BR11* plays a critical role in BR signaling and perception and concluded that *Arabidopsis bri1* mutant has many deficiencies like thick leaves of dark green in color, male sterility, and short stature. In rice, the severe mutant of *BR11* (*d61-4*) also shows phenotypes with short stature and twisted leaves (Nakamura et al. 2006). In *Arabidopsis*, the BR-insensitive mutants (*bin2*) are dark green in color, dwarf, and curly leaves (Li et al. 2001). In another study, the rice BR-deficient mutant (*brd2*) was dwarf, dark green, erect leaves, and shortened leaf sheaths (Hong et al. 2005). Similarly, *BZR1* is the main transcription factor in the BR signaling pathway and necessary for anther and pollen development, as quadruple, pentuple, and hexuple *bzr* mutants were unable to produce seeds (Chen et al. 2019). The BR biosynthesis pathway comprises many genes in which *DWF4*, *CPD*, and *CYP* (P450) are the key enzymes biosynthesis genes (Ohnishi et al. 2012; Si et al. 2016; Zheng et al. 2018). Tanabe et al. (2005) characterized a dwarf and short seeds rice mutant which was associated with BR biosynthesis-related gene *CYP724B1* (a novel cytochrome P450). The BR signalling pathway involves several genes in which *BR11* is the receptor kinase which further activated *BAK1* and forms the *BR11/BAK1* complex by transphosphorylation. Subsequently, phosphorylation of *BSK* and activation of *BSU1*, and nuclear accumulation of *BZR1* and *BES1* transcription factors occur. These transcription factors directly bind to the promoters of BR responsive genes for its regulation (Wang et al. 2006; Kim and Wang 2010).

Furthermore, BR play diverse roles throughout the plant life cycle. The exogenous application of BR significantly improves the growth and development of various crop plants. Foliar spraying of BR hamper leaf senescence, enhanced photosynthesis, number of kernels, improved source and sink capacity, and ultimately yield in maize (Gao et al. 2017). Tong et al. (2014) showed that BR regulates cell elongation in rice via gibberellin metabolism. In another study, BR application increases the plant height and delay senescence in older leaves than young leaves of papaya (de Assis-Gomes et al. 2018). The growth of apple tree is also regulated by foliar application of BR with the integration of auxin and gibberellin (Zheng et al. 2019a, b). Similarly, BR also plays an important role in adaptation and tolerance to various abiotic stresses. The exogenous application of BR confer tolerance to drought stress in maize (Anjum et al. 2011) and pepper plants (Kaya et al. 2019) and temperature stress in *Brassica juncea* (Kaur et al. 2018). Furthermore, the EBL foliar

application also confers salt tolerance via modulation of osmolyte biosynthesis, enzymatic and non-enzymatic antioxidant defense system in apple (Soliman et al. 2020), soybean (Alam et al. 2019; Su et al. 2020), and tomato (Ahanger et al. 2020; Ahmad et al. 2018a, b). Moreover, the exogenous BR application also mitigates the heavy metal toxicity and confer tolerance via the activation of the antioxidant enzyme defense system, higher antioxidant contents, and more proline content accumulation in *Brassica juncea* (Soares et al. 2020), pepper (Kaya et al. 2020), tomato (Jan et al. 2020), pea (Jan et al. 2018), and chickpea (Ahmad et al. 2018b).

There are few studies in tobacco regarding the role of BR as it promotes seed germination (Leubner-Metzger 2001), confers tolerance to various abiotic stresses in *Nicotiana benthamiana* (Deng et al. 2015), enhance resistance to tobacco mosaic virus in *Nicotiana benthamiana* (Deng et al. 2016), and finally also enhance resistance in *Nicotiana tabacum* to a broad range of diseases (Nakashita et al. 2003). Leaves are central to the plant's function. Leaves are initiated from the flanks of the shoot apical meristem and its development is flexible and adjustable according to species, developmental stage, and environmental responses which are regulated by various hormones, transcriptional regulators, and mechanical properties of the tissue (Bar and Ori 2014). The tobacco leaf axis arises from approximately 100 cells in the shoot apex and the pattern of cell growth in the lamina is complex (Poethig and Sussex 1985). For stem elongation and cambial proliferation in leaf is essential for GA accumulation and to send a mobile signal to the stem (Dayan et al. 2012). Therefore, a deeper understanding of leaf growth and development contributes to the overall comprehension of plant biology, and this understanding can be used to improve crop production. At present, keeping in view the role of BR, no such study has been done in the *Nicotiana tabacum* to elucidate the role of exogenously applied brassinosteroids on leaf growth and development. Therefore, this study is designed to clarify the role of exogenous EBL on tobacco leaf growth at morphological, biochemical, anatomical, and molecular levels.

Methods

Plant materials, growth conditions, and EBL treatment

Tobacco (*Nicotiana tabacum*) K326 variety was used in this study. This variety was selected by Northup King Seed Company (USA) and introduced by the Yunnan Branch of China Tobacco Company. K326 variety was kept under the national serial number “00002266” after the DUS test and

certification from the National Tobacco Variety Certification Committee. This variety was provided by the National Infrastructure for Crop Germplasm Resources (Qingdao, China) as a test material to explore the role of brassinosteroids in the tobacco seedling leaf area and expansion. The 2,4-epibrassinolide (EBL) was purchased from Solarbio Science and Technology Company Limited (Beijing, China). The EBL was applied as, T1 (0.5×10^{-7} mol L⁻¹), T2 (0.5×10^{-4} mol L⁻¹) along with a control (CK, 0 mol L⁻¹). The control plants were sprayed with ddH₂O. After germination, the seedlings were planted in 5 cm × 7 cm (diameter × depth) plastic pots containing peat and vermiculite (V/V, 1:1) with one seedling per pot. The seedlings were held in a greenhouse under normal conditions (26 °C with 16 h light, 24 °C with 8 h dark, and 70% relative humidity). The tobacco seedlings grew to the four fully expanded leaves (length > 5 cm) and one new leaf (length < 1 cm) stage was used in this study. A total of 24 seedlings with no disease and pests and uniform growth were selected for each treatment. The EBL was sprayed once a day at 16:00 on the adaxial side of the leaves for consecutive five days. The sketch of the entire experimental setup is portrayed in Fig. 1.

Measurement of leaf morphological parameters

After exogenous EBL application for five days, ten seedlings with uniform growth were selected from each treatment. The fifth individual leaf of each seedling was excised with a razor blade. Leaf digital images were acquired by the camera (Nikon SMZ1000, Japan) and the leaf length, leaf width, and leaf area were measured using ImageJ software (<http://rsbweb.nih.gov/ij/>).

