The Characterization of Columnar Apple Gene MdCoL Promoter and Its Response to Abscisic Acid, Brassinosteroid and Gibberellic Acid

Tingting Han 1,†, Jiahui Yu 1,†, Jie Zhuang 1, Ziyu Wang 1, Xin Sun 1 and Yugang Zhang 1,2,∗

1 College of Horticulture, Qingdao Agricultural University, Qingdao 266109, China
2 Engineering Laboratory of Genetic Improvement of Horticultural Crops of Shandong Province, Qingdao 266109, China
∗ Correspondence: ygzhang@qau.edu.cn
† These authors contributed equally to this work.

Abstract: Columnar apple was an important germplasm resource to develop compact cultivars for labor-saving cultivation and to study fruit tree architecture. MdCol is a strong candidate gene for controlling the columnar phenotype in apple. In this study, a 2000 bp upstream region of MdCol was cloned as a full-length promoter, named MdColp1. To gain a better understanding of the characterization of the MdCol promoter, cis-acting elements and the binding sites of transcription factors were predicted and analyzed, and four binary expression vectors consisting of the GUS reporter gene under the control of the MdCol promoter was transformed into Arabidopsis thaliana to analyze the response to abscisic acid (ABA), brassinosteroid (BR) and gibberellic acid (GA3) of MdCol promoters. Multiple transcription factors involving TCP, BEL1 and BES1/BZR1 and other transcription factor (TF) binding sites were predicted on the promoter of MdCol. Histochemical staining showed that both full-length and 5′ truncated promoters could initiate GUS expression. The GUS activity was the most in leaf and stem, and mainly concentrated in the fibrovascular tissue, followed by root, and the least activity was observed in silique and flower. In addition, MdCol expression was mainly localized in the quiescent center (QC) and lateral root growing point of root tip and the vascular tissue of stem and leaf by in situ hybridization. The results of exogenous hormones treatment showed that ABA and BR could activate the activity of the MdCol promoter, while GA3 had opposite effects. In columnar apple seedlings, ABA treatment could upregulate the expression of MdCol, but GA3 and BR restrained the transcription level of MdCol. These results provide the foundation for deciphering the regulatory network of hormones affecting MdCol transcription.

Keywords: ABA; BR; columnar apple; GA; MdCol promoter

1. Introduction

Plant architecture is a major determinant of photosynthesis, resource allocation, cultivation pattern and crop productivity [1]. Columnar apple tree exhibits short internodes, limited side branches and increased spurs, which were initially identified in the 1960s as a sort of normal structure varieties of ‘McIntosh’ (Mc) named ‘McIntosh Wijcik’ (Wi) [2]. The columnar type was considered as an ideal architecture that could improve photosynthetic utilization and facilitate mechanized management. Classical genetic studies proved that the columnar growth habit is a quality trait controlled by a single dominant gene Co [3–6]. Genetic linkage maps had been constructed and DNA molecular markers had been developed for identifying the Co gene and the columnar phenotype. Finally, the Co gene was located on linkage group 10 [4,5,7–10]. Later studies have found that there is a DNA element/Gypsy retrotransposon that was inserted in the Co region of columnar apples, and the expression of MdCoL/MdCo31/91071-gene located 16kb downstream of
insertion in columnar apples is significantly upregulated compared with standard apples [10–13]. There were other candidate genes in or around the Co region; RT-PCR analysis showed that MdCoL was expressed in the buds, shoot apexes and leaves of ‘McIntosh Wijcik’ in aseptic culture [10–13]. Furthermore, Arabidopsis and tobacco overexpressed MdCoL and are more dwarfed than their wild type, and both have short internodes which are similar to columnar apples [10,12]. Therefore, MdCoL was considered a strong candidate gene for controlling the columnar phenotype in apple.

Phytohormones play a principal role in regulating plant architecture. ABA was well-established as a stress-response hormone. Recently, some studies showed that ABA also regulates plant branching. The strigolactones (SL) signaling pathway could upregulate the expression of OsHOX12 by increasing the expression level of OsNCED1, a key gene for ABA synthesis, and then promoting the synthesis of ABA in shoot base, thereby inhibiting the elongation of lateral buds [3]. BRC1, specifically expressed in lateral buds, could promote the expression of ABA biosynthetic genes to increase the content of ABA in buds, thus causing inhibition of bud development [14–16]. GA has provided multiple biological functions, the most important of which is to promote the elongation of internodes [17]. An SNP mutation occurred in GA receptor GID1, resulting in a GA insensitive dwarf type in peach [18]. BR is a group of steroid phytohormones that also plays an important role in regulating plant architecture [19,20]. Several reports showed that mutations in BR synthesis and signaling genes result in lower tiller number [21,22]. BR directly repressed the transcription of BRC1 though the key transcription factor BZR1 and relieved its inhibitory effect on lateral bud activation [23]. In addition to this, some studies have shown that there are complex regulatory relationships among BR, GA and ABA. BR could promote GA accumulation by regulating the expression of GA metabolic genes GA3ox-2 [22]. ABA could induce the expression of OsGSR1, a positive regulator of GA signaling, and OsGSR1 could activate BR synthesis by regulating a BR biosynthetic enzyme at the post-translational level directly [24,25]. MdBZR1, a transcription factor of the BR signal transduction pathway, directly binds to the promoter of MdABI5 and suppresses its expression to mediate ABA response [26].

