PhNAL1 Is Involved in Regulating Branch Development of Petunia

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ABSTRACT. The branch number of plants is an important agronomic trait that directly influences the ornamental characters and production costs of ornamental plants. Shoot branching has always been a hot topic for Petunia hybrida. During our research, we isolated the homologous gene of narrow-leaf 1 (NAL1), denoted as PhNAL1. The expression level of PhNAL1 was higher in leaves and axils than in roots, stems, and flowers. Pertinent to shoot apex removal and 6-benzyladenine treatments, both interventions demonstrated a suppressive effect on the expression of PhNAL1. Through subcellular localization analysis, we found that PhNAL1 predominantly localized in the nucleus. By using RNA interference targeting PhNAL1, we induced a noticeable increase in branch number while concurrently reducing plant height of petunia. These findings demonstrate that PhNAL1 is involved in regulating branch development within petunia. This study provides genetic resources for the subsequent cultivation of new cultivars of petunia endowed with distinct branching characteristics.

Branching is a pivotal determinant of plant architecture (Umehara et al. 2008), not only influencing the capacity of the plant to adapt to its environment but also significantly impacting crop yield, ornamental characteristics, and production economics of horticultural plants. Therefore, delving into the molecular regulation mechanisms governing shoot branching holds significant theoretical and practical importance.

The initiation of axillary meristems (AMs), also termed lateral meristems, serves as the fundamental process underlying branching. During AM initiation, a morphologically detectable bump forms in the leaf axil and subsequently matures into a bud (Wang and Jiao 2018). These axillary buds possess the capacity to enter the dormant state, forming dormant buds, or continue their growth trajectory to develop into branches. Moreover, dormant buds can be activated to initiate branching; however, the process of early activation can also be reversed, causing the dormant buds to return to their quiescent state (Dun et al. 2006). Therefore, factors that affect these processes will ultimately affect the number of branches.

Petunia hybrida serves as an excellent model organism that proves invaluable for both fundamental and applied research because of its array of appealing characteristics. These characteristics encompass a well-defined genetic background, a brief growth cycle, and a well-established transformation system (Tran et al. 2021). Significant research progress has been achieved in understanding branch development within petunia. Strigolactone, a newly identified class of plant hormones involved in the inhibition of shoot branching (Gomez-Roldan et al. 2008; Umehara et al. 2008), in petunia was studied. Decreased apical dominance (Dad1) and Dad3 encode carotenoid cleavage dioxygenase 8 (CCD8) and CCD7, respectively, which cleave the carotenoid 9-cis-b-carotene to carlactone (Drummond et al. 2009). Petunia ATP-binding cassette transporter PLEIOTROPIC DRUG RESISTANCE 1 is the first known component in stri-golactone transport, providing new opportunities for investigating and manipulating strigolactone-dependent processes (Kretzschmar et al. 2012; Shirakata et al. 2019). In addition, some genes that regulate branch development have been identified. The constitutive overexpression of petunia x-expansin 1 exhibited precocious timing of axillary meristem development (Dal Santo et al. 2011). Transgenic petunia plants expressing petunia cytokinin binding protein in antisense orientation displayed profuse branching, indicating the key role in shoot branching (Godge et al. 2008). Overexpression of lateral shoot-inducing factor, a zinc-finger protein, resulted in a dramatic increase in lateral shoots and reduced plant height of petunia (Nakagawa et al. 2005). Moreover, investigations of the influence of environmental factors on petunia branching showed that the nutrient (phosphate) availability and light quality (crowding and the red-to-far-red light ratio) were integrated within the axillary bud and could additively affect the growth of branches (Drummond et al. 2015). These aforementioned studies have substantially enriched our comprehension of plant branching mechanisms.