Scanning electron microscopy

Samples from the fifth leaf of seedlings from different treatments were detached and fixed in formalin acetic acid alcohol (FAA) solution [5% (v/v) formalin, (5% (v/v) acetic acid, and 70% (v/v) ethanol)] for 1 h. Following a brief rinse in 0.1 M sodium phosphate solution (PBS) (pH 7.4) (Servicebio, Beijing, China), the leaves were post-fixed by washing with 0.1 M PBS (pH 7.4) for 3 times, 15 min each. Then the leaf samples were transferred into 1% OsO₄ (Ted Pella Inc. CA, USA) in 0.1 M PBS (pH 7.4) for 1–2 h at room temperature. After that, the samples were dehydrated in an ethanol series (30, 50, 70, 80, 90 and 95%) for 15 min at each gradation. Similarly, the samples were also dehydrated two times with 100% ethanol and in

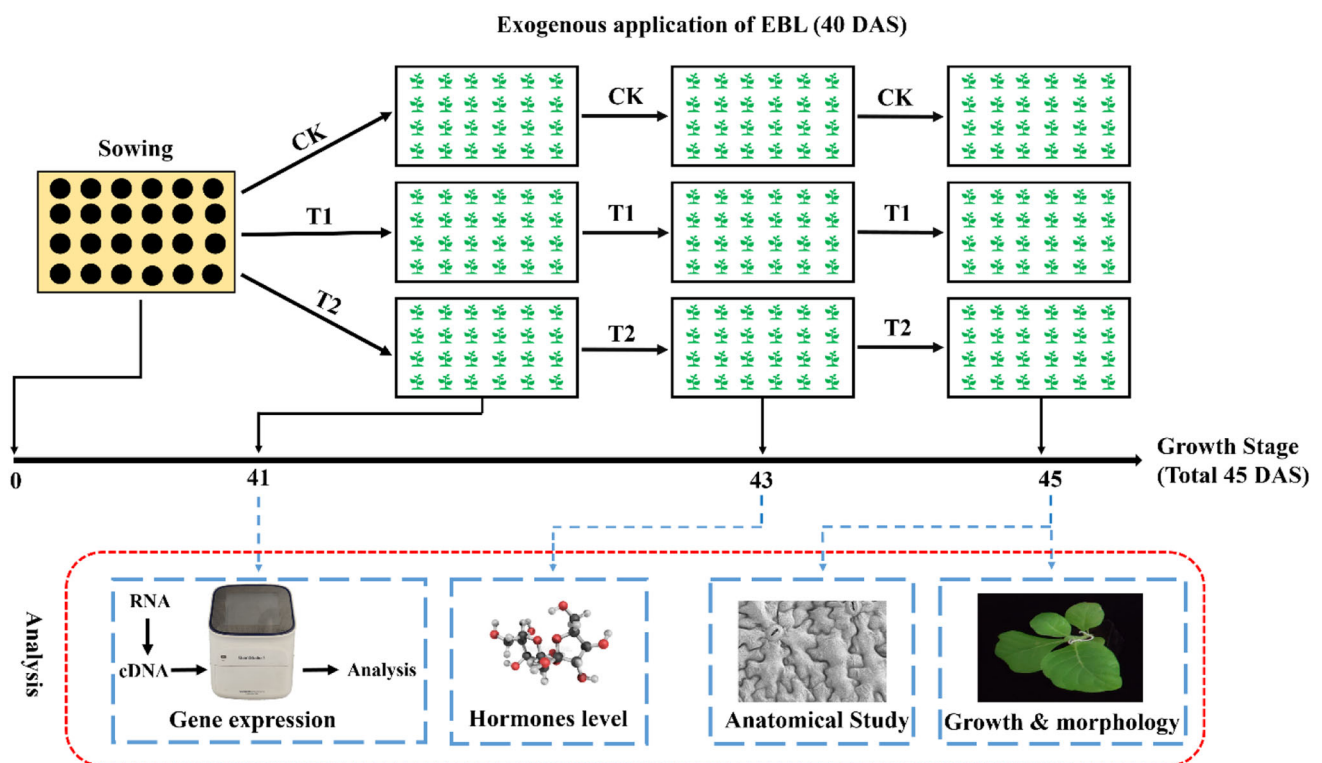


Fig. 1 Setup of the experiment. This schematic flow shows a brief introduction to the entire experiment from start to end. 2,4-epibrassinolide (EBL) was exogenously applied to the leaves of tobacco seedlings with different concentrations. CK symbolizes control

(spraying of seedlings with ddH₂O); T1 represents seedlings sprayed with EBL of 0.5×10^{-7} mol L⁻¹ concentration; T2 denotes seedlings sprayed with EBL of 0.5×10^{-4} mol L⁻¹ concentration; DAS shows days after sowing

isoamyl acetate (Sigma, USA) for 15 min. The dehydrated samples were dried in a critical point dryer (Quorum, K850, England) with liquid CO₂ as transitional fluid, after which the samples were sputter-coated with gold and examined in a scanning electron microscope (HITACHI, SU8100, Japan) (Kim et al. 2003). The area of the epidermal cells was determined using Image J software (<http://rsbweb.nih.gov/ij/>). Cell numbers were calculated by the total leaf area and the average cell number in 4.9152 mm².

BR, IAA, and GA₃ hormone contents

On the third day of EBL treatment, the fifth positioned-leaf from each treatment was collected and immediately frozen in liquid nitrogen and store at – 80 °C. Endogenous hormone content was extracted and purified using the modified procedure as described by Dobrev and Kamínek (2002). Approximately one g of fresh leaf weight for IAA and GA₃ measurement and 2 g for BR measurement was firstly frozen in liquid nitrogen, grounded into fine powder. BRs were extracted with 80% methanol solution (4 °C) for 2 h and purified by Bond Elut column (Agilent Company, Beijing, China) and Strata-X column (Phenomenon Company, Cambridgeshire, England). Then the remaining methanol phase was evaporated to dryness by N₂ and the residue was dissolved in methanol (200 µL). IAA and GA₃ were extracted with 2-propanol/H₂O/concentrated HCl extraction buffer (2:1:0.002, v/v/v, 10 mL) and internal standard solution (8 µL, 4 °C) vibrate for 30 min, and dissolved in 400 µL methanol (0.1% formic acid). The sample solution (2 µL) was injected into the reverse-phase C18 Gemini HPLC column for HPLC–ESI–MS/MS (Qtrap 6500, AB, USA) quantitative analysis (Ding et al. 2013). Hormone levels were measured using a high-performance liquid chromatography system. An external standard was used for quantitative analysis. BR, IAA, and GA₃ standards were purchased from Sigma (USA).