MdCoL encodes a putative 2OG-Fe(II) oxygenase or downy mildew resistant 6 (DMR6) [10,11]. The homologous gene in Arabidopsis thaliana, AtDMR6 (AT5G245301.1), also encodes a putative 2OG-Fe(II) oxygenase that is defense-associated but required for susceptibility to downy mildew [27]. Studies have shown that dmr6 was resistant to the bacterium Pseudomonas syringae and the Oomycete Phytophthora capsici and has a cross-talk between the flavone and salicylic acid (SA) pathways in Arabidopsis [28,29]. Further studies revealed that DMR6 could act as an SA 5-hydroxylase (SSH) that catalyzes the formation of 2,5-DHBA by hydroxylating SA at the C5 position of its phenyl ring [30]. At the same time, MdCoL was also confirmed to restrain the synthesis of bioactive GAs via leading to 12-hydroxylation of GA12 in the process of GAs synthesis while the formation of columnar characteristics due to GA deficiency in columnar apple occurred [13–16]. Our previous studies had shown that MdCoL could improve the biosynthesis of ABA via upregulating the expression of MdNCED6/9, which may be one of the reasons for inhibiting the branch growth of columnar apple [13]. Multiple hormones regulated the columnar phenotypes in apple. Although the MdCoL could affect hormone levels in columnar apple, it was unclear whether it was also regulated by a variety of hormones.

In the current study, we report the characterization of the columnar gene MdCoL promoter in apple firstly and revealed that GA and ABA could regulate the expression of MdCoL in a feedback regulatory loop to further affect the formation of columnar traits. These efforts will provide significant information for the analysis of MdCoL function and regulatory network.
2. Results

2.1. Isolation and Bioinformatics Analysis of MdCoL Promoter

To identify the promoter sequence for regulatory motifs, the sequence of 2000 bp upstream of the MdCoL gene was cloned and submitted to the PlantCARE database and PlantRegMap for bioinformatics analysis. As shown in Figure 1, some cis-regulatory elements and TF binding sites were mapped in the MdCoL promoter. TATA-box and CAAT-box were 252 bp and 113 bp upstream of the start codon. In addition, we identified two ABA response elements ABRE (−1481 bp, −1054 bp), six MYB binding sites (−201 bp, −226 bp, −415 bp, −646 bp, −1139 bp, −1499 bp), three TCP binding sites (−897 bp, −1316 bp, −1934 bp), and twelve BES1/BZR1 binding sites (−226 bp, −415 bp, −646 bp, −79 bp, −1139 bp, −1499 bp, −1510 bp, −1490 bp, −1510 bp, −1673 bp, −1769 bp, −1934 bp).

![Figure 1](image_url). The nucleotide sequence and bioinformatics analysis of MdCoL promoter by PlantCARE and PlantRegMap. Different colored blocks represent different transcription factors’ binding sites or cis-acting elements. The short black line shows the start codon of MdCoL.

2.2. The Tissue Expression Characteristics of MdCoL in Columnar Apple

The expression of MdCoL was examined in various tissues of columnar apple Wi and standard apple Mc through qRT-PCR in our group in 2021; the stronger expression of MdCoL was observed in leaves, roots, shoot tips (stems) and buds of Wi, with the highest expression was in stems [13]. In order to facilitate the tissue positioning characteristics of MdCoL in columnar apple, and thus to analyze the relationship between the target gene and the columnar habits of apple more clearly, we performed in situ hybridization in leaves, roots, stems and buds of Wi in this study. The immunolocalization showed that the expression of MdCoL was observed in leaves, roots, shoot tips (stems) and buds of Wi, with the highest expression in stems, leaves, buds and root tips, but the signal mainly reflected in the QC and lateral root growing point of root tips, spongy and palisade tissue of leaves, and vascular tissue of shoots and leaves (Figure 2).
Figure 2. In situ hybridization of mRNA of *MdCoL* in leaf, root, stem and bud of columnar apple 'Wijcik McIntosh'. “Bright” means the fluorescent signals were observed directly, “DAPI” means the fluorescence signal after DAPI (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride) staining. “Merge” represents the superposition image of the above two fluorescence signals.

2.3. GUS Staining in Different Transgenic Arabidopsis Plants

To determine whether the full-length *MdCoL* promoter and its 5’ truncated versions had transcriptional activity in Arabidopsis, GUS-reporter vectors containing different *MdCoL* promoter fragments or 35S promoter were prepared to transform Arabidopsis, obtaining five groups of transgenic Arabidopsis (Figures 3 and S1). A variety of transgenic Arabidopsis organs (siliques, stems, leaves and flowers) with different truncated versions were stained by GUS staining. The staining assays in Arabidopsis showed that all truncated versions in transgenic lines exhibited stronger GUS signals than full-length promoters (Figures 4 and 5), especially in Figure 5. Therefore, it is speculated that there is a sequence for an active repressor between the full-length promoter *MdCoLp1* and the 5’ truncated promoter *MdCoLp2*. Furthermore, leaves and stems had stronger GUS immunostaining than the other three organs (Figures 4 and 5). It can be also seen that the activity level of GUS signals induced by *MdCoLp1* were the lowest in all truncated versions’ transgenic lines (Figures 4 and 5). The highest GUS activity was observed in the stems of *MdCoLp2* and *MdCoLp3* (Figure 5). According to the results mentioned above, the GUS staining in transgenic Arabidopsis with different *MdCoL* promoter fragments was observed, and the GUS activity was the most in leaves and stems, in which, GUS staining was mainly concentrated in the fibrovascular tissue, followed by roots, and the least activity was...
observed in siliques and flowers. The activity of GUS in leaves was strong and stable, so we chose leaves as materials in the next hormones treatment test.

Figure 3. The scheme of MdCol promoter constructs for Arabidopsis transformation. (a) The constructs diagram of MdCol promoter with different length. (b) Schematic diagram of the expression vector for promoter activity detection, in which the expression vector pCambia1301 was used to clone the MdCol promoter at the BamHI and NcoI sites. The transcription factors’ binding sites and cis-acting elements located on the MdCol promoter were indicated by squares of different colors.

Figure 4. Histochemical staining of GUS in Arabidopsis plants transformed with the MdCoLp1/2/3/4::GUS construct. The age of Arabidopsis seedlings in (a–e) were 2 days, 5 days, 12 days, 18 days and 25 days, respectively. The scale bar of (a–e) is 2 mm.