Numerous genes have been implicated in the intricate orchestration of branching, yet narrow-leaf 1 (NAL1) stands out as a pivotal player in various aspects of plant development. Initially identified within an Oryza sativa nal mutant because of its influence on vascular patterning, NAL1 encodes a putative trypsin-like serine/cysteine protease (Qi et al. 2008). It also affects the hybrid vigor of rice (Huang et al. 2018), leaf width (Chen et al. 2012), total spikelet number per panicle, nitrogen (N) content, leaf chlorophyll content, and leaf photosynthesis (Fujita et al. 2013; Hirotsu et al. 2017; Takai et al. 2013; Wang et al. 2015; Zhang et al. 2014). Additionally, NAL1 assumes a regulatory role in plant height by influencing cell division (Jiang et al. 2015), crop yield, adventitious root formation, and source–sink relationships of rice (Cho et al. 2014; Xu et al. 2015). Recent studies have found that NAL1 mutants in O. sativa ssp. indica significantly increase the number of tillers (Ouyang et al. 2022). In another investigation, the
interaction of NAL1 with the FRIZZY PANICLE (FZP) gene was uncovered, facilitating the degradation of FZP. Upregulating NAL1 or suppressing FZP expression emerged as potential strategies to amplify grain yield by augmenting secondary branch numbers and grain count (Huang et al. 2018). However, although the significance of NAL1 in plant branch development is acknowledged, its characterization within the context of ornamental plants remains underexplored.

In summary, the intricacies of the branching of petunia have long captured research attention (Dong et al. 2022; Drummond et al. 2015; Rashidi et al. 2023; Yao et al. 2022). To further enrich the regulatory mechanism of petunia branch development, we cloned the full-length sequence of the PhNAL1 gene and studied the expression patterns of PhNAL1 across various treatments and tissues as well as its subcellular localization. In addition, the research explored the functions of PhNAL1, thereby laying a foundational basis for unraveling the mechanisms underlying shoot branching of petunia.

Materials and Methods

PLANT MATERIALS AND GROWTH CONDITION. During this study, Petunia ×hybrida cv. Mitchell Diploid was used as the chosen experimental plant species. Petunia seedlings were cultivated in square pots (10 cm × 10 cm × 7.8 cm) filled with sphagnum peat soil (Findstrup, Ryomgaard, Denmark). These seedlings were nurtured within a controlled tissue culture room, maintained at a temperature of 23 ± 2°C with an illumination intensity of 100 mmol·m⁻²·s⁻¹, and subjected to the photoperiod (day/night) of 16 h/8 h.

ISOLATION OF PHNAL1. Total RNA was extracted from pooled leaves of 30-d-old petunia plants (n = 10) using the RNAprep Pure Plant Plus Kit (TIANGEN Biotech, Beijing, China). Subsequently, DNA removal and first-strand complementary DNA (cDNA) synthesis were conducted using the Prime-Script™ RT reagent Kit with gDNA Eraser (TaKaRa, Beijing, China). Guided by the petunia genome sequence accessible from the Solanaceae Genome Network (Bombarely et al. 2016), the amplified products were cloned using the NoveRec™ plus One step PCR Cloning Kit (Novoprotein, Shanghai, China) and MegaFi™ Fidelity 2× PCR MasterMix (Toyobo, Shanghai, China) to determine the sequence of PhNAL1 (accession number: OR400704).

BIOMINFORMATIC ANALYSIS. Multiple sequence alignment was performed using DNAMAN software (version 5.2.2; Lynnon BioSoft, Quebec, Canada). The identification of conserved domains was conducted using Pfam online software. The basic chemical and physical properties of the protein sequences were analyzed using the ProParam online tool available on ExPASy (Gasteiger et al. 2005). The hydrophilicity of the protein was evaluated using ExPASy-Protscale software. Secondary structure predictions were accomplished using the SOPMA software program (Geourjon and Deleage 1994).

EXPRESSION ANALYSIS. To conduct tissue expression experiments, five plant organs encompassing flowers, axils, leaves, stems, and roots from 75-d-old petunia seedlings were collected. Unless specified, each experiment included three biological replicates and three technical replicates. For all tissue samples, 40 plants were pooled for each biological replicate. Notably, leaves, axils, and stems were sampled within the range of node 2 to node 6 from the shoot tip downward. Additionally, tender roots and fully open flowers were selected. After harvest, the tissues were immediately immersed in liquid nitrogen and stored at −80°C.

Cytokinin, recognized as the second messenger of auxin, has the ability to induce bud outgrowth upon its application to a bud (Müller and Leyser 2011). The growth of shoot tips can inhibit the outgrowth of axillary buds located along the stem below, and decapitation of the shoot apex results in the triggering of bud outgrowth. To analyze whether cytokinin and decapitation regulate the expression of NAL1, we conducted expression analysis experiments. The 6-benzyladenine (6-BA) treatment and decapitation experiment used 60-d-old petunia seedlings as the experimental material. The plants were systematically divided into three groups. The first group served as an untreated control, and RNA was extracted from the fourth axillary bud starting from the top and moving downward. In the second group, the fourth axillary bud from the top was subjected to 6-BA treatment (50 μM). The formulation and application of the solution adhered to the methodology established by Kerr et al. (2021). The third group was designated for the decapitation experiment (Supplemental Fig. 1). Both the 6-BA application and decapitation were sampled after a 6-h interval.