Real time-qPCR (RT-qPCR) analysis

We examined BR signaling pathway-related genes, BR, IAA, GA₃ biosynthesis-related genes in response to exogenous EBL application. Additionally, the expression levels of cell size- and cell division-related genes were also analyzed in leaves after EBL treatments. The National Centre for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/>) for cDNA sequences of *Arabidopsis thaliana* genes were used as queries to identify tobacco homologs via a BLASTN search using e-value as < 1e – 5 of the NCBI. Only homologs with high scores, percentage nucleotide identity to the gene of interest, and high coverage of the query sequence were considered. The genes-specific primers were designed using Primer Premier

5.0. The gene-specific primers detail were provided in Additional file 1 (Table S1) and *NtActin* was used as the internal reference gene (Li et al. 2018). After 24 h of EBL treatments, the total RNA was extracted from the samples using MiniBEST Plant RNA Extraction Kit (TaKaRa, Shiga, Japan), according to the manufacturer's instructions. The concentration of the RNA samples was determined using the NanoPhotometer (IMPLEN, CA, USA) and its quality was evaluated on an agarose gel. The first-strand cDNA was synthesized from 1.0 µg of total RNA using the Prime Script 1st Strand cDNA Synthesis kit (TaKaRa, Shiga, Japan) following the manufacturer guidelines. The reaction volume was 20 µL, including cDNA 1 µL, SYBR RT-qPCR master mix 10 µL (TaKaRa, Shiga, Japan), forward primer 0.4 µL, reverse primer 0.4 µL, and ddH₂O 8.2 µL. The relative expression of each gene was analyzed using the 2^{–ΔΔCt} method (Livak and Schmittgen 2001).

Statistical analysis

Statistical analysis was carried out using one-way ANOVA in SPSS 16.0 (SPSS Inc., Chicago, Ill., USA) and an LSD test ($p < 0.05$) was used to identify the significant differences in leaf dimensional parameters, cell size and cell area, hormones content, and gene expression under different EBL concentration treatments. Figures were generated using OriginPro 9.1 (OriginLab Corporation, Northampton, MA, USA).

Results

Changes in leaf morphology

Exogenous application of EBL with different concentrations produces a prominent effect on tobacco seedlings and exhibit better growth performance than control (Fig. 2A). The leaf length, leaf width, and leaf area of the fifth leaf are significantly increased in response to EBL as compared with the control. The leaf is 9.47% and 16% longer in T1 and T2 compare with control, respectively (Fig. 2B & C), while it is 9.5% and 8.05% wider in T1 and T2 in comparison with control, respectively (Fig. 2B & D). Similarly, T1 results in 17.29% while T2 has 25.99% more leaf area compared with the control (Fig. 2B & E). Overall, the results show that the exogenous application of EBL promoted the leaf area via increment in the leaf length and width.

Changes in leaf anatomy

Leaf size is determined by cell proliferation and cell enlargement. The anatomical analysis reveals that

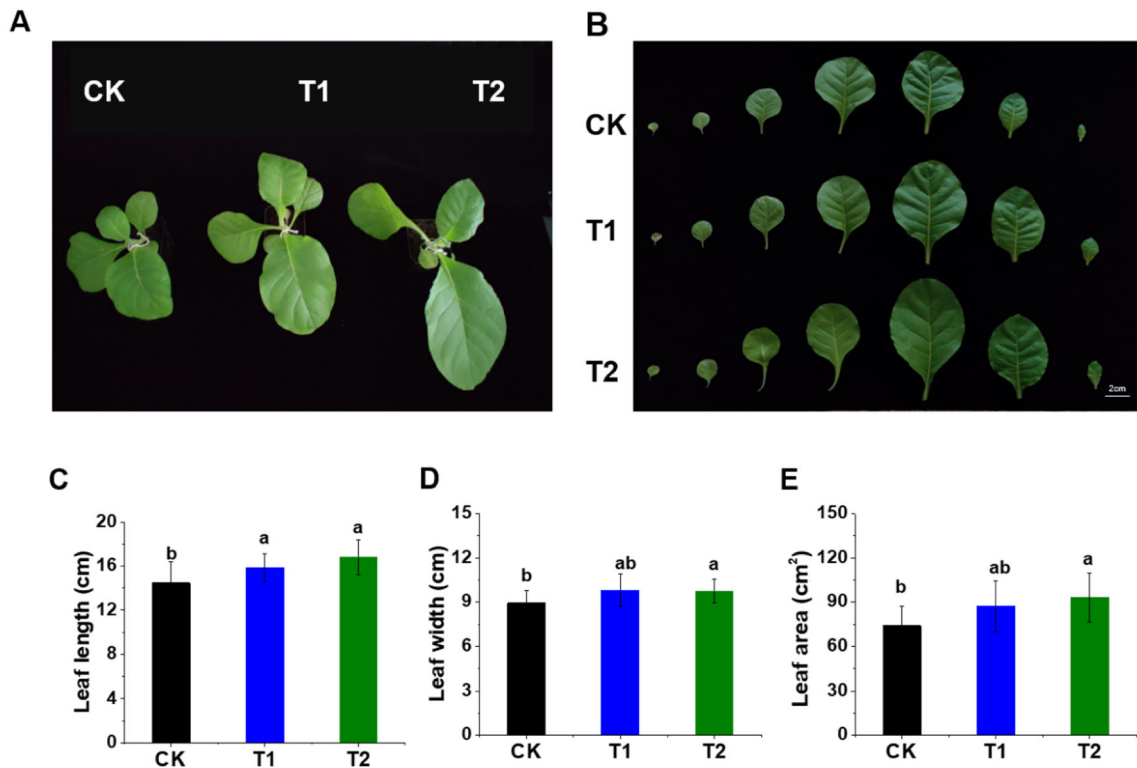


Fig. 2 Effect of exogenous application of 2,4-epibrassinolide (EBL) on leaf growth and morphology. Growth performance under various concentrations of EBL along with a control (A and B). Leaf length, leaf width, and leaf area after EBL treatments (C, D and E, respectively). CK represents control seedlings sprayed with ddH₂O,

T1 represents seedlings sprayed with EBL of 0.5×10^{-7} mol L⁻¹ concentration, and T2 represents seedlings sprayed with EBL of 0.5×10^{-4} mol L⁻¹ concentration. Values represent the mean \pm standard deviation (SD). Different letters indicate significant differences (LSD test; $p < 0.05$)

exogenous EBL applications significantly affected the leaf structure (Fig. 3). The leaf epidermal cells are larger and more in number in the EBL-treated seedlings relative to control (Fig. 3A–C). 24.40% and 17.13% increment is observed in the epidermal cell area of T1 and T2, respectively, in comparison with control (Fig. 3D). Similarly, the number of epidermal cells is also increased with a 7.06% and 21.06% rise in T1 and T2, respectively, compared with control (Fig. 3E). In conclusion, the EBL application promoted the leaf area by an increase in the area and the number of epidermal cells.