2.4. GUS Activity in Different Transgenic Arabidopsis Plants under Hormones Treatment

The plant architecture, especially apple columnar traits, was closely related to plant hormones. A series of hormone-related cis-regulatory elements and TF binding sites were predicted on the MdCol promoter (Figure 1) to further explore the relationship between plant hormones and columnar traits and clarify the response of MdCol to plant hormones. Five groups of transgenic Arabidopsis with different promoters were stimulated by ABA (200 µM), GA3 (500 µM), BR (200 µM) and staining by GUS staining solution (Figure 6). Four groups of transgenic Arabidopsis with different lengths of MdCol promoters in them were collected to measure GUS activity after ABA, GA3 and BR treated (Figure 7). Our results show that ABA and BR treatment...
significantly enhanced the GUS activity of all transgenic lines, whereas the GUS activity after GA$_3$ treatment was inhibited (Figures 6 and 7).

Figure 5. Histochemical staining of GUS in roots (a), stems (b), cauline leaves (c), inflorescences (d) and siliques (e) of Arabidopsis transformed with the construct of MdCoLp1/2/3/4::GUS and 35S::GUS at 35 days of seedlings. Scale bar is 2 mm.
Figure 6. Effect of exogenous hormones treatment on the GUS histochemical staining of transgenic Arabidopsis plants transformed with the construct of MdCoLp1/2/3/4::GUS and 35S::GUS. The leaves used for 200 µM ABA, 200 µM BR and 500 µM GA_{3} treatment were rosette leaves of transgenic Arabidopsis growing normally for 30 days. A set of three leaves represent three transgenic Arabidopsis lines, respectively.
Figure 7. The effects of different hormones treatments on GUS activity under the control of different *MdCol* promoter deletion segments in transgenic Arabidopsis lines. The GUS activity was detected in transgenic lines at 0, 3, 6, 9 and 12 h after treatment with 200 μM ABA, 200 μM BR and 500 μM GA3. Error bars indicate standard deviation from three independent biological replicates (*p* < 0.05; **p** < 0.01; ***p*** < 0.001).

GUS staining results of *MdColp0*, the positive control plants, fused with 35S promoter were stable after treat with ABA for 1–12 h (Figure 6). *MdColp1* and *MdColp2* showed the deepest GUS staining at 6 h of ABA treatment, while *MdColp4* had the highest increase at 3 h of ABA treatment (Figure 6). There was no significant difference in GUS staining of *MdColp3* from 3 to 9 h after ABA treatment, and both of them were deeper than 0 h (Figure 6). The results of GUS activity showed that *MdColp1*, *MdColp3* and *MdColp4* were significantly enhanced at 3–9 h compared with 0 h, and *MdColp2* was significantly enhanced at 6–9 h (Figure 7). After 12 h of ABA treatment, GUS activity in four transgenic lines presented downregulation to varying degrees (Figure 7). The changes of GUS activity in all lines were consistent with the results of GUS histochemical staining.
BR and ABA treatments were similar in four promoter deletions’ transgenic lines, all of which could increase the expression of GUS (Figures 6 and 7). The GUS staining of MdCoLp0 did not change after BR treatment, and the staining levels of the other four Arabidopsis groups were deepened after BR treatment (Figure 6). The GUS activity of MdCoLp1 treated with BR for 3 h and 9 h was significantly higher than that of control at 0 h (Figure 7). In MdCoLp2 and MdCoLp4, the GUS activity increased after 3 h of BR treatment and recovered after 12 h of BR treatment (Figure 7). The trend of change in MdCoLp3 started rising after 3 h until it began to recover after 9 h, and its GUS activity was less upregulated after BR treatment: only after 3 h of treatment were significant differences from untreated controls shown (Figure 7).

Unlike ABA and BR treatment, GA3 treatment could reduce GUS expression in different 5’ truncated versions’ transgenic lines (Figures 6 and 7). GUS staining results of MdCoLp0 were stable after treatment with GA3 (Figure 6). In MdCoLp1, the GUS staining degree after GA3 treatment was weakened and gradually disappeared compared with 0 h (Figure 6). The GUS staining of MdCoLp2, MdCoLp3 and MdCoLp4 decreased first and then recovered gradually with the extension of treatment time (Figure 6). The GUS activity of MdCoLp1 and MdCoLp2 also decreased first and then increased after GA3 treatment and reached the minimum value after GA3 treatment for 6 h and 9 h, respectively (Figure 7). The GUS activity in MdCoLp3 and MdCoLp4 decreased significantly after 3 h of GA3 treatment and resumed after 6 h (Figure 7). These results were consistent with the results of GUS staining.

2.5. MdCoL Promoter Affected MdCoL Expression under Hormones Treatment

Consistent with the above results, the columnar apple seedlings treated with ABA, BR and GA3 showed time-dependent changes in MdCoL expression (Figure 8). After 3 h of ABA treatment, the levels of MdCoL expression increased 3.2 times compared to that of the baseline, slowly increased to the peak, and was followed by a downward trend after 9 h (Figure 8). The change of MdCoL expression in columnar apple seedlings after BR treatment was contrary to GUS activity analysis results of transgenic Arabidopsis. After 3 h with BR treatment, the expression of MdCoL was significantly decreased, and the subsequent treatment period remained basically unchanged (Figure 8). On the other hand, GA3 could downregulate MdCoL levels relative to the baseline after 3 h significantly; then, they gradually decreased and showed an upward trend after 12 h (Figure 8).

