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The GATA Factor *HANABA TARANU* Promotes Runnering by Regulating Axillary Bud Initiation and Outgrowth in Cultivated Strawberry

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SUMMARY

A runner, as an elongated branch, develops from axillary bud (AXB) in the leaf axil and is crucial for clonal propagation of cultivated strawberry (*Fragaria* × *ananassa* Duch.). Runner formation occurs in at least two steps: AXB initiation and AXB outgrowth. *HANABA TARANU* (*HAN*) encodes a GATA transcription factor that affects AXB initiation in Arabidopsis and promotes branching in grass species, but the underlying mechanism is largely unknown. Here, the function of a strawberry HAN homologue FaHAN in the runner formation was characterized. *FaHAN* transcripts can be detected in the leaf axils. Overexpression (OE) of *FaHAN* increased the number of runners mainly by enhancing AXB outgrowth in strawberry. The expression of the strawberry homolog of *BRANCHED1*, a key inhibitor of AXB outgrowth in many plant species, was significantly down-regulated in the AXBs of *FaHAN*-OE lines, whereas the expression of the strawberry homolog of *SHOOT MERISTEMLESS*, a marker gene for AXB initiation in Arabidopsis, was up-regulated. Moreover, several genes of gibberellin biosynthesis and cytokinin signaling pathways were activated, while the auxin response pathway genes were repressed. Further assays indicated that *FaHAN* could be directly activated by FaNAC2, whose overexpression in strawberry also increased the number of runners. Silencing of *FaNAC2* or *FaHAN* inhibited AXBs initiation and led to higher proportion of dormant AXBs confirming their roles in the control of runner formation. Taken together, our results revealed a FaNAC2-FaHAN pathway in the control of runner formation and provided means to enhance vegetative propagation of cultivated strawberry.

INTRODUCTION

Axillary buds (AXBs) that form in the axils of leaves can develop into shoot branches that shape plant architecture and increase the reproductive potential of the plant (McSteen, 2009, Shen et al., 2019). The AXBs can adopt a range of fates during vegetative and reproductive development in different species (McSteen and Leyser, 2005). For example, in a perennial rice species *Oryza longistaminata*, AXBs can form long underground shoots called rhizomes instead of tillers for vegetative reproduction (Fan et al., 2020). Comparable to perennial rice, the cultivated strawberry (*Fragaria* × *ananassa* Duch.), which is one of the most economically-important berry crops in the world, can generate long aerial stolons (runners) from AXBs for clonal reproduction during vegetative phase or new leaf rosettes called "branch crowns" that can bear inflorescences and produce fruits (Hytönen and Elomaa, 2011, Martins et al., 2018). Because cultivated strawberry cultivars are hybrids, they are clonally propagated from runners to maintain their desirable characteristics (Vallejo-Marin et al., 2010, Li et al., 2018, Hytönen and Kurokura, 2020). Therefore, it is important to explore mechanisms controlling AXB development into runners in strawberry.

In Arabidopsis, development of an AXB includes at least three stages; initiation of an axillary meristem (AXM) in the leaf axil, development of the AXM into an AXB, and subsequent outgrowth or dormancy of the AXB (Figure 1) (Tantikanjana et al., 2001, Yang and Jiao, 2016, Wang and Jiao, 2018). AXM initiation requires the activation of the meristem marker gene *SHOOT MERISTEMLESS* (*STM*) and is marked by the formation of a morphologically detectable bump in the leaf axil (Grbic and Bleecker, 2000, Shi et al., 2016, Wang and Jiao, 2018). The newly initiated AXM has the same developmental potential as the shoot apical meristem (SAM) (Wang et al., 2017, Xin et al., 2017). The AXM then produces its own leaf primordium changing its identity to AXB (Wang and Jiao, 2018). Thus, AXM initiation is the prerequisite and the key step to AXB initiation. The outgrowth or dormancy of AXB is under hormonal control as well as environmental signals (Ongaro and Leyser, 2008, McSteen, 2009).

Auxin moves downwards in the stem and suppresses AXB outgrowth indirectly by repressing cytokinin and activating strigolactone biosynthesis (Chatfield et al., 2000, Brewer et al., 2009, Young et al., 2014). *BRANCHED 1* (*BRC1*), that is specifically expressed in AXB, leaf primordia and provascular tissue underlying the AXB, integrates these hormonal signals to inhibit AXB outgrowth (Aguilar-Martínez et al., 2007, Dun et al., 2009, González-Grandío et al., 2013, Rameau et al., 2015).

Compared with Arabidopsis, strawberry AXB development has its own characteristics. AXBs in the leaf axils may remain dormant or develop into branch crowns or runners (Andrés et al., 2021, Hytönen and Elomaa, 2011). Runner formation occurs through at least two stages: AXB initiation and subsequent AXB outgrowth. *LOSS OF AXILLARY MERISTEM* (*LAM*) is essential for AXB initiation in wild strawberry (Feng et al., 2021), while gibberellin (GA) promotes AXB outgrowth (Thompson and Guttridge, 1959, Hytönen et al., 2009, Feng et al., 2021). Natural mutation of the GA biosynthesis gene *FveGA20ox4* leads to a runnerless phenotype (Tenreira et al., 2017), while a loss of function of DELLA gene, *FveRGA1* encoding an inhibitor of the GA signaling pathway, restores runner formation in runnerless accessions (Caruana et al., 2018, Li et al., 2018). In addition to the AXBs arising from the main crowns, the development of AXBs on strawberry runner has also been explored. Strawberry runner contains two long internodes followed by a daughter plant produced by the SAM of the runner. The AXB in the first node of the runner (the first runner AXB) typically remains dormant, while the second runner AXB produces a new unit of two internodes followed by a daughter plant (Hytönen et al., 2009, Qiu et al., 2019). Compared with non-dormant buds, higher auxin activity, higher level of *BRC1* expression and lower cytokinin activity are present in dormant buds. Qiu et al. (2019) proposed that auxin and cytokinin act antagonistically to regulate the outgrowth of AXBs in strawberry runner. Given the important role of strawberry runners in the clonal propagation of the plant, more genes involved in AXB development need to be identified.