Changes in the hormones content

After EBL treatment, the various hormones like BR, IAA, and GA₃ content are significantly affected and increased gradually with the increase in its concentration (Fig. 4). The BR content is 7.5 times and 68.4 times more in T1 and T2, respectively, in comparison with control (Fig. 4A). The IAA content is also elevated in the EBL-treated seedlings with 4.03% and 25.29% increment in T1 and T2, respectively, compared with control (Fig. 4B). Similarly, an 84.42% and 91.76% increase in the content of GA₃ is

observed in T1 and T2, respectively, compared with control (Fig. 4C).

BR, IAA, and GA₃ biosynthesis-related gene expression

In this study, the BR synthesis- and signal transduction-related genes, IAA and GA₃ biosynthesis-related genes are investigated in tobacco seedlings after exogenous EBL application (Figs. 4 and 5). As shown in Fig. 5A, the BR signalling genes, *NTBR11* expression is found non-significant while the transcript levels of *NtBIN2* and *NTBES1* are significantly increased in response to EBL application. *NtBIN2* shows an approximately 1.4 fold change higher expression in T2 compared with control (Fig. 5B). Similarly, *NtBES1* shows 0.5 and 1.8 fold change expression in T1 and T2, respectively, relative to control (Fig. 5C). The BR, IAA, and GA₃ biosynthesis-related genes like *NtDWF4*, *NtYUCCA6*, and *NtGA3ox-2*, respectively, show a rise in their transcript levels in response to EBL application (Fig. 4D–F) while the response observed in the *NtGA2ox-3* gene expression is opposite (Fig. 4G).

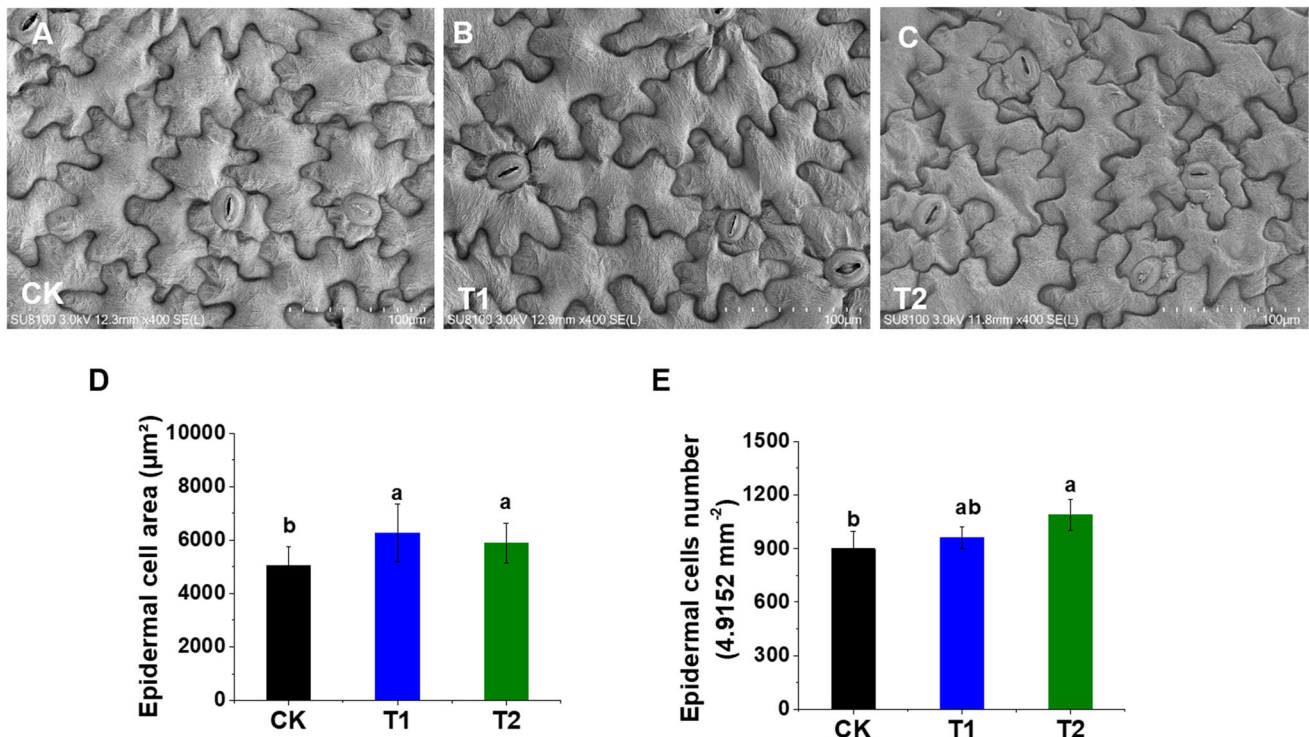


Fig. 3 Leaf structure changes in response to exogenous application of 2,4-epibrassinolide. Representative scanning electron microscopic images of the fifth leaf of control and EBL-treated seedlings (A, B and C). Epidermal cell area (D) and epidermal cell numbers (calculated in 4.9152 mm^2) (E) in response to exogenous application of EBL. CK represents control seedlings sprayed with ddH_2O , T1

represents seedlings sprayed with EBL of $0.5 \times 10^{-7} \text{ mol L}^{-1}$ concentration, and T2 represents seedlings sprayed with EBL of $0.5 \times 10^{-4} \text{ mol L}^{-1}$ concentration. Values represent the mean \pm SD. Different letters indicate significant differences (LSD test; $p < 0.05$)

Cell division- and cell expansion-related gene expression

The cell area and cell numbers are increased in response to the EBL application. Therefore, to know the molecular mechanism related to enhancement in the cell area and number, we seek to analyze the expression levels of cell division- and cell expansion-related genes in response to exogenous EBL application. The expression of *NtCYCD3;1*, *NtXTH*, *NtARGOS*, *NtGRF5*, and *NtGRF8* are significantly higher in EBL-treated than in control tobacco seedlings (Fig. 6A–E). The transcript levels of *NtXTH* and *NtARGOS* are more in T1 while the expression levels of *NtCYCD3;1*, *NtGRF5*, and *NtGRF8* are higher in T2 treatment (Fig. 6A, D & E). *NtXTH* and *NtARGOS* gene expression are notably increased in T1 by about 0.9- and 6.6-time, respectively, while *NtCYCD3;1*, *NtGRF5*, and *NtGRF8* gene expression are distinctly higher in T2 by approximately 3.8-, 1-, and 0.7-time, respectively. Overall, the results show that exogenous EBL application promoted the expression of cell division- and cell expansion-related genes.