![Figure 8. The expression of MdCoL in columnar apple ‘Wijcik McIntosh’ under different hormones treatments. The expression levels were detected in ‘Wijcik McIntosh’ seedlings at 0, 3, 6, 9 and 12 h after treatment with 200 µM ABA (a), 200 µM BR (b) and 500 µM GA3 (c). Error bars show standard deviation from three independent biological replicates (* p < 0.05; ** p < 0.01; *** p < 0.001; “ns” means no significant difference).](image)

3. Discussions

Since its discovery in the last century, columnar apple has been an important germplasm resource for scientists to study the structure of apple trees and breeding varieties which are suitable for high-density, low-cost and labor-saving cultivation. Although breeders have developed many new varieties by combining columnar habits with other advantageous characteristics through traditional crossbreeding, the formation mechanism of the columnar...
Phenotype is still unclear. Previous studies shown that MdCoL is a potential candidate gene involved in this process, and it was accumulated higher in aerial parts of columnar than in standard apple [10–13]. In this study, in situ hybridization results showed that the expression of MdCoL was abundant in leaves, stems, shoots and roots of Wi (Figure 3). Similar results were also observed in the GUS staining of transgenic Arabidopsis with different promoter fragments (Figure 5). These results could further prove that MdCoL is related to the formation of columnar traits, including short internodes, poor lateral branch formation, increased spurs, thick green leaves and a well-developed root system.

Some studies had shown that the significant upregulation expression of MdCoL in aerial parts of columnar apple was due to the insertion of a retrotransposon upstream of the promoter [10–12]. Interestingly, previous studies, including ours and other groups, found that the transcription of MdCoL also accumulated highly in the roots of standard apple [13,31]. The MdCoL promoter was active in all tissues of transgenic Arabidopsis, and the above results indicated that there were other regulatory elements that affect the expression of MdCoL. In the current study, ABA, BR and GA3 could affect the activity of MdCoL promoter with different length in transgenic Arabidopsis, and the above results indicated that there were other regulatory elements that affect the transcription of MdCoL (Figures 6–8). The analysis of promoter showed that MdCoL promoter contained the binding sites of TCP, BEL1 and BES1/BZR1, and some TFs are relevant to phytohormone (Figure 1). Therefore, it is speculated that the transcription of MdCoL should be regulated by a series of TFs. Some studies have shown that genes of these transcription factor families may be involved in the formation of plant architecture by affecting hormone synthesis and signal transduction [15,23,32,33]. The branching regulators, the TCP transcription factor TB1 could restrict the elongation of stem internodes and also induce bud suppression through increasing the production of ABA and JA and reducing sugar levels and energy balance [34,35]. The cucumber TCP family gene CsBRC1 could reduce the accumulation of auxin in buds by directly repressing the expression of CsPIN3, a transporter of auxin, resulting in inhibition of the lateral bud outgrowth [36]. BZR1, the BR signaling component, could bind to BCRI directly, regulating its transcription and then promoting the growth of tomato buds through integrating GA, CK, SL and other hormonal pathways by it [23]. Thus, the transcription factor binding site on the MdCoL promoter region is likely to regulate the formation of apple columnar traits by recruiting specific TFs in response to plant hormones. The prediction of cis-acting elements and the transcription factor binding site of the MdCoL promoter is helpful to improve the regulatory mechanism and network of MdCoL in the formation of columnar traits.

The GUS activity of the full-length promoter was the weakest among the four promoters mentioned above; compared with the full-length promoter MdColp1 and the three short promoters MdColp2, MdColp3 and MdColp4, there was an extra 533 bp promoter sequence in MdColp1. Except for ABRE and the TCP binding site, this region may contain a binding site for an inhibitor that reduces the activity of MdColp1.

Phytohormones play a vital role in the formation and regulation of plant architecture, including columnar habits in apple [6,13,37,38]. GA and ABA were key factors in regulating plant architecture [16,18,39]. The MdCol could lead the formation of columnar traits through decreasing the biosynthesis of biologically active GAs and improving the biosynthesis of ABA, resulting in internode shortening and lateral branch growth in a slow manner [13,37,40]. The expression of GUS driven by those promoters, or the degree of GUS staining, showed a downward trend after GA treatment, and an upward trend after ABA treatment, although there were some differences in details. In other words, ABA could upregulate the activity of the MdCol promoter with different lengths, and GA3 could downregulate the activity of the MdCol promoter (Figures 6 and 7). The results of qRT-PCR show that ABA upregulated the expression of MdCol, and GA3 downregulated the expression of MdCol, which also confirmed the above conclusion (Figure 8). On the whole, MdCol did not only influence the formation of apple columnar traits by regulating the biosynthesis of ABA and GA, but also was upregulated by ABA and downregulated by GA3 in a feedback regulatory loop.
Promoters do not regulate gene expression themselves but play a role by binding to TFs. There are many cis-elements and TF binding sites on promoters, which could help to predict their expression characteristics and functions. ABRE was predicted at two locations (−519 bp, 946 bp) in MdCoL promoters (MdCoLp1 and MdCoLp2) (Figures 1 and 3). Therefore, the presence of ABRE might be caused by the strong response of MdCoLp1 and MdCoLp2 to ABA treatment. Some MYB genes’ expression was induced by ABA but was repressed by GA, and these genes regulated cell-wall biosynthesis and phytohormone signaling [41,42]. Although the ABRE elements were absent at MdCoLp3 and MdCoLp4, they had multiple MYB binding sites (Figures 1 and 3); this activity could also be induced by ABA. The inhibition of GUS activity by GA3 in four promoter fragments as well as MdCoL expression may also be due to the presence of MYB sites. CANNTG were cis-elements associated with BR and were binding sites of BZR1 [38,43], though they were predicted in four promoter fragments. Therefore, in transgenic Arabidopsis, BR could promote the activity of promoters (Figures 6 and 7). Interestingly, the expression of MdCoL was decreased after treatment of BR on the columnar apple ‘Wijcik McIntosh’ seedlings, and the expression of MdCoL was decreased (Figure 8). This may be due to the high content of ABA and low content of GA in columnar apple and the presence of coregulation of other genes.