HANABA TARANU (*HAN*) encodes a GATA transcription factor (TF) that affects AXB initiation in Arabidopsis and promotes branching in grass species (Wang et al., 2009; Whipple et al., 2010, Tian et al., 2019), but its regulatory mechanisms are not well understood. Previous studies on HAN mainly focused on its function in the SAM during reproductive growth. It is

mainly expressed at the boundaries between SAM and leaf primordia, SAM and floral organ primordia, and floral whorls, and is required for establishing organ boundaries in shoots and flowers by controlling *WUSCHEL*-expressing cells (Zhao et al., 2004, Tian et al., 2019). During the formation of the meristem boundary, HAN interacts with several meristem regulators and maintains boundary morphology through *CYTOKININ OXIDASE 3* (*CKX3*)-mediated cytokinin homeostasis (Ding et al., 2015). Furthermore, *HAN* overexpression represses hundreds of genes involved in hormone responses, and gibberellin content is decreased in *han* mutant (Zhang et al., 2013, Ding et al., 2015). These reports indicate that HAN plays an indispensable role in meristem development by participating in hormone networks. Nevertheless, how HAN regulates AXB initiation through hormonal networks, and whether it is involved in AXB outgrowth are still open questions. In addition, not much is known about its upstream regulators.

Here, a strawberry *HAN* homolog, *FaHAN*, was isolated and characterized in strawberry. *FaHAN* was expressed in the leaf axils, and was induced by exogenous cytokinin and gibberellin. Based on *in vitro* assays, overexpression (OE) lines and virus-induced gene silencing (VIGS) experiments, FaNAC2 was identified as an activator of *FaHAN* that enhanced runner formation by affecting AXB initiation and AXB outgrowth. Gene expression analysis of *FaHAN*-OE lines indicated that FaHAN plays a role as a positive regulator of AXB outgrowth through its effect on cytokinin and auxin signaling, and gibberellin biosynthetic pathway.

RESULTS

FaHAN encodes a homolog of the GATA transcription factor HAN

To identify a HAN homolog from the strawberry cultivar 'Benihoppe', we used the coding sequence (CDS) of Arabidopsis HAN (AT3G50870) as a BLAST query in searches against *Fragaria* × *ananassa* Camarosa Genome Assembly (v1.0.a1) Transcripts. Based on BLAST results, a 762 bp CDS of strawberry *HAN* homolog was amplified from 'Benihoppe' and named *FaHAN*. A multiple sequence alignment of the translated FaHAN protein sequence and HAN homologs of cucumber, Arabidopsis, rice and maize showed that FaHAN shared a highly conserved GATA zinc finger domain and a HAN motif with other species (Figure 2a). Further phylogenetic analysis of the predicted HAN proteins from various species showed that FaHAN

was more closely related to proteins from other Rosaceous species than to those from other eudicots (Figure 2b). Moreover, HAN homologs of monocots, called NECKLEAF1 (NL1)/Tassel sheath1 (Tsh1)/THIRD OUTER GLUME (TRD) and their paralogs (Ntt-like), formed a distinct clade shown earlier by Whipple et al. (2010). In addition, the subcellular localization of FaHAN was examined in *Nicotiana benthamiana* (*N. benthamiana*) leaf cells, and the results showed that the FaHAN-GFP fusion protein was mainly localized in the nucleus, with a weak signal in the cytoplasm (Figure 2c). These results suggested that FaHAN is a GATA TF primarily localized in the nucleus.

FaHAN/FvHAN is expressed in the leaf axils and axillary buds

Using qRT-PCR assays, we found that *FaHAN* mRNA accumulated not only in the shoot apex of strawberry, but also in the AXBs and flowers, whereas the expression level was relatively low in roots, leaves and fruits (Figure 3a). To better understand the expression pattern of *FaHAN* in 'Benihoppe', *in situ* hybridization was performed. The signals of *FaHAN* mRNA accumulation slightly above potential background were observed at the axils of leaf primordia (Figure 3b-d, Figure S1a-b), and AXMs (the morphologically detectable bump indicated by the arrows) (Figure 3e-f, Figure S1a-b). We also carried out *in situ* hybridization in the AXBs in diploid woodland strawberry (*Fragaria vesca*; genotype FIN56) that is one of the subgenome donors of the cultivated strawberry (Edger et al., 2019). Weak signals of *FvHAN* (the *HAN* homolog of FIN56) were found in the meristematic dome and in the axils of young leaf primordia of the developing AXBs (Figure 3g-i). These results implied that HAN might play roles in the AXB development in strawberry.

Overexpression of *FaHAN* increases runner numbers

To further understand the function of FaHAN in strawberry, *FaHAN* was overexpressed in the cultivar 'Benihoppe'. Fifteen independent *FaHAN*-OE lines that were regenerated from cross-sectioned leaf slices were selected on the screening tissue culture medium containing kanamycin, and the presence of the transgene was confirmed using RT-PCR assays. *FaHAN*-OE plants produced runners already during the tissue culture stage, whereas the control plants did not produce runners on the same tissue culture medium. The number of runners per plant ranged

from 0.0 ± 0.0 in 'Benihoppe' to 0.8 ± 0.42 in the weakest OE line #18, and to 1.3 ± 0.48 in the strongest OE line #24 (Figure 4a). However, the plant height of *FaHAN*-OE lines was nearly 54% of that of the control lines during the tissue culture stage (Figure 4b). After removing all the runners from the tissue culture plants and transplanting them into the soil, *FaHAN*-OE lines again produced more runners from the main crown than the control lines (Figure 4c-d), and the results showed that the increase of runner numbers correlated with the overexpression level of *FaHAN* (Figure S2). Also, in *N. benthamiana*, the ectopic expression of *FaHAN* enhanced branching (Figure S3a-b). Furthermore, the results revealed that the proportion of dormant AXBs was lower in *FaHAN*-OE plants than in the control (Figure 4e). This was further supported through histological observations of plants grown on tissue culture, in which AXBs in the axil of the second youngest expanded leaf had begun to elongate in *FaHAN*-OE plants that produced runners, but not in the control plants (Figure 4f-g). Considering these results, overexpression of *FaHAN* increased the number of runners mainly by promoting AXB outgrowth. Interestingly, almost 45% of the first runner AXBs in *FaHAN*-OE plants developed into new runners, while the buds at the same location in the control plant runners were dormant (Figure S4), suggesting that overexpression of *FaHAN* also promoted the outgrowth of the first runner AXB.