Discussion

Brassinosteroids are the steroidal class hormones that play decisive roles in various biological processes during the plant life cycle (Saini et al. 2015; Planas-Riverola et al. 2019). Earlier studies confirm that BR regulates various developmental processes like germination (Steber and McCourt 2001), root growth and development (Müssig et al. 2003; Wei and Li 2016; Lv et al. 2018; Li et al. 2020), organ boundary formation in the shoot apical meristem (Gendron et al. 2012), root meristem development (Li et al. 2020), cell division and cell expansion (Nakaya et al. 2002; Zhiponova et al. 2013), stomatal opening (Inoue et al. 2017), and floral induction (Li et al. 2010).

EBL activates BR signaling pathway

Previously, it is well-documented that BR played crucial roles to improve plant growth and development in various plant species (Que et al. 2017; Zheng et al. 2019a, b; Trevisan et al. 2020) and it is important to know the BR perception and signalling in plants. *BR11*, *BIN2*, and *BES1* genes are the key components of the BR signalling pathway which regulated the bulk number of genes (Ye et al.

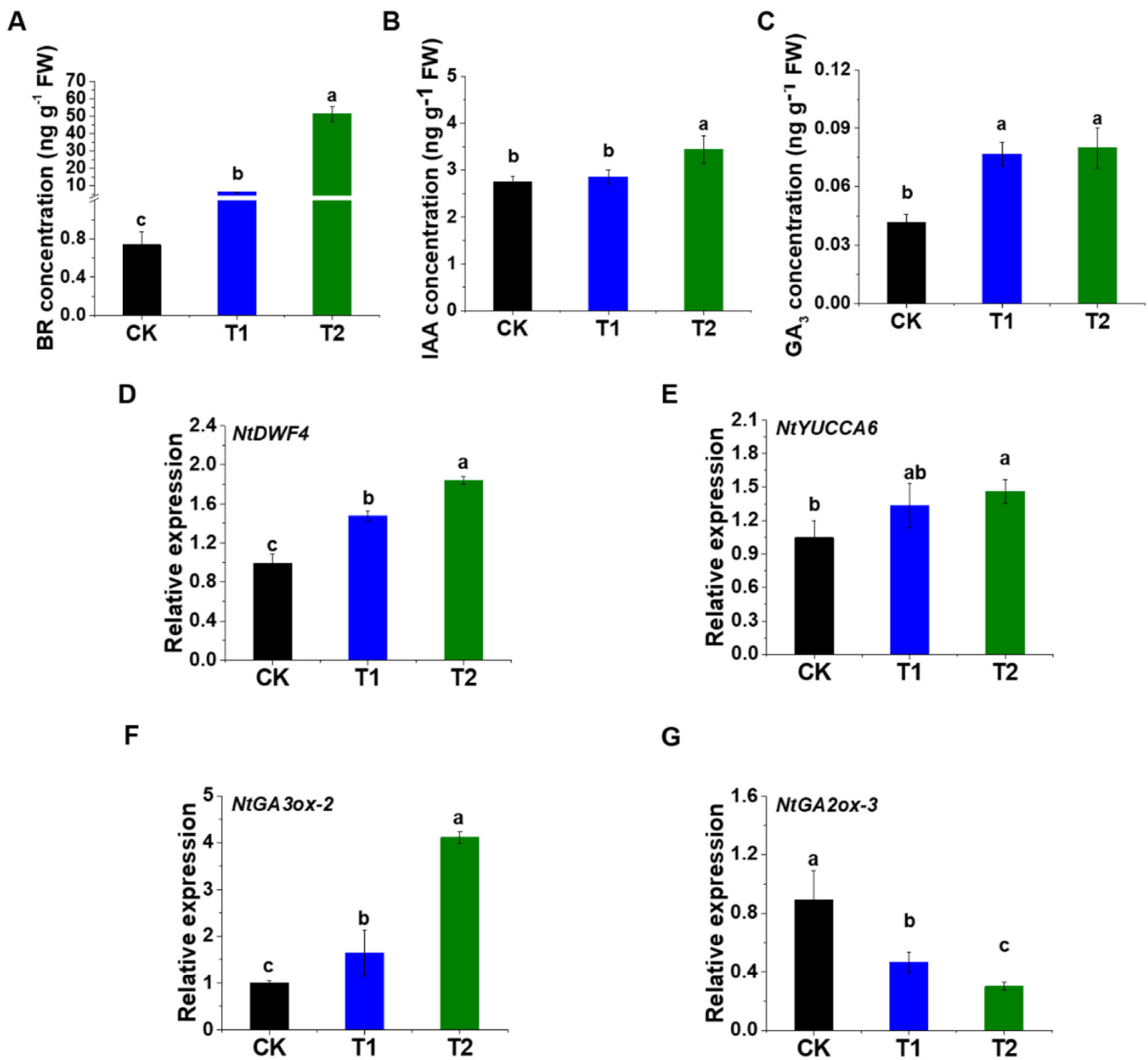


Fig. 4 The endogenous hormones content and its biosynthesis-related genes expression in response to EBL spraying. Brassinosteroids (BR) concentration (A), auxin (IAA) concentration (B), and gibberellin (GA₃) concentration (C) under various EBL concentrations along with a control. *NtDWF4* (BR biosynthesis gene) (D), *NtYUCCA6* (IAA biosynthesis gene) (E), *NtGA3-ox2* (GA₃ biosynthesis gene) (F), and *NtGA2-ox3* (GA₃ inhibitor gene) (G) differentially expressed

under various EBL treatments. Values represent mean ± SD. Different letters indicate significant differences (LSD test; *p* < 0.05). CK represents control seedlings sprayed with ddH₂O, T1 represents seedlings sprayed with EBL of 0.5 × 10⁻⁷ mol L⁻¹ concentration, and T2 represents seedlings sprayed with EBL of 0.5 × 10⁻⁴ mol L⁻¹ concentration