In this study, the promoter of columnar gene MdCoL was cloned, and a series of analyses and evaluations was conducted. The MdCoL promoter was able to respond to ABA, BR and GA3, and these hormones could regulate the expression of MdCoL (Figures 6–8). A lack of active GAs and high levels of ABA could lead to the emergence of columnar traits. Moreover, GA3 and ABA could regulate the expression of MdCoL in a feedback loop to affect the formation of columnar traits. Further studies are in progress to explain the detailed mechanism of different hormones, especially BR, which regulated the formation of columnar type by influencing the expression of MdCoL.

4. Materials and Methods

4.1. Plant Materials and Treatments

The apple materials were obtained from tissue cultured columnar apple Wi. The wild type Columbia Arabidopsis thaliana (Col-0) was used for genetic transformation in this study. Transgenic Arabidopsis and Wi seedlings were treated with ABA (200 μM), GA3 (500 μM) and BR (200 μM). Then, rosette leaves of transgenic Arabidopsis were sampled at 0, 3, 6, 9 and 12 h, and were used for histochemical GUS staining and GUS activity assays. In addition, MdCoL gene expression was performed in leaves of Wi seedlings at 0, 3, 6, 9 and 12 h through qRT-PCR after treatment.

4.2. In Situ Hybridization

The MdCoL-specific probe labelled with FAM (carboxyfluorescein) was prepared from the unique region of coding sequence, and its nucleotide sequence was 5’-TGGGCATGGCGGATAAGTGGTGGACAG-3’. Apical tips, leaves, stems and buds approximately 5.0 mm in length were collected from the tissue cultured seedling of columnar apple Wi, which were grown approximately one month in rooting medium after subculturing for about one month and fixed with RNase-free FAA solution at 4 °C overnight, respectively. Tissue sections and RNA hybridization were performed according to previous experimental methods with minor modifications [44]. Paraffin blocks were trimmed to make slices 10 μm thick; among them, stems and leaves were cut crosswise, and root tips and buds were cut longitudinally. The hybridization was carried out at 55 °C for 16 h, then observed and photographed by CaseViewer.

4.3. Isolation and Bioinformatics Analysis of MdCoL Promoter

The genomic DNA was extracted from columnar apple Wi with TIANGEN Plant Genomic DNA Kit (TIANGEN BIOTECH, Beijing, China). The 2000 bp sequence upstream of the start codon of MdCoL was used as the promoter region [45]. Both the full-length and three 5′ truncated promoter fragments were isolated by PCR amplification using specific primers.
MdCoLp1-F, MdCoLp2-F, MdCoLp3-F, MdCoLp4-F and MdCoLp1-R (Supplementary Table S1). PCR products were cloned into the pMD-19T-vector and sequenced. The full-length promoter was submitted to PlantRegMap (http://plantregmap.gao-lab.org/binding_site_prediction.php accessed on 5 March 2020) to predict the binding site of transcription factors [46]. The online tool PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ accessed on 5 March 2020) was used to predict cis-regulatory elements.

4.4. Construction of GUS Reporter Expression Vectors and Genetic Transformation of Arabidopsis

The full-length MdCol promoter and three 5’ truncated promoter fragments were ligated into pCAMBIA1301, a binary expression vector with GUS reporter gene, to replace CaMV 35S promoter. Recombinant plasmids and pCAMBIA1301 were transformed into Agrobacterium strain EHA105 and then introduced into Arabidopsis by Agrobacterium-mediated floral dip method [47]. The hygromycin-resistant positive transgenic individuals were screened from progeny seeds. T3 lines were obtained by screening generationally and used as the follow-up experimental materials after identification performed by PCR and qRT-PCR, whose identification primers were GUS-F/R and Q.GUS-F/R (Supplementary Table S1).

4.5. Histochemical GUS Staining

The seedlings and plant organs of each of the transgenic Arabidopsis lines were immersed in GUS staining solution (Solarbio LIFE SCIENCES, Beijing, China) for 4 h at 37 °C. Before staining, they were placed in a decompression chamber for 30 min. After staining, they were immersed in 70% alcohol for 3 h and then soaked in 95% alcohol until chlorophyll was completely removed at 37 °C. The rosette leaves of transgenic Arabidopsis treated with hormones were stained and decolorized in the same way. Some stained seedlings and leaves were observed and photographed with the Pixera Pro600ES digital CCD camera system.

4.6. The Analysis of GUS Activity

The analysis of GUS activity was performed as described in previous study [48]. The rosette leaves of transgenic Arabidopsis treated with phytohormones collected at different periods of time were ground in liquid nitrogen to extract samples to be tested. The 4-methyl-umbelliferyl-glucuronide (4-MUG) (Sangon Biotech, Shanghai, China) was used as a substrate. Fluorescence was determined using a multifunctional microplate reader (PerkinElmer, Shanghai, China) at excitation and emission wavelengths of 365 and 455 nm, respectively.

4.7. RNA Extraction and qRT-PCR Analysis

The total RNA was isolated from columnar apple Wi seedlings with RNA Pure Plant Plus Kit (TIANGEN BIOTECH, Beijing, China). HiScriptII 1st strand cDNA synthesis kit (Vazyme, Nanjing, China) was used for synthesizing cDNA. qRT-PCR was performed with AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). The MdActin gene, which was stably expressed in all of the tissue, was selected as a reference gene [13]. Relative expression levels were calculated using the comparative Ct method [48].

4.8. Statistical Analysis

The data were shown as the means ± SE of three independent experiments with at least three replicates in each experiment. Differences among means were analyzed by one-way analysis of variance (ANOVA) combined with Duncan’s multiple range test: *p < 0.05, **p < 0.01 and ***p < 0.001.