Total RNA was extracted using the TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa) according to the manufacturer’s instructions. For the quantitative real-time polymerase chain reaction assay, an ABI 7500 Fast real-time PCR system was used along with the SYBR Green PCR Master Mix (Toyobo, Shanghai, China). The reaction procedure encompassed initial denaturation at 95°C for 1 min, succeeded by 45 cycles of denaturation at 95°C for 20 s, cooling at 50 to 60°C for 20 s, and extension at 72°C for 30 s. The expression level of PhNAL1 was assessed using the NAL1-RT-F and NAL1-RT-R primers. The normalization control was petunia Glyceraldehyde-3-phosphate dehydrogenase (PhGAPDH), and its expression was determined using the GAPDH-F and GAPDH-R primers (Supplemental Table 1). The relative 2⁻ΔΔCT method was used to calculate the relative expression of the target genes (Livak and Schmittgen 2001).

SUBCELLULAR LOCALIZATION ANALYSIS. For the generation of the PhNAL1-enhanced green fluorescent protein (eGFP) fusion protein, the PhNAL1 lacking the stop codon was amplified using the NAL1-F-SL and NAL1-R-SL primers. Then, these amplified fragments were integrated into the pCAMBIA1300 vector containing the 35S promoter of cauliflower mosaic virus and eGFP (GenBank accession number: U55761) using the ClonExpress™ II One Step Cloning Kit (Vazyme, Nanjing, China). This yielded the pSuper1300-PhNAL1-eGFP construct. Both fusion plasmids were subsequently transformed into Agrobacterium tumefaciens GV3101, followed by the selection of positive clones for transformation into Nicotiana benthamiana leaves, using a previously detailed procedure (Hoshikawa et al. 2019). After 48 h of cultivation, the GFP fluorescence was observed using a confocal microscope (Olympus, Tokyo, Japan).

CONSTRUCTION AND TRANSFORMATION OF THE PHNAL1-RNA INTERFERENCE VECTOR. Guided by the PhNAL1 gene sequence, primers were designed to generate a target fragment of ~300 bp incorporating KpnI and XhoI restriction endonuclease sites. The amplified PhNAL1 fragment and pJL10 vector plasmid were both digested using KpnI and XhoI and then connected with T4 ligase. After positive clone identification and sequencing, a recombinant plasmid containing the sense insertion fragment of PhNAL1 was obtained. The restriction endonuclease sites of the upstream and downstream amplification primers of the aforementioned
PhNAL1 fragment were replaced with XbaI and ClaI, respectively. This amplified product was then ligated into the recombinant plasmid containing the sense insertion fragment. After the positive clones were identified, the PhNAL1-RNAi plasmid was ultimately obtained.

Leaves (0.5 cm × 0.5 cm) from sterile petunia seedlings were placed on Murashige and Skoog (MS) medium [MS + 30 g·L⁻¹ sucrose + 5 g·L⁻¹ agar powder + 0.1 mg·L⁻¹ 1-Naphthylacetic acid (Aladdin, Shanghai, China); optical density (OD₆₅₀) = 0.8]. After 10 min, the leaves were removed from the infection solution and dried. Then, the leaves were placed adaxially (face down) in the medium for further cultivation. After an additional 2 d of dark cultivation, the leaves underwent five to six sterile water rinses before being transferred to the screening medium [MS + 30 g·L⁻¹ sucrose + 5 g·L⁻¹ agar powder + 0.1 mg·L⁻¹ 1-Naphthylacetic acid + 2 mg·L⁻¹ Zeatin (Aladdin, Shanghai, China) + 200 mg·L⁻¹ Timentin (Aladdin, Shanghai, China) + 50 mg·L⁻¹ kanamycin (Aladdin, Shanghai, China)] for further cultivation. The culture medium was renewed every 20 d. Upon the emergence of resistant buds measuring ~2.0 cm, they were excised to stimulate rooting. Upon reaching a height of 8 to 10 cm, the resilient seedlings were transplanted into pots. Statistical analyses encompassing plant height and branch number were conducted using 90-d-old plants cultured to the T₃ generation. Plant height, measured from ground level to the shoot tip on the main stem,