2011). The BR directly binds to its receptor BRI1 at the cell membrane through an extracellular domain (Wang et al. 2001). The BRI1 physically interacts with another receptor kinase BAK1 and activates their kinase activity via transphosphorylation (Chinchilla et al. 2009; Nam and Li, 2002). The formation of the BRI1-BAK1 complex initiates the BR signalling (Wang et al. 2014). The signal is then transduced from the cell membrane to the cytoplasm via the activation BRI1-BAK1 complex and indirectly

regulates the activity of BIN2 which then directly regulates the phosphorylation of BZR1 and BES1 transcription factors and subsequently BR-responsive gene expression (Wang et al. 2006). Overexpressed *SIBRI1* lines enhance the BR signalling thereby increase the germination rate, plant height, and flower size (Nie et al. 2017). *BIN2*, a BR signalling component that is involved in the regulation of pavement cells and leaf growth via its involvement in mediating the stabilization of microtubules (Liu et al.

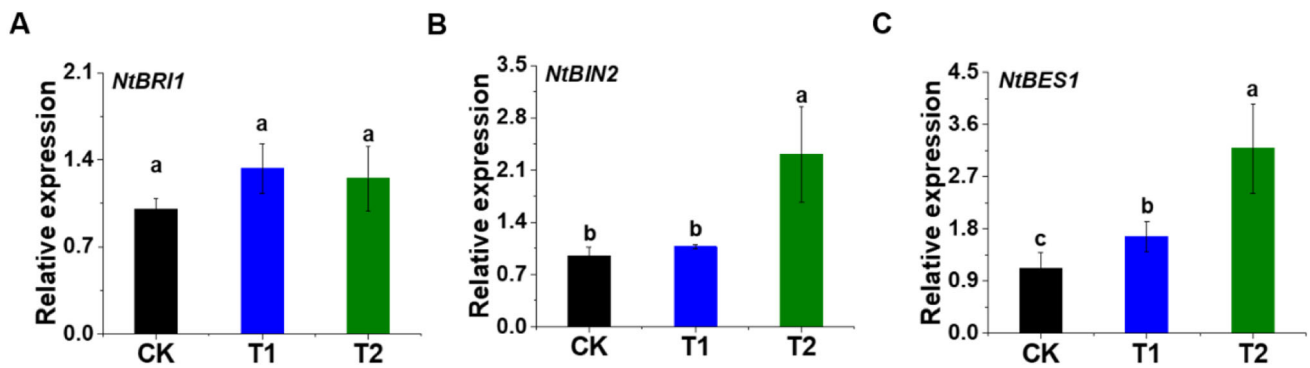


Fig. 5 Differential regulation of BR signaling pathway-related genes under the exogenous EBL application. Expression levels of the corresponding genes-related to the BR signaling pathway are *NiBR1* (A), *NiBIN2* (B), and *NiBES1* (C). Values represent mean \pm SD. Different letters indicate significant differences (LSD test; $p < 0.05$).

CK represents control seedlings sprayed with ddH₂O, T1 represents seedlings sprayed with EBL of 0.5×10^{-7} mol L⁻¹ concentration, and T2 represents seedlings sprayed with EBL of 0.5×10^{-4} mol L⁻¹ concentration

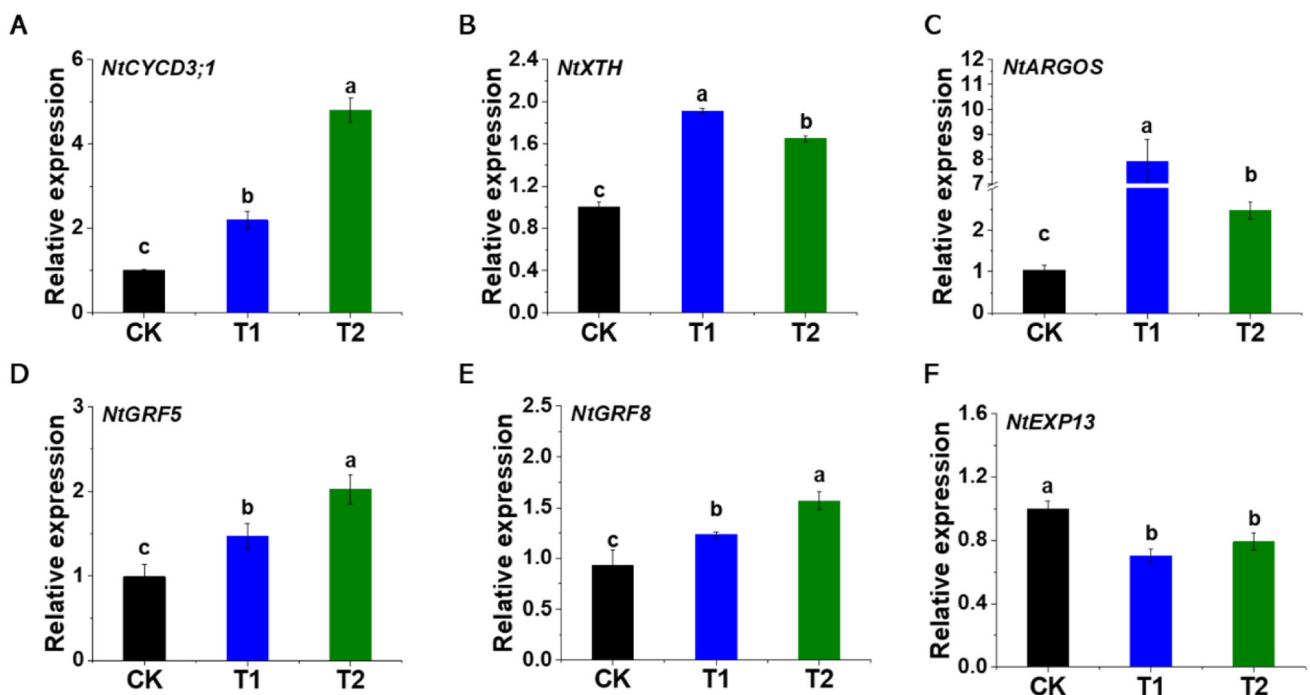


Fig. 6 EBL application significantly regulates the expression of cell growth-related genes. The differential expression of cell growth-related genes; *NiCYCD3;1* (A), *NiXTH* (B), *NiARGOS* (C), *NiGRF5* (D), *NiGRF8* (E), and *NiEXP13* (F) are presented relative to the control samples. Different letters above vertical bars indicate

significant differences using the LSD test at $p < 0.05$. CK represents control seedlings sprayed with ddH₂O, T1 represents seedlings sprayed with EBL of 0.5×10^{-7} mol L⁻¹ concentration, and T2 represents seedlings sprayed with EBL of 0.5×10^{-4} mol L⁻¹ concentration

2018a, b). Yin et al. (2002) show that the *bes1-D bri1-119* double mutants possess long and bending petioles with curly leaves formation. Similarly, Saito et al. (2018) demonstrate that *BES1* is involved in the regulation of xylem and phloem cell differentiation from vascular stem cells. The expression levels of these genes are significantly enhanced by the exogenous EBL application, proposing that they can elevate the endogenous levels of BR and

signaling activity to promote tobacco leaf growth and development.