5. Conclusions

Multiple transcription factors involving TCP, BEL1 and BES/BZR1 and other transcription factor binding sites were predicted on the promoter of MdCoL. The MdCoL promoter
was able to respond to ABA, BR and GA₃, and these hormones could regulate the MdCoL expression. Moreover, GA and ABA could regulate the expression of MdCoL in a feedback regulatory loop to further affect the expression of columnar traits.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms231810781/s1.

Author Contributions: Y.Z. and X.S. designed all the experiment as well as revised the draft of the manuscript. T.H. and J.Y. wrote the manuscript. T.H. performed the experiments and analyzed the data. J.Y., J.Z. and Z.W. helped analyze data. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Sciences Foundation of China (31901977), Natural Sciences Foundation of Shandong (ZR2019BC002, ZR2019MC003), National Key Research and Development Program Foundation (2019YFD1001403), Special fund project for guiding local scientific and technological development by the central government (YDZX2021091) and China Agriculture Research System Foundation (CARS-27).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article and the Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Wang, B.; Smith, S.M.; Li, J.Y. Genetic regulation of shoot architecture. *Annu. Rev. Plant Biol.* 2018, 69, 437–468. [CrossRef] [PubMed]
2. Kelsey, D.F.; Brown, S.K. ‘McIntosh Wijcik’: A columnar mutation of ‘McIntosh’ apple proving useful in physiology and breeding research. *Fruit Var. J.* 1992, 46, 83–87. [CrossRef]
3. Lapins, K.O. Segregation of compact growth types in certain apple seedling progenies. *Can. J. Plant Sci.* 1969, 49, 765–768. [CrossRef]
4. Tian, Y.K.; Wang, C.H.; Zhang, J.S.; James, C.; Dai, H.Y. Mapping Co, a gene controlling the columnar phenotype of apple, with molecular markers. *Euphytica* 2005, 145, 181–188. [CrossRef]
5. Bai, T.; Zhu, Y.; Fernández-Fernández, F.; Keulemans, J.; Brown, S.; Xu, K. Fine genetic mapping of the Co locus controlling columnar growth habit in apple. *Mol. Genet. Genom.* 2012, 287, 437–450. [CrossRef] [PubMed]
6. Petersen, R.; Krost, C. Tracing a key player in the regulation of plant architecture: The columnar growth habit of apple trees (*Malus × domestica*). *Planta* 2013, 238, 1–22. [CrossRef]
7. Moriya, S.; Okada, K.; Haji, T.; Yamamoto, T.; Abe, K. Fine mapping of Co, a gene controlling columnar growth habit located on apple (*Malus × domestica* Borkh.) linkage group 10. *Plant Breed.* 2012, 131, 641–647. [CrossRef]
8. Baldi, P.; Wolters, P.J.; Komjanc, M.; Viola, R.; Salvi, S. Genetic and physical characterisation of the locus controlling columnar habit in apple (*Malus × domestica* Borkh.). *Mol. Breed.* 2013, 1, 429–440. [CrossRef]
9. Savel’ev, N.I.; Lyzhin, A.S.; Kudryavtsev, A.M.; Boris, K.V.; Shamshin, I.N. Use of molecular markers for identification of genotypes of columnar apple trees. *Russ. Agric. Sci.* 2015, 41, 323–325. [CrossRef]
10. Wolters, P.J.; Schouten, H.J.; Velasco, R.; St-Amour, A.; Baldi, P. Evidence for regulation of columnar habit in apple by a putative 2OG-Fe(II) oxygenase. *New Phytol.* 2013, 200, 993–999. [CrossRef]
11. Otto, D.; Petersen, R.; Brauksiepe, B.; Braun, P.; Schmidt, E.R. The columnar mutation (“Co gene”) of apple (*Malus × domestica*) is associated with an integration of a Gypsy-like retrotransposon. *Mol. Breed.* 2014, 33, 863–880. [CrossRef]
12. Okada, K.; Wada, M.; Moriya, S.; Katayose, Y.; Fujisawa, H.; Wu, J.Z.; Kanamori, H.; Kurita, K.; Sasaki, H.; Fujii, H.; et al. Expression of a putative dioxygenase gene adjacent to an insertion mutation is involved in the short internodes of columnar apples (*Malus × domestica*). *J. Plant Res.* 2016, 129, 1109–1126. [CrossRef] [PubMed]
13. Sun, X.; Wen, C.P.; Xu, J.H.; Wang, Y.H.; Zhu, J.; Zhang, Y.G. The apple columnar gene candidate MdCoL and the AP2/ERF factor MdDREB2 positively regulate ABA biosynthesis by activating the expression of MdNCED6/9. *Tree Physiol.* 2021, 41, 1065–1076. [CrossRef] [PubMed]
14. Yao, C.; Finlayson, S.A. Abscisic acid is a general negative regulator of Arabidopsis axillary bud growth. *Plant Physiol.* 2015, 169, 611–626. [CrossRef] [PubMed]
15. González-Grandío, E.; Pajoro, A.; Franco-Zorrilla, J.M.; Tarancón, C.; Immink, R.G.H.; Cubas, P. Abscisic acid signaling is controlled by a BRANCHED1/HD-ZIP I cascade in Arabidopsis axillary buds. *Proc. Natl. Acad. Sci. USA* 2017, 114, E245–E254. [CrossRef] [PubMed]
16. Zhan, J.J.; Chu, Y.; Wang, Y.; Diao, Y.Y.; Zhao, Y.Y.; Liu, L.S.; Wei, X.; Meng, Y.; Li, F.G.; Ge, X.Y. The miR164-GhCUC2-GhBRC1 module regulates plant architecture through abscisic acid in cotton. *Plant Biotechnol. J.* 2021, 19, 1839–1851. [CrossRef]