Boundaries where cells usually display reduced growth activity that results in separation of adjacent organs or tissues, may act as a reference point for AXM initiation (Aida and Tasaka, 2006). We observed the morphology of the axils of leaf primordium and the boundary zone to explore the effects of *FaHAN* overexpression on SAM and young AXM. We found that SAMs of *FaHAN*-OE#21 appeared smaller than in the control plants, were not as clearly dome-shaped as the control SAMs, and were less clearly separated from the surrounding tissues (Figure 4h-i). The boundaries between SAMs and stipules in the shoot apex (Figure 4j-k), as well as between shoot apices and young leaves during the AXM initiation of *FaHAN*-OE lines were significantly wider (Figure 4l-m), increased by ~40% compared with that of the control plants (Figure 4n). Besides, the cell densities in the AXMs of *FaHAN*-OE lines were higher than in the control in the AXM initiation stage (Figure 4o), indicating that overexpression of *FaHAN* may affect the development of the boundary zone during the AXM initiation.

It was also obvious that, in contrast to the regularly serrated leaf margins in control plants,

leaves of *FaHAN*-OE plants were small and wrinkled with more serrated margins (Figure S5a-c), and were darker than that of control plants because they contained more chlorophyll (Figure S5d). Similar to the *FaHAN*-OE plants in strawberry, *N. benthamiana 35S-FaHAN* plants exhibited small and lobed leaves (Figure S5e). In addition, the *FaHAN*-OE lines produced 2~3 times more trichomes on the leaves and runners (Figure S6a-i), indicating that *FaHAN* might be involved in leaf and trichome development in addition to its role in AXM.

Promotion of runnering by FaHAN involves changes in hormonal pathways

To explore how *FaHAN* affects the AXB development, the AXBs smaller than 2 mm long were sampled from the main crowns of two *FaHAN*-OE strawberry lines and the 'Benihoppe' control line, and transcriptome sequencing was carried out in a single simple pool for each line. Among the several genes related to AXB initiation and AXB outgrowth, we found that the Fragments Per Kilobase per Million (FPKM) value of AXM initiation marker gene *STM* was increased in the *FaHAN*-OE lines, while the FPKM level of AXB outgrowth inhibitor, *BRC1* was decreased (Figure S7). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and Gene Ontology (GO) term enrichment showed that many differentially expressed genes (DEGs) were enriched in plant hormone signal transduction pathways (Figure S8). Based on the enrichment analysis, several DEGs, including auxin, cytokinin responsive genes, and GA biosynthetic genes were discovered with the overexpression of *FaHAN* (Figure S9), indicating that FaHAN might promote AXB outgrowth involving hormone action.

Further, qRT-PCR was performed to confirm these findings. Although the sampled AXBs had passed the AXB initiation stage, the expression level of *STM* was up-regulated with the overexpression of *FaHAN* (Figure 5a). As expected, *BRC1* expression was significantly suppressed in *FaHAN*-OE lines, suggesting that FaHAN might promote AXB outgrowth by down-regulating this key inhibitor. GA metabolic pathway genes were also analyzed due to the importance of GA in AXB outgrowth (Feng et al., 2021). The expression of GA biosynthetic gene *GA20ox1* was increased in both *FaHAN*-OE lines, while no clear differences in the expression of *GA3ox* were found between the control and the transgenic lines. In the GA catabolism pathway, the expression level of *GA2ox* was significantly decreased with the

overexpression of *FaHAN* (Figure 5b). Moreover, the expression of some strawberry homologs of auxin-responsive genes and -signaling pathway genes, such as *AUXIN RESPONSE FACTOR 18-LIKE* (*ARF18-like*) and auxin-responsive protein *IAA4-like* were generally repressed (Figure 5c), while cytokinin-responsive genes and -signaling pathway genes, such as *CYTOKININ RESPONSE 1* (*CRE1*) and *ARABIDOPSIS THALIANA RESPONSE REGULATOR* (*ARR5*) were induced by *FaHAN* overexpression (Figure 5d). These results indicated the possible effects of FaHAN on the GA metabolism and auxin and cytokinin signalling. In addition, the results of hormone content determination showed that the contents of two kinds of auxin, two types of cytokinin and GA_{15} were decreased in the AXBs of *FaHAN*-OE lines compared with those of the control lines, while the contents of GA_4 and GA_{19} were significantly increased (Figure S10). Taken together, these results implied that FaHAN might take part in the control of homeostasis of auxin, cytokinin and gibberellin to regulate AXB outgrowth.