EBL enhances the endogenous BR, IAA, and GA₃ hormone contents and its biosynthesis-related gene expression

The three main hormones auxin, brassinosteroids, and gibberellin play important roles in plant growth in general

while specifically in cell elongation (Depuydt and Hardtke 2011). In *Arabidopsis*, the BR, auxin and gibberellin regulate the hypocotyl elongation. The cell elongation in the hypocotyl region is also regulated by the signalling network of BR, auxin and ethylene phytohormones which integrate the light and hormone signal via BZR-ARF-PIF/DELLA-ERF module (Liu et al. 2018a, b). The endogenous BR contents increase in response to the EBL application which is supported by the up-regulation of its biosynthesis-related gene *NtDWF4*. In this study, the EBL application enhances the IAA content as it is also witnessed by the up-regulation of *NtYUCCA6*, the biosynthesis gene of IAA. These results are in line with (Mao et al. 2017) in terms of the elevated IAA content due to the exogenous EBL application. Similarly, exogenous EBL greatly induces the expression of *NtGA3ox-2* and ultimately enhances the GA₃ contents and suppresses the expression of *NtGA2ox-3* which is the inactivation gene of GA₃ (He et al. 2019), thus enhances its synthesis and accumulation and inhibits its deactivation. The results of this study are supported by Tong et al. (2014) which demonstrate that BR modulates the GA metabolism by targeting *D18/GA3ox-2* to promote cell expansion in the coleoptile elongation of rice. Similarly, Unterholzner et al. (2015) show that BR is a master regulator of GA biosynthesis. The expression of *GA2ox1* in a BR signalling deficient mutant (*bri1-301*) restore GA biosynthesis and other developmental process. Other studies also find higher content of GA₃ which is induced by brassinosteroid application (Que et al. 2018). The final size of the leaf is strongly controlled by cell division and cell elongation via independently different pathways by analyzing various hormone levels, transcriptomes, and metabolomes (Gonzalez et al. 2010). Similarly, the size and shape of the leaves are also dependent upon the Spatio-temporal regulation of cell division and expansion (Kalve et al. 2014). Hormones regulate the final size of the organ by crosstalk synergistically or antagonistically to stimulate cell proliferation (Wolters and Jürgens 2009). Previous studies have shown that exogenous application of EBL promoted the leaf area of Coleus (Swamy and Rao 2011), mung bean under nickel toxicity (Yusuf et al. 2014), and under normal and high-temperature conditions in *Leymus chinensis* (Niu et al. 2016). Yuan et al. (2012) have also shown a promotion effect of exogenous EBL application on a leaf under salt stress in cucumber. The results of this study show an increase in the epidermal cell numbers, area, and ultimately leaf area under exogenous EBL application as BR is required for cell elongation and cell division (Azpiroz et al. 1998; Nolan et al. 2020; Oh et al. 2020). Nakaya et al. (2002) also demonstrate that the BR application restores cell proliferation and cell expansion in brassinosteroid-related mutants. Auxin is involved in plant shaping architecture by regulating cell division and

elongation (Jiang et al. 2020). GA enhances cell elongation in the leaf sheath of the dwarf mutant of rice (Matsukura et al. 1998) while it also enhances the coleoptile length and seedling leaf one length and area in *Rht12* dwarf bread wheat lines (Chen et al. 2014). The results of this study in terms of higher endogenous gibberellin and auxin levels are congruent with (Talarek-Karwel et al. 2020). Similarly, Sun et al. (2020) results are also analogous to our study which have higher auxin and GA₃ contents under foliar application of BR. BR and GA coordinate to regulate plant growth and development and controlling cell elongation through mediating crosstalk between them (Li and He 2013). Kozuka et al. (2010) also notice crosstalk between auxin and brassinosteroid to promote petiole elongation via cell elongation to shade stimulus in *Arabidopsis*. The growth of the apple tree is also regulated by the interactions of auxin, gibberellin, and brassinosteroid which further activate their signaling pathways and cell growth-related gene expression (Zheng et al. 2019a, b). In the light of the above reports, we speculate that exogenous application of EBL promotes the tobacco leaf area along with increment in cell numbers and cell area might be due to higher endogenous BR, IAA and GA₃ contents and its biosynthesis related-genes and further regulate the cell growth-related genes expression.

EBL induces cell growth-related genes

Plant development is controlled by environmental and endogenous signals, BR and GA signaling pathways involve in cell elongation are supported by physiological and molecular mechanisms (Gallego-Bartolomé et al. 2012). Similarly, auxin and brassinosteroids are also involved in both cell expansion and proliferation which is confirmed at physiological levels and might be at molecular levels via the involvement of the upstream connection of calcium–calmodulin and phosphoinositide signaling in both hormones signaling pathways (Hardtke et al. 2007). Goda et al. (2002) provide a comprehensive view of cell elongation and cell wall organization-related genes induced by BR in *Arabidopsis*. Miyazawa et al. (2003) also speculate that BR is involved in the regulation of genes related to the cell cycle and its multiplication in tobacco BY-2 cells. Xu et al. (2020) demonstrate that brassinosteroid controls the expression of *XTH19* and *XTH23* via *BES1*, as it acts directly upstream of these genes and regulates the lateral root development in *Arabidopsis* under salt stress. Analogous to our results, Zheng et al. (2019a, b) also show that exogenous application of EBL significantly increases the expression levels of *CYCD*, *XTH*, and *GRF* genes in apple plants. *ARGOS-LIKE* (*ARL*) gene is involved in cell expansion during organogenesis in *Arabidopsis* by mediating BR-related cell expansion signals, as it acts