Fig. 1. Sequence analysis of Petunia hybrida narrow-leaf 1 (PhNAL1). (A) Sequence analysis of PhNAL1 in three petunia species (Petunia × hybrida cv. Mitchell Diploid, P. inflata, and P. axillaris). (B) Prediction of hydrological and hydrological regions of PhNAL1. (C) Prediction of the secondary structure of PhNAL1.
and the count of basal branches (bud length ≥ 10 mm) assessed for petunia plants. The trial was replicated three times.

**Statistical analysis.** All the data in this study were expressed as the mean value ± SD. Tukey’s tests were performed for statistical analyses.

**Results**

Cloning of PhNAL1 and protein structure analysis. Using the published genome sequences of *P. inflata* and *P. axillaris*, primers were designed to amplify and acquire the complete sequence of PhNAL1. In petunia, PhNAL1 was 1800 bp and encoded a protein composed of 599 amino acids (Fig. 1A). The molecular formula of PhNAL1 was C_{2871}H_{4516}N_{786}O_{905}S_{15}, presenting a relative molecular weight of 65 kDa and an isoelectric point of 4.95. The PhNAL1 protein exhibited relatively heightened concentrations of leucine (9.8%; 59) and glycine (9.7%; 58) coupled with low concentrations of tryptophan (1.0%; 6) and cysteine (1.2%; 7). The overall average hydrophobic index was -0.304, and the aliphatic amino acid index was 83.67, suggesting the hydrophilic nature of the PhNAL1 (Fig. 1B). The sequence of the PhNAL1 predominantly encompassed alpha helix, extended strand, beta turn, and random coil. Specifically, the alpha helix comprised 148 amino acids (24.71%), the extended strand had 127 amino acids (21.20%), the beta turn had 40 amino acids (6.68%), and the random coil had 284 amino acids (47.41%).

Tissue-specific expression analysis of PhNAL1. To assess the tissue-specific expression pattern of PhNAL1, the relative expression levels across various tissues, namely, root, stem, leaf, axil, and flower, were measured (Fig. 2). The findings indicated that the PhNAL1 gene exhibited the highest expression within leaves and axils, whereas its expression was minimal in flowers.

Expression analysis of PhNAL1 after decapitation and 6-BA treatment. To investigate the responsiveness of PhNAL1 to decapitation, the expression level of PhNAL1 was evaluated (Fig. 3). After decapitation, the expression level of PhNAL1 decreased to 0.2-times that of control plants, indicating the negative regulatory effect of decapitation on PhNAL1 expression.

To clarify whether the PhNAL1 gene responds to cytokinin, the expression of PhNAL1 was monitored after 6-BA treatment. The results showed that 6-BA treatment led to a reduction in the expression level of the PhNAL1 gene to one-sixth of the control, indicating that cytokinin can regulate the influence on the expression of PhNAL1.

Subcellular localization of PhNAL1. To elucidate the role of the PhNAL1, a comprehensive subcellular localization analysis was conducted. In our study, we devised a pCAMBIA1300 vector that encompassed fusion between GFP and PhNAL1 at the N terminus. The pCAMBIA1300-GFP vector served as a control counterpart. The results showed that the fusion protein involving PhNAL1 and GFP exhibited distinct green fluorescence exclusively within the nucleus, suggesting that PhNAL1 is localized in the nucleus (Fig. 4).

RNAi if PhNAL1 increased the branch number in petunia. To elucidate the effects through RNAi, the expression level of PhNAL1 was determined using a quantitative real-time polymerase chain reaction. The results showed that the suppression of PhNAL1 was successfully achieved because the average expression level of PhNAL1 in control plants was approximately four-times greater than that in transgenic seedlings (Fig. 5B). Furthermore, an intriguing observation was made in terms of the number of branches of petunia, which increased from 2.1 branches to 5.1, 4.6, and 4.7 branches (Fig. 5C). Concurrently, the plant height demonstrated a reduction from 38.3 cm to 18.7, 19.3, and 19.7 cm (Fig. 5D). These findings provide evidence of a noteworthy correlation existing between the number of branches of petunia and the expression level of PhNAL1.