FaNAC2 activates *FaHAN* by binding to its promoter

According to yeast-one hybrid screening results (Table S1), a member of NAC family from strawberry, FaNAC2, which was very closely related to RhATAF1 from rose (Figure S11a-b), was able to bind the *FaHAN* promoter (Figure 6a). Additionally, several NAC-binding sites (5'-CGT[G/A]-3') were present in the *FaHAN* promoter (Figure S12; Olsen et al., 2004). FaNAC2 was localized into nucleus (Figure 6b). Transcriptional activity analysis was performed in yeast to examine the transactivation ability of FaNAC2 and FaHAN. On selection medium, yeast cells producing GAL4 (positive control) or FaNAC2 grew well, whereas yeast cells transformed with binding domain (BD; negative control) or FaHAN could not grow normally (Figure 6c). Furthermore, fusion of FaHAN with the VP16 activation domain inhibited the transactivation function of VP16, and these findings were confirmed by the detection of the β-galactosidase activity in transformed yeast cells (Figure 6d). A transient luciferase (LUC) expression system was used to further analyze FaNAC2 and FaHAN transcriptional activity in *N. benthamiana* leaves. Our results revealed that FaNAC2 enhances the activity of *LUC*, while FaHAN represses LUC activity (Figure 6e-f). Taken together, these results indicated that FaNAC2 had transcriptional activation capacity, while FaHAN did not, and might be a transcriptional repressor.

A dual-luciferase assay was performed to analyze the regulation of *FaHAN* by FaNAC2 in *N. benthamiana* leaves. It was observed that co-infiltration of FaNAC2-SKII (effector) and Pro*FaHAN*-LUC (reporter) exhibited significantly higher LUC activity than leaves infiltrated with the SKII and Pro*FaHAN*-LUC (Figure 6g-h), which suggested FaNAC2 might activate the expression of *FaHAN*. To obtain additional support for the role of FaNAC2, transient transactivation assays using Pro*FaHAN*-GUS-expressing transgenic *N. benthamiana* plants were performed. Darker GUS staining and higher level of GUS expression were found in leaves agroinfiltrated with the *35S*-FaNAC2-1300 construct to overexpress FaNAC2 compared with control leaves infiltrated with *35S*-1300 construct (Figure 6i-j). In an additional experiment, *35S-FaNAC2*-1300 and *35S*-1300 were agroinfiltrated separately along with the Pro*FaHAN*-GUS reporter construct into degreening strawberry fruits about 18 days after anthesis (Jia et al., 2011). As expected, the fruits co-infiltrated with *35S-FaNAC2*-1300 and Pro*FaHAN*-GUS showed darker GUS staining and higher GUS expression than the control fruits (Figure 6k-l). All these results indicated that FaNAC2 positively regulated *FaHAN* expression, possibly through direct binding to *FaHAN* promoter.

FaNAC2 promotes runner production

QRT-PCR assays showed that *FaNAC2* mRNA accumulation also occurred in the shoot apex, AXB and flower in strawberry (Figure 7a). The results of *in situ* hybridization in 'Benihoppe' showed that *FaNAC2* anti-sense probe signals were detected in the shoot apices, leaf primordia axils and AXMs (Figure 7b-e, Figure S13). To investigate whether FaNAC2 influences runner production during vegetative growth, overexpression of *FaNAC2* was performed in 'Benihoppe'. The number of runners increased ranging from 0.0 ± 0.0 in 'Benihoppe' to 2.2 ± 0.63 in strongest line #11 as shown in Figure 7f. Moreover, the expression level of *FaHAN* was up-regulated in *FaNAC2*-OE lines (Figure 7g). In addition, ectopic expression of *FaNAC2* increased the number of branches in *N. benthamiana* (Figure S14a - b).

Since AXB outgrowth is regulated by a complex hormonal network in plants (Aguilar-Martínez et al., 2007, Chatfield et al., 2000, Ferguson and Beveridge, 2009, Qiu et al., 2019), hormone treatments were applied to explore hormonal regulation of *FaNAC2* and *FaHAN* in the

AXBs. Hormones including 6-Benzylaminopurine (6-BA), Gibberellic Acid 3 (GA_3) and Indole-3-Acetic Acid (IAA) were used to exogenously treat 2.5-months-old 'Benihoppe' tissue cultured plants (Figure S15). The results showed that both *FaNAC2* and *FaHAN* were transiently induced by 6-BA, and were gradually induced by GA_3. Furthermore, *FaNAC2* responded to IAA significantly, but there was no effect on *FaHAN*. Because both GA_3 and 6-BA influenced the expression of *FaNAC2* and *FaHAN* in the AXBs, GA and cytokinin pathways might take part in the control of FaNAC2-FaHAN module-mediated regulation of AXB outgrowth. Considering all these results, FaNAC2 might play a positive role in runner production through up-regulation of *FaHAN* expression.

Virus-induced gene silencing of *FaNAC2* and *FaHAN* inhibits runner formation in strawberry

To obtain more support for the role of FaNAC2-FaHAN module in runner formation, VIGS (Virus-induced gene silencing) was utilized to silence *FaNAC2* and *FaHAN* in 'Benihoppe'. After 8 weeks of growth on soil, the number of runners was decreased 29% and 53% in *FaNAC2*-TRV2 and *FaHAN*-TRV2 lines, respectively (Figure 7h). The proportion of leaf axils lacking AXBs in *FaNAC2*-TRV2 and *FaHAN*-TRV2 VIGS lines was ~2.75 and ~4.75 times higher than in control lines, respectively, suggesting that silencing of *FaNAC2* or *FaHAN* inhibited the AXB initiation. In addition, the ratio of the number of dormant AXBs to runners in the *FaNAC2*-TRV2 and *FaHAN*-TRV2 lines was ~2.35 and ~1.78 times higher than that of the control lines in the nodes 2-5, and ~3.79 and ~5.68 times higher than that of control lines in the nodes 4-5, suggesting that the outgrowth of AXBs was also suppressed with the silencing of *FaNAC2* or *FaHAN*. Furthermore, qRT-PCR results showed that the expression levels of both *FaNAC2* and *FaHAN* were down-regulated in the *FaNAC2*-TRV2 lines (Figure 7i). Surprisingly, the expression of *FaNAC2* was decreased also in the *FaHAN*-TRV2 lines (Figure 7i), while it was not significantly changed in the *FaHAN*-OE lines (Figure S16), indicating that FaNAC2 could positively regulate *FaHAN* in the AXB, and FaHAN might also affect the *FaNAC2* expression.