17. Bümke, C.; Tudzynski, B. Diversity, regulation, and evolution of the gibberellin biosynthetic pathway in fungi compared to plants and bacteria. *Phytochemistry* 2009, 70, 1876–1893. [CrossRef]

18. Cheng, J.; Zhang, M.M.; Tan, B.; Jiang, Y.J.; Zheng, X.B.; Ye, X.; Guo, Z.J.; Xiong, T.T.; Wang, W.; Li, J.D.; et al. A single nucleotide mutation in *GID1c* disrupts its interaction with DELLA1 and causes a GA-insensitive dwarf phenotype in peach. *Plant Biotechnol. J.* 2019, 17, 1723–1735. [CrossRef]

19. Singh, A.P.; Savaldi-Goldstein, S. Growth control: brassinosteroid activity gets context. *J. Exp. Bot.* 2015, 66, 1123–1132. [CrossRef]

20. Hu, J.; Sun, S.Y.; Wang, X.L. Regulation of shoot branching by strigolactones and brassinosteroids: Conserved and specific functions of Arabidopsis BES1 and rice BZR1. *Mol. Plant* 2020, 13, 808–810. [CrossRef]

21. Mori, M.; Nomura, T.; Ooka, H.; Ishizaka, M.; Yokota, T.; Sugimoto, K.; Okabe, K.; Kajiwara, H.; Satoh, K.; Yamamoto, K.; et al. Isolation and characterization of a rice dwarf mutant with a defect in brassinosteroid biosynthesis. *Plant Physiol.* 2002, 130, 1152–1161. [CrossRef]

22. Tong, H.N.; Jin, Y.; Liu, W.B.; Li, F.; Fang, J.; Yin, Y.H.; Qian, Q.; Zhu, L.H.; Chu, C.C. DWARF AND LOW-TILLERING, a new module regulates plant architecture through abscisic acid in cotton. *Plant Biotechnol. J.* 2015, 81, 210–222. [CrossRef] [PubMed]

23. Xia, X.J.; Dong, H.; Yin, Y.L.; Song, X.W.; Gu, X.H.; Sang, K.Q.; Zhou, J.; Shi, K.; Zhou, Y.H.; Foyer, C.H.; et al. Brassinosteroid signaling integrates multiple pathways to release apical dominance in tomato. *Plant Cell Rep.* 2021, 40, 1127–1139. [CrossRef] [PubMed]

24. Wang, L.; Wang, Z.; Xu, Y.Y.; Joo, S.H.; Kim, S.K.; Xue, Z.; Xu, Z.H.; Wang, Z.Y.; Chong, K. OsGSR1 is involved in crosstalk between gibberellins and brassinosteroids in rice. *Plant J.* 2009, 57, 498–510. [CrossRef] [PubMed]

25. Li, Q.Q.; Xu, F.; Chen, Z.; Teng, Z.F.; Sun, K.; Li, X.C.; Yu, J.Y.; Zhang, G.X.; Liang, Y.; Huang, X.H.; et al. Synergistic interplay of ABA and BR signal in regulating plant growth and adaptation. *Nat. Plants* 2021, 7, 1108–1118. [CrossRef]

26. Liu, Y.J.; An, J.P.; Wang, X.F.; Wang, Z.; Gao, N.; Wang, X.; Zhang, S.; Gao, W.S.; Hao, Y.J.; You, C.X. MdBZR1 regulates ABA response by modifying the expression of *MdABIS* in apple. *Plant Cell Rep.* 2021, 40, 1127–1139. [CrossRef] [PubMed]

27. Van Damme, M.; Huibers, R.P.; Elberse, J.; Van den Ackerveken, G. Arabidopsis DMR6 encodes a putative 2OG-Fe(II) oxygenase that is defense-associated but required for susceptibility to downy mildew. *Plant J.* 2008, 54, 785–793. [CrossRef]

28. Zeilmaier, T.; Ludvig, N.R.; Elberse, J.; Seidl, M.F.; Berke, L.; Van Doorn, A.; Schuurink, R.C.; Snel, B.; Van den Ackerveken, G. Downy mildew resistant 6 and dmr6-like oxygenase 1 are partially redundant but distinct suppressors of immunity in Arabidopsis. *Plant J.* 2015, 81, 210–222. [CrossRef] [PubMed]

29. Falcone, M.L.; Emilian, J.; Rodriguez, E.J.; Campos-Bermudez, V.A.; Grotewold, E.; Casati, P. The Identification of Maize and Arabidopsis Type I Flavone Synthases Links Flavones with Hormones and Biotic Interactions. *Plant Physiol.* 2015, 169, 1090–1107. [CrossRef]

30. Zhang, Y.; Zhao, L.; Zhao, J.; Li, Y.; Wang, J.; Guo, R.; Gan, S.; Liu, C.J.; Zhang, K. SSH/DMR6 Encodes a Salicylic Acid 5-Hydroxylase That Fine-Tunes Salicylic Acid Homeostasis. *Plant Physiol.* 2017, 175, 1082–1093. [CrossRef]

31. Wada, M.; Iwanami, H.; Moriya, S.; Hanada, T.; Moriya-Tanaka, Y.; Honda, C.; Shimizu, T.; Abe, K.; Okada, K. A root-localized isoform of *OsGID1c* disrupts its interaction with DELLA1 and causes a GA-insensitive dwarf phenotype in peach. *Plant Physiol.* 2021, 185, 1163–1174. [CrossRef] [PubMed]

32. Rutjen, B.; Bao, D.P.; Van Eck-Stouten, E.; Brand, M.; Smeekens, S.; Proveniers, M. Shoot apical meristem function in Arabidopsis requires the combined activities of three BEL1-like homeodomain proteins. *Plant Physiol.* 2009, 58, 461–644. [CrossRef] [PubMed]