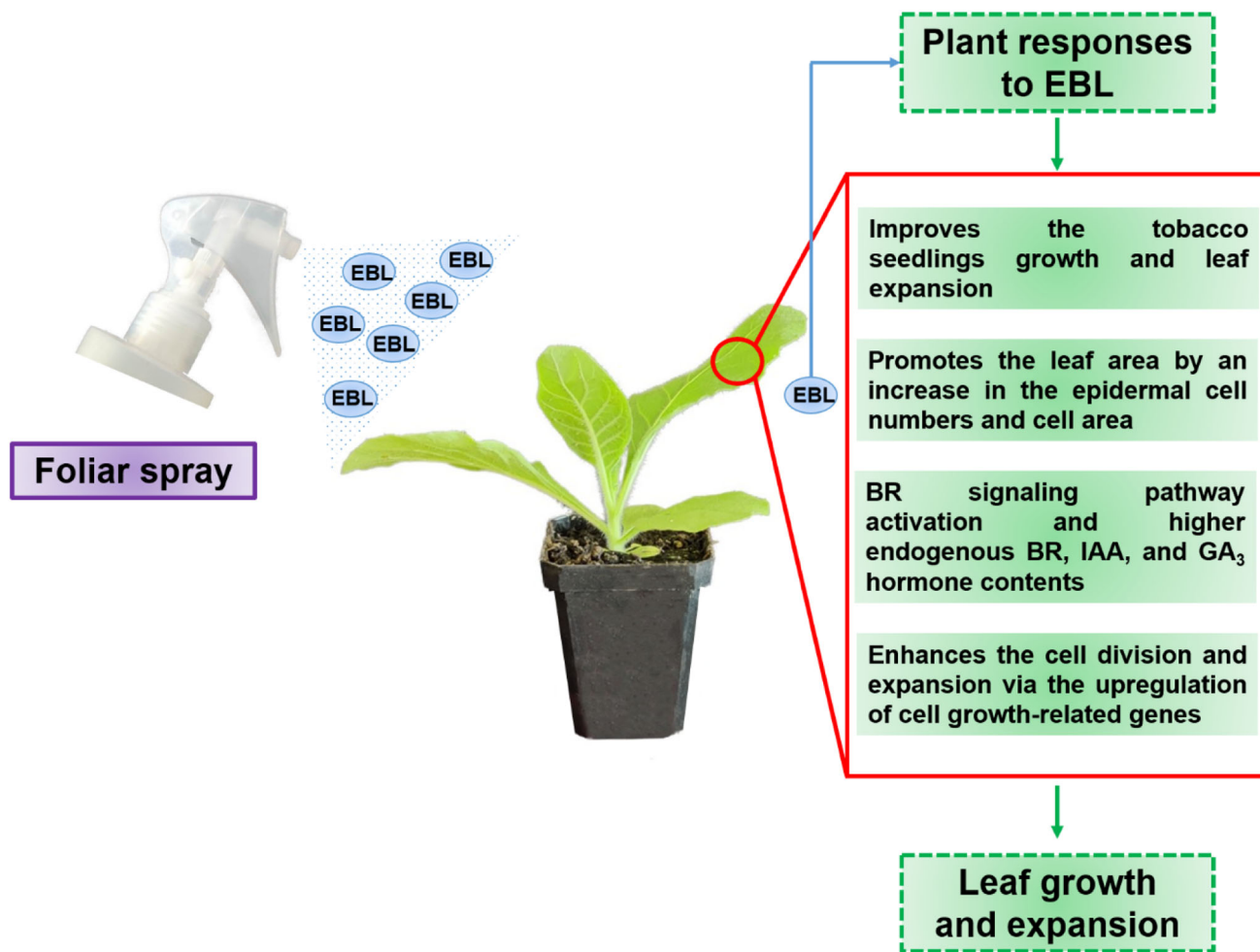


Fig. 7 Promotion effect of the exogenous 2,4-epibrassinolide application on tobacco leaf growth and expansion. This schematic model shows the proposed mechanism of the involvement of EBL in growth and expansion from a morphological level to cellular and molecular levels

downstream of *BRI1* (Hu et al. 2006). In another study, the *ARGOS* gene is also involved in cell proliferation during organ growth which might induce auxin signaling pathway. Overexpression of *ARGOS* gene increases the organ size in *Arabidopsis* and also its ectopic expression induces higher expression of *CYCD3;1* which show that the organ size increase is due to cell proliferation (Hu et al. 2003). It is well established that brassinosteroids and auxin are the key regulators in plant growth and induce morphogenesis (Zheng et al. 2019a, b). As it is mentioned above that *CYCD3;1* and *ARGOS* genes are mediated by both auxin and BR signaling pathways, so in this study the exogenous application results in higher *CYCD3;1* and *ARGOS* genes expression as well as higher IAA levels indicating crosstalk between them and helps in promoting leaf area. The transcript levels of cell growth-related genes (*NtCYCD3;1*, *NtXTH*, *NtARGOS*, *NtGRF5*, and *NtGRF8*) are increased in response to exogenous EBL application with different concentrations, thus imply that BR promoted the

expression of these genes which results in an increment in cell numbers, cell area, and finally the leaf area is increased.

Conclusions

Our study provides a systematic analysis of the tobacco leaf growth at morphological, biochemical, anatomical, and molecular levels in response to exogenous application of EBL. Based on the current study reports (Fig. 7), it is concluded that (i) EBL application improves the tobacco seedlings growth by having a greater leaf area. (ii) EBL application promotes the leaf area by an increase in the epidermal cell numbers and cell area. (iii) The BR signaling pathway is activated and higher endogenous BR, IAA, and GA₃ hormone contents are biosynthesized in response to the EBL application. (iv) Exogenous EBL application enhances the cell division and expansion via the up-regulation of cell growth-related genes. Overall,

these results suggested that the exogenous EBL application promoted the tobacco leaf growth and its expansion by modulating cell division and expansion via higher endogenous BR, IAA, and GA₃ hormones content and regulation of cell growth-related gene expression.

Authors Contribution XM conceived, designed, and supervised the experiment. JZ, YZ, and RK performed the experiments, finalized the figures and tables and their interpretation, and wrote the manuscript. XW and LZ contributed to qRT-PCR studies; NX and SD took part in anatomical studies. All authors read and approved the final manuscript. Juan Zhang, Yan Zhang, and Rayyan Khan contributed equally to this work.

Funding This work was supported by the Central Public-Interest Scientific Institution Basal Research Fund (No. Y2019PT13) and the Agricultural Science and Technology Innovation Project of the Chinese Academy of Agricultural Sciences (ASTIP-TRIC03).

Declaration

Conflict of interest The authors have no conflicts of interest to declare.

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