DISCUSSION

FaNAC2 functions as a direct positive regulator of *FaHAN* for AXB development

The NAC family is a class of TFs found only in plants (Ooka et al., 2003). Previous studies have reported that multiple NAC members are involved in the formation of organ boundaries and meristems, such as NAM in *Petunia* (Souer et al., 1996), CUC1-3 in Arabidopsis (Aida et al., 1997, Vroemen et al., 2003, Raman et al., 2008), and CUP in *Antirrhinum majus* (Weir et al., 2004). Studies on FaNAC2 homologs in other species and in strawberry have focused on its role in resistance to biotic and abiotic stresses, such as ATAF1 in Arabidopsis, OsNAC6 in rice and FaNAC2 in strawberry (Nakashima et al., 2007, Wu et al., 2009, Liang et al., 2020). In this study, we found for the first time that FaNAC2 was involved in runner formation and could directly activate *FaHAN* by binding to its promoter (Figure 6a, Figure 7f). *FaNAC2* and *FaHAN* showed similar expression patterns in AXBs (Figure 3, Figure 7a - c), suggesting that they might function together to control AXB development. This was also supported by the results of our overexpression and gene silencing experiments. The overexpression of *FaNAC2* or *FaHAN* resulted in more runners than in the control (Figure 4a, Figure 7f), while the silencing of *FaNAC2* or *FaHAN* reduced runner formation in strawberry (Figure 7h). These results indicate that FaNAC2-FaHAN module might promote runner formation in strawberry.

***FaHAN* promotes runnering during vegetative phase of strawberry**

In strawberry, AXBs in the leaf axils have three possible fates: they can remain dormant or develop into runners or branch crowns depending on either environmental or endogenous factors (Hytönen et al., 2004, Hytönen and Kurokura, 2020, Andrés et al., 2021). GA is an essential factor for runner formation, promoting AXB outgrowth rather than AXB initiation, and its biosynthetic gene *FvGA20ox4* and signaling pathway gene *FvRGA1* have been shown to play key roles in this process (Hytönen et al., 2009, Tenreira et al., 2017, Caruana et al., 2018, Li et al., 2018, Andrés et al., 2021, Feng et al., 2021). In *FaHAN*-OE lines, *GA20ox1* expression was up-regulated rather than *GA20ox4* (Figure 5b, Figure S17), and *GA2ox* was down-regulated (Figure 5b), whereas genes encoding DELLA proteins, the well-known growth repressors in GA signaling pathway, showed no obvious differences (Figure S17). In consistence with the gene expression data, the levels of GA_4 and GA_{19} were increased (Figure S10). Although the function

of *GA20ox1* in the strawberry AXBs fate requires further investigation, the expression of *GA20ox1* in dormant AXBs is lower than that in non-dormant AXBs in runner (Qiu et al., 2019). Thus, our results suggest that changes in the GA biosynthesis and catabolism may promote runner outgrowth in *FaHAN* overexpression lines. However, neither *FaHAN*-OE nor control lines produced branch crowns during the vegetative phase with 23±2□ and long day (16 h/8 h light/dark cycle) (Figure 4c-e). Therefore, our results suggest that *FaHAN* may promote AXB outgrowth as a runner in the vegetative phase. Additional studies are needed to explore FaHAN function during reproductive phase, in which AXBs mainly develop into branch crowns that can bear additional inflorescences, resulting in a sharp decrease in the number of runners (Hytönen and Elomaa, 2011, Martins et al., 2018, Heide et al., 2013). A recent study showed that woodland strawberry *FLOWERING LOCUS T 3* (*FvFT3*) increases branch crown number when overexpressed in strawberry (Gaston et al., 2021). Further studies using the knock-out lines of these AXB development related genes through the CRISPR/Cas9 system (Feng et al., 2021) would be a promising way to discover their functions during the formation of branch crowns.

FaHAN may be involved in hormonal signaling pathways during the axillary bud outgrowth

In many plants, the growing shoot apex inhibits the outgrowth of AXBs, which is called 'apical dominance'. Auxin is a major signal promoting apical dominance, and cytokinin represents an auxin-regulated secondary messenger that promotes AXB outgrowth (Mueller and Leyser, 2011). Also in strawberry, antagonistic effects of auxin and cytokinin on the outgrowth of the AXB in the first node of the runner were found, where auxin maintains the dormancy of the AXB and cytokinin promotes dormancy release (Qiu et al., 2019). Interestingly, either decapitation or application of auxin inhibitors released dormancy and caused the outgrowth of the first runner AXBs. Similarly, we found outgrowth of the first runner AXBs in our *FaHAN*-OE lines (Figure S4). In the main crown AXBs, the auxin-responsive genes and -signaling pathway genes of *FaHAN*-OE plants were down-regulated and the levels of two auxins were decreased (Figure 5c, Figure S9-10). Although the levels of two types of cytokinin were also decreased (Figure S10), the cytokinin-responsive genes and -signaling pathway genes were generally up-regulated in *FaHAN*-OE lines (Figure 5d, Figure S9). Combining the result of the decreased proportion of

dormant AXBs in the main crown of *FaHAN*-OE strawberry plants (Figure 4e), elongated AXBs in *FaHAN*-OE plants grown on the tissue culture (Figure 4f-g), and increased branching in *35S-FaHAN* tobacco plants (Figure S3), it is hypothesized that FaHAN may reduce apical dominance by affecting the auxin and cytokinin signalling in the AXBs.