33. Braun, N.; de Saint Germain, A.; Pillot, J.P.; Boutet-Mercey, S.; Dalmais, M.; Antoniadi, I.; Li, X.; Maia-Grondon, A.; Le Signor, C.; Bouteiller, N.; et al. The pea TCP transcription factor *PsBRC1* acts downstream of strigolactones to control shoot branching. *Plant Physiol.* 2012, 158, 225–238. [CrossRef] [PubMed]

34. Dong, Z.B.; Xiao, Y.G.; Govindaraju, R.; Feil, R.; Siddoway, M.L.; Nielsen, T.; Lunn, J.E.; Hawkins, J.; Whipple, C.; Chuck, G. The regulatory landscape of a core maize domestication module controlling bud dormancy and growth repression. *Nat. Commun.* 2019, 10, 3810. [CrossRef]

35. Dixon, L.E.; Pasquaillio, M.; Boden, S.A. TEOSINTE BRANCHED1 regulates height and stem internode length in bread wheat. *J. Exp. Bot.* 2020, 71, 4742–4750. [CrossRef]

36. Shen, J.J.; Zhang, Y.Q.; Ge, D.F.; Wang, Z.Y.; Song, W.Y.; Gu, R.; Che, G.; Cheng, Z.H.; Liu, R.Y.; Zhang, X.L. CsBRC1 inhibits axillary bud outgrowth by directly repressing the auxin efflux carrier *CsPIN3* in cucumber. *Proc. Natl. Acad. Sci. USA* 2019, 116, 17105–17114. [CrossRef] [PubMed]

37. Watanabe, D.; Takahashi, J.; Jaroensanti-Tanaka, N.; Miyazaki, S.; Jiang, K.; Nakayasu, M.; Wada, M.; Asami, T.; Mizutani, M.; Okada, K.; et al. The apple gene responsible for columnar tree shape reduces the abundance of biologically active gibberellin. *Plant J.* 2021, 105, 1026–1034. [CrossRef] [PubMed]

38. Hill, J.L.; Hollender, C.A. Branching out: New insights into the genetic regulation of shoot architecture in trees. *Curr. Opin. Plant Biol.* 2019, 47, 73–80. [CrossRef]

39. Wang, H.J.; Tang, J.; Liu, J.; Hu, J.; Liu, J.J.; Chen, Y.X.; Chen, Z.Y.; Wang, X.L. Abscisic acid signaling inhibits brassinosteroid signaling through dampening the dephosphorylation of BIN2 by ABI1 and ABI2. *Mol. Plant* 2018, 11, 315–325. [CrossRef]
40. Okada, K.; Wada, M.; Takebayashi, Y.; Kojima, M.; Sakakibara, H.; Nakayasu, M.; Mizutani, M.; Nakajima, M.; Moriya, S.; Shimizu, T.; et al. Columnar growth phenotype in apple results from gibberellin deficiency by ectopic expression of a dioxygenase gene. *Tree Physiol.* 2020, 40, 1205–1216. [CrossRef]
41. Schmidt, R.; Schippers, J.H.M.; Mieulet, D.; Obata, T.; Fernie, A.R.; Guiderdoni, E.; Mueller-Roeber, B. MULTIPASS, a rice R2R3-type MYB transcription factor, regulates adaptive growth by integrating multiple hormonal pathways. *Plant J.* 2013, 76, 258–273. [CrossRef]
42. Zhu, N.; Cheng, S.F.; Liu, X.Y.; Du, H.; Dai, M.Q.; Zhou, D.X.; Yang, W.J.; Zhao, Y. The R2R3-type MYB gene OsMYB91 has a function in coordinating plant growth and salt stress tolerance in rice. *Plant Sci.* 2015, 236, 1205–1216. [CrossRef]
43. Zhu, N.; Cheng, S.F.; Liu, X.Y.; Du, H.; Dai, M.Q.; Zhou, D.X.; Yang, W.J.; Zhao, Y. The R2R3-type MYB gene OsMYB91 has a function in coordinating plant growth and salt stress tolerance in rice. *Plant Sci.* 2015, 236, 1205–1216. [CrossRef]
44. Zhu, N.; Cheng, S.F.; Liu, X.Y.; Du, H.; Dai, M.Q.; Zhou, D.X.; Yang, W.J.; Zhao, Y. The R2R3-type MYB gene OsMYB91 has a function in coordinating plant growth and salt stress tolerance in rice. *Plant Sci.* 2015, 236, 1205–1216. [CrossRef]
45. Zhu, N.; Cheng, S.F.; Liu, X.Y.; Du, H.; Dai, M.Q.; Zhou, D.X.; Yang, W.J.; Zhao, Y. The R2R3-type MYB gene OsMYB91 has a function in coordinating plant growth and salt stress tolerance in rice. *Plant Sci.* 2015, 236, 1205–1216. [CrossRef]
46. Zhu, N.; Cheng, S.F.; Liu, X.Y.; Du, H.; Dai, M.Q.; Zhou, D.X.; Yang, W.J.; Zhao, Y. The R2R3-type MYB gene OsMYB91 has a function in coordinating plant growth and salt stress tolerance in rice. *Plant Sci.* 2015, 236, 1205–1216. [CrossRef]
47. Zhu, N.; Cheng, S.F.; Liu, X.Y.; Du, H.; Dai, M.Q.; Zhou, D.X.; Yang, W.J.; Zhao, Y. The R2R3-type MYB gene OsMYB91 has a function in coordinating plant growth and salt stress tolerance in rice. *Plant Sci.* 2015, 236, 1205–1216. [CrossRef]
48. Zhu, N.; Cheng, S.F.; Liu, X.Y.; Du, H.; Dai, M.Q.; Zhou, D.X.; Yang, W.J.; Zhao, Y. The R2R3-type MYB gene OsMYB91 has a function in coordinating plant growth and salt stress tolerance in rice. *Plant Sci.* 2015, 236, 1205–1216. [CrossRef]