**Discussion**

Recently, a spectrum of narrow-leaf-related genes has surfaced in rice, such as NAL1 (Ouyang et al. 2022), NAL2/NAL3 (Ishiwata et al. 2013), NAL7 (Fujino et al. 2008), NAL9 (Li et al. 2013), NAL21 (Uzair et al. 2021), and NAL22 (Xu et al. 2023). It is noteworthy that although these genes do not belong to the same gene family, they encode different proteins. For instance, NAL7 encoded a flavin-containing monoxygenase, and its mutation altered the content of endogenous auxin in plants. NAL7 played important roles in leaf narrowing, vascular tissue development, and indole-3-acetic acid biosynthesis (Fujino et al. 2008). NAL2 and NAL3 encoded a WUSCHEL-related homeobox 3A transcriptional activator homologous to PRESSED FLOWER in Arabidopsis and were proven to be mainly involved in lateral-axis outgrowth and vascular patterning during leaf development, lemma and palea morphogenesis in spikelets, and development of lateral roots (Cho et al. 2013). NAL22, encoding a Maf-like nucleotide triphosphate pyrophosphatase protein, regulated the width of...
veins and cell division (Xu et al. 2023). These genes exhibit involvement not only in leaf development but also in branch development. This is evident from the mutations observed in NAL1, NAL9, and NAL21, which lead to narrow leaves and increased tillers (Li et al. 2013; Ouyang et al. 2022; Uzair et al. 2021).

NAL1 encodes a putative trypsin-like crystal/serine protease. Although plant cysteine protease plays significant roles in various aspects of plant development and growth, aging, and stress response (Liu et al. 2018), its involvement in branch development has received limited attention. During our research, we cloned the homologous gene of NAL1 from Petunia × hybrida cv. Mitchell Diploid. Through sequence alignment, we found minimal amino acid variations in the PhNAL1 sequences across the three species, with only one amino acid distinction within the conserved domain region. This indicates the high conservation of the gene among different petunia species. We detected the expression level of PhNAL1 across five petunia tissues and found that PhNAL1 was highly expressed in leaf axils, whereas expression was lowest in flowers, suggesting that PhNAL1 might play a vital part in the branch development of petunia. However, no indications have emerged to suggest the involvement of NAL1 in flower development, which could account for the lowest expression of PhNAL1 in flowers. The nuclear localization of PhNAL1 has been established. In rice, NAL1-GFP fusion protein formed nuclear protein bodies, showcasing considerable variations in number, size, and brightness among various cells (Qi et al. 2008). Therefore, the complexity of NAL1 localization underscores the need for further research in the future.

Decapitation activates multiple downstream pathways involving hormones and genes, ultimately leading to axillary bud germination. Cytokinin influences axillary bud germination by regulating the transcription of multiple genes. We found that both decapitation and application of 6-BA inhibited the expression of PhNAL1, indicating their regulatory influence. It is plausible that the impact of decapitation on cytokinin might subsequently...
affect the expression of PhNAL1. However, more experiments are needed to ascertain whether the regulation of PhNAL1 by decapitation and 6-BA is indirect or direct.

The construction and application of the PhNAL1-RNAi vector in petunia yielded a noteworthy reduction of PhNAL1 expression by approximately four- to five-times compared with the control through RNAi. This correlated with a significant increase in branch number and reduction in plant height, underlining the role of PhNAL1 in regulating the branch development of petunia. There are few reports of the regulation of branch development by crystal/serine protease; currently, only NAL1 has been identified in plants. In rice, NAL1, also known as PLANT ARCHITECTURE AND YIELD 1, influences plant architecture by affecting polar auxin transport activity and altering endogenous indole-3-acetic acid distribution (Zhao et al. 2015). Furthermore, NAL1 interacted with FZP to regulate branching (Huang et al. 2018). However, beyond its influence on branch development, whether NAL1 regulates other hormone pathways and branching-related genes warrants further exploration. Whether there are more crystal/serine proteases involved in regulating plant branching will be our future focus.

In conclusion, we have cloned the full length of PhNAL1 from petunia, detected the expression of PhNAL1 in various tissues and treatments, and verified the function of PhNAL1 through RNAi. The results generated during our study augment our understanding of shoot branching of plants and lay the groundwork for selecting candidate genes for branch regulation in petunia.

References Cited