In Arabidopsis, cytokinin and GA inhibit, and auxin and strigolactones indirectly activate the expression of a signaling hub *BRC1*, which represses AXB outgrowth according to these hormonal signals (Ferguson and Beveridge, 2009, Lantzouni et al., 2017, Wang et al., 2019). Here, we found that the expression of *BRC1* was significantly down-regulated in *FaHAN*-OE lines (Figure 5a), indicating that FaHAN may promote AXB outgrowth by affecting the expression of *BRC1*. In contrast, *STM*, a marker gene for AXM initiation (Long and Barton, 2000), was up-regulated in the AXBs of *FaHAN*-OE plants (Figure 5a). This finding, together with the result of the inhibited AXB initiation in *FaHAN*-silencing lines (Figure 7h), suggested that FaHAN may affect the initiation of AXB by up-regulating the expression of *STM*, but this requires further verification.

Taking all the results into consideration, we propose a model on the role of FaNAC2-FaHAN module in the control of AXB initiation and AXB outgrowth in strawberry. FaNAC2 activates *FaHAN* by directly binding to its promoter. *FaHAN*, in turn, may regulate AXB initiation by affecting the expression of *STM*, and influence *BRC1* expression through precise regulation of homeostasis of auxin, cytokinin and GA, thus indirectly regulating AXB outgrowth (Figure 8).

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

All the growth experiments using 'Benihoppe' and *N. benthamiana* grown in soil were maintained in a plant culture room (23±2□, 16 h/8 h light/dark cycle). Plants materials for tissue culture were obtained from AXBs on the runner that were collected from actively growing plants and disinfected with 75% ethanol (30 s) and 1% NaClO (10 min). Tissue culture medium contained: 4.43 g/l MS+ 20g/l sucrose+ 7g/l Agar+ 0.2 mg/l 6BA+ 0.1 mg/l IBA. The environment of the tissue culture room is the same as that of the plant culture room. A Finnish

Fragaria vesca accession FIN56 (PI551792, National Clonal Germplasm Repository, Corvallis, OR, USA) was used for *in situ* hybridization experiments. The FIN56 plants were propagated from runner cuttings and grown in a growth chamber (23□, 18 h/6 h light/dark cycle).

Gene isolation and sequence alignment

Total RNA samples were extracted from strawberry shoot apices using the E.Z.N.A.® Total RNA Kit (Omega, R6834-01, USA) according to the manufacturer's instructions. DNA contaminations were removed and the first-strand cDNA was synthesized from 1 μg RNA using HIScript® II Reverse Transcriptase (Vazyme, R233-01, China). The CDS of *FaNAC2* and *FaHAN* were amplified from 'Benihoppe' cDNA library using Polymerase Chain Reaction (PCR) using the primers that were designed according to the *Fragaria* × *ananassa* Camarosa Genome v.1.0.a1 Transcripts (https://www.rosaceae.org/), and *FvHAN* (FvH4_1g13230) was identified from FIN56 cDNA library using the primers that were designed according to the *Fragaria vesca* Genome v4.0.a2. Alignments of the predicted full-length amino acid sequences of the two proteins with their homologues from other plant species (Figure 2a, Figure S11a) were performed using BioEdit (https://www.bioedit.com/) and ClustalW (http://www.clustal.org/). Phylogenetic analyses were performed using MEGA 7.0 (https://www.mega.com/) with the analysis methods of construct/test neighbor-joining tree (No. of bootstrap replications:100; Substitution model/method: P-distance; Rates among sites: Uniform rates; Gaps/Missing data treatment: Pairwise deletion). The sequences of HAN and NAC2 homologues from other plant species were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). The primers are listed in Table S2. The accession number of proteins are shown in Table S3.

Subcellular localization

The pCAMBIA1300-GFP vector was used for subcellular localization experiments. *FaNAC2* and *FaHAN* CDS were cloned into pCAMBIA1300-GFP respectively under the control of *35S* promoter to produce C-terminal-GFP fusion proteins. The recombinant plasmids and the empty vector were transformed separately into *Agrobacterium tumefaciens* strain GV3101 and grown in Luria-Bertani liquid culture overnight with 50 μg/ml kanamycin and 25 μg/ml rifampicin. Bacteria cells were collected by centrifuging 5000 rpm for 5 min and resuspended in

resuspension solution (1 M $MgCl_2$, 1 M 2-Morpholinoethanesulfonic acid, and 0.05 M Acetosyringone) with OD_{600} = 1.0. Bacterial resuspension solution was kept at room temperature for 2 h before infiltration into leaves of *N. benthamiana* for transient gene expression. The GFP fluorescence signals were observed using a confocal laser-scanning microscope (FV3000, Olympus, Japan) at 3 d post-infiltration. The primers are listed in Table S2.

Quantitative RT-PCR analysis

The qRT-PCR reactions with 20 µl volume containing 1 µl cDNA as template were run using SYBR Premix ExTaq (Takara, RR82WR, Japan) on an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, USA). The $2^{-\Delta\Delta CT}$ method was used to analyze the qRT-PCR expression data according to Gong et al. (2014). *FaACTIN* and *NbACTIN* were used as internal controls for normalization of gene expression in strawberry and *N. benthamiana*, respectively. For the qRT-PCR in Figure 7g, the samples were taken from the AXBs of *FaNAC2* -OE lines that were subcultured for half a month and were grown on the tissue culture. Gene-specific primers are given in Table S2.

***In situ* hybridization**

The probes for *FaNAC2* (399 bp) and *FaHAN* (450 bp) were designed based on regions of unique sequences, and sense and anti-sense probes were synthesized by PCR amplification using SP6 and T7 RNA polymerases, respectively, with the DIG RNA Labeling Kit (SP6/T7) (Sigma, 11175025910, USA). The shoot apices of 'Benihoppe' were collected from 2-months-old plants after planting in the soil and fixed in 3.7% formalin–acetic acid–alcohol (FAA) (Kurokura et al., 2005). Sectioning and hybridization steps were performed as described by Zhang et al. (2013) with minor modification. A prehybridization step was added according to Zhao et al. (2020). Sections were examined and photographed using the Leica DM500 Microscope equipped with the RisingCam industrial digital camera (E3ISPM20000KPA, China). For in situ hybridization in *F. vesca* accession FIN56, synthesis of *FvHAN* probe (227 bp), preparation, sectioning, and hybridization of AXBs of FIN56 were performed according to Elomaa et al. (2003) and Zhao et al. (2020). Sections were examined and photographed using the Leitz Laborlux S Microscope

equipped with the Leica DFC420C Digital Camera (Wetzlar, Germany). The primers are given in Table S2.

Stable transformation of strawberry and *N. benthamiana*

For both strawberry and *N. benthamiana*, the vector pCAMBIA2300 including kanamycin resistance gene was used for stable transformation. The CDS of *FaNAC2* and *FaHAN* were inserted into pCAMBIA2300 using the Trelisf™ SoSoo cloning Kit (TsingKe, TSV-S1, China). The resulting *35S-FaHAN* (*FaNAC2*) vector was introduced into *A. tumefaciens* strain EHA105 and transformed into strawberry (Gao et al., 2020). The kanamycin-resistant (25 mg/l) plants were screened for the presence of the construct by RT-PCR analysis. The primers listed in Table S2. The biological replicates of each line were subcultured by tissue culture from the original positive plant. When positive plants were identified, the tissue culture plants (including overexpression and control plants) were transferred to the same medium for observation.

Similar to strawberry transformation, the *FaNAC2* and *FaHAN* CDSs were introduced into pCAMBIA2300 under the control of the *35S* promoter and transformed into *N. benthamiana* as described by Wydro et al. (2006).

Scanning electron microscope

Shoot apices were fixed in 4% glutaraldehyde at 4□ overnight, treated sequentially with 30%, 50%, 70%, 80%, 90%, 95%, 100%, 100% ethanol for 10 min each, critical point dried for 4.5h, coated with gold for 1min, and photographed by scanning electron microscope (Hitach SU3500, Japan).

Determination of chlorophyll content

Samples were collected from the second youngest expanded leaves consisted of 1 cm squares with symmetrical veins taken from the center of the leaves. Three individual plants from *FaHAN*-OE#21 and control line were sampled for each line. Samples were ground using mortar and pestle, and the chlorophyll analyses were done as described in Sartory and Grobbelaar

(1984). The chlorophyll a (Ca), chlorophyll b (Cb), and total chlorophyll (Ct) contents were then calculated using the formula $Ca = 13.95A_{665}-6.88A_{649}$; $Cb = 24.96A_{649}-7.32A_{665}$; $Ct = Ca + Cb$.

RNA-seq

The AXBs (less than 2 mm) of 'Benihoppe' and two lines of *FaHAN*-OE were sampled from plants grown for 2 months on soil at constant temperature of 23±2℃ in LD conditions (18 h/6 h; light/dark). AXBs of 9 individual plants from each line were used as a sample mixture. RNA extraction was carried out as described above and RNA sequencing was performed on an Illumina 4000 sequencing platform. Hisat software (USA) was used for alignment and valid data were aligned to the reference octoploid strawberry genome (https://datadryad.org/stash/dataset/doi:10.5061/dryad.b2c58pc) (Edger et al., 2019). Data analysis was performed by Stringtie software (https://ccb.jhu.edu/software/stringtie/); R program (https://www.r-studio.com/data-recovery-software/) and TBtools (https://github.com/CJ-Chen/TBtools/releases.) were used for display the results graphically. FPKM value were used to estimate quantification of gene expression levels. A corrected *P* value <0.05 and |log2foldchange| ≥ 1 were set as the threshold for significantly differential expression. The raw sequence data have been submitted to the NCBI Sequence Read Archive with accession code PRJNA632583. The number of reads per sample was supplemented in Table S5. The genomic position of genes in Figure S7, Figure S9 and Figure S17 are shown in Table S4. For the qRT-PCR verification of the expression of the genes mentioned in RNA-seq, two independent experiments were conducted with a sample mixture containing AXBs from 9 plants per line in each experiment. The samples from the first independent experiment were used for the preliminary analysis of RNA-seq and the verification of qRT-PCR, and the samples from the second independent experiment are used for further qRT- PCR verification.

Detection of phytohormones

AXBs from *FaHAN*-OE#18 and control plants were harvested from 2-month-old plant grown in the soil. Sampling was done with a total of two biological replicates, with each replicate consisting of a mixture of AXBs from 9 plants. The fresh weight of each biological replicate was

about 0.2 g. Samples were extracted with methyl alcohol/H_2O/formic acid (15:4:1), purified with 80% methyl alcohol/H_2O and 0.22 μm filter membrane. The sample extracts were analyzed using an UPLC-ESI-MS/MS system (UPLC, ExionLC™ AD, https://sciex.com.cn/; MS, Applied Biosystems 6500 Triple Quadrupole, https://sciex.com.cn/). AB 6500+ QTRAP® LC-MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in both positive and negative ion modes and controlled by Analyst 1.6 software (AB Sciex).

Yeast one-hybrid screening and assays

Based on the distribution of *cis*-elements on the *FaHAN* promoter (Figure S7) and yeast one-hybrid screening methods (Deplancke et al., 2006), the fragment (-714 to -475) including CAT-box (*cis*-acting regulatory element related to meristem expression) was selected and inserted into the pDEST-HISi-2 vector by Gateway cloning as bait vector and was transformed into yeast strain YM4271 using PEG/LiAC method after linearization by *Apa*I. The Arabidopsis transcription factor library (Mitsuda et al., 2010) containing about 1400 transcription factors (TFs) was transformed into yeast strain EGY48. EGY48 yeast cells with bait vector were selected for background expression of the HIS3 as 20 mM 3-AT (3-amino-1,2,4-triazole). Positive colonies were sorted on the screening synthetic dropout (SD) nutrient medium (SD-Ura-His-Leu + 20 mM 3-AT) after yeast mating.

By doing BLAST against *Fragaria*×*ananassa* Camarosa Genome Assembly v1.0.a1 using candidate interacting proteins of Arabidopsis as query, the candidate interacting proteins from strawberry were obtained for further verification. The vectors pGADT7 and pHIS2.1 were used to test whether these candidate genes can bind to the *FaHAN* promoter. The *FaHAN* promoter was inserted into pHIS2.1 and *FaNAC2* were cloned into pGADT7, respectively. Two recombinant vectors were co-transformed into yeast strain Y187, and successful transformants were selected by growth on screening plates (SD-Leu-Trp-His + 20 mM 3-AT) at 30□ for 3 days. The primers are listed in Table S2.

Transcriptional activity assay

The yeast system was used for transcriptional activation activity assays. The pGBKT7 (BD) and GAL4 vectors were used as the negative and positive controls, respectively. The CDS of *FaNAC2* and *FaHAN* were inserted into pGBKT7 to produce fusion proteins with the GAL4 DNA-binding domain, and the resulting constructs were designated BD-FaNAC2 and BD-FaHAN. All plasmids were transferred to yeast strain AH109 and transcriptional activation activity was evaluated by spot assays and LacZ-filter lift assays. β-Galactosidase activity was measured according to Gong et al. (2014). pEAQ vector was used in the transient luciferase expression system, FaNAC2 or FaHAN was fused to the GAL4 DNA-binding domain to construct an effector, with the *LUC* gene driven by the 5×*USA GAL* promoter as a reporter plasmid. 48h after infection, luciferase activity was observed and quantitative analysis of the DNA-protein assay was conducted by determining the LUC/REN ratio. Primers used for vector construction are shown in Table S2.

Promoter isolation, analysis, and GUS activity assays

Genomic DNA was extracted from the strawberry cultivar 'Benihoppe' using a TIANquick Midi Purification Kit (TianGen, DP360, China). The primers for amplifying the promoters of *FaHAN* were designed based on the *Fragaria* × *ananassa Camarosa* Genome v1.0.a1, and fragments were amplified using the 'Benihoppe' DNA as a template and sequenced. The 1245 bp fragment (Fvb1-4: 6474897..6476142) before the start codon of *FaHAN* were cloned into the pCAMBIA1391 vector to generate Pro*FaHAN-GUS* reporter constructs. The constructs were used for stable transformation of *N. benthamiana* by *Agrobacterium*-mediated transformation as described above.

For transient transactivation assays, the *FaNAC2* CDS was cloned into pCAMBIA1300 driven by the *35S* promoter to generate an effector construct and was transiently introduced into the Pro*FaHAN*-GUS transgenic *N. benthamiana* lines by vacuum infiltration (-0.1 atm, 1 min), or co-transfection with Pro*FaHAN-GUS* constructs into strawberry fruits by agroinfiltration (Jia et al., 2011, Li et al., 2009, Marion et al., 2008). 48h after infection, a part of the samples (*N. benthamiana* seedlings or strawberry fruits) was frozen in liquid nitrogen for detection of GUS gene expression, and the remaining samples were incubated with GUS staining buffer (2 M

ferri/ferrocyanide, 0.1% Triton X-100, 0.1 M sodium phosphate buffer, 0.5 mg.ml^{-1} X-gluc, pH 7) at 37□ for 9 h, after which the stained samples were decolorized using 75% ethanol for decolorization and photographed. The primers are listed in Table S2.

Dual-luciferase reporter assay in *N. benthamiana*

Two pGreenII vectors, pGreenII 0800-LUC and pGreen 62-SKII were used to detect the interaction between FaNAC2 and the *FaHAN* promoter *in vivo* (Wei et al., 2017). The *FaNAC2* CDS was cloned into pGreen 62-SKII to generate the effector vector, and 1245 bp promoter of *FaHAN* was cloned into pGreenII 0800-LUC to generate the reporter vector. The empty vector was used as the negative control. These constructs were transformed into *A. tumefaciens* strain GV3101 harboring the pSoup helper plasmid. The *N. benthamiana* leaves were infiltrated with a mixed bacterial suspension (OD_{600} = 1.0, effector: reporter = 4:1) for induction analysis. The LUC fluorescence of the agroinfiltrated leaves was measured after 48 h, luciferase activity was observed using a charge-coupled device camera (Tanon 5200, China), and LUC and REN activities were measured using the dual-luciferase reporter assay reagents and a GloMax 20/20 luminometer (Promega, USA). The primers used are given in Table S2.

Silencing of *FaNAC2* and *FaHAN* in strawberry by virus-induced gene silencing

For silencing of *FaNAC2* and *FaHAN*, 243 bp and 213 bp fragments from unique regions were amplified, respectively. The fragments were then inserted into the TRV2 vector using the Trelisf™ SoSoo cloning Kit as mentioned above. TRV1, TRV2, and its derivatives were introduced into *A. tumefaciens* strain GV3101 (the vectors are the same as the vectors in Tian et al. (2014)). A mixture of *Agrobacterium* cultures containing pTRV1 & pTRV2, pTRV1 & pTRV2-*FaNAC2*, and pTRV1 & pTRV2-*FaHAN* (OD_{600} = 1.0) in 1:1 (v/v) ratios with 0.01 % Silwet L-77 were placed in the dark at room temperature for 3 h before inoculation. Plant culture room-grown three-week-old runner-propagated daughter plants at the same growth stage without runners were selected for the experiments. The plants were cleaned and small holes were poked into the main crowns using pins to facilitate efficient infiltration of *Agrobacterium* carrying the TRV plasmids (Wu et al., 2015). The strawberry plants were then infiltrated with the *Agrobacterium* strains under vacuum at -0.4~-0.5 atm for 2 min, and this operation was repeated

once. The surfaces of the treated plants were wiped off with filter paper to remove excess *Agrobacterium* culture and then transferred to soil (Tian et al., 2015). Each independent plant represents an individual line. For the qRT-PCR, AXBs on the main crown of independent plant were sampled, and 3 individual lines of TRV2-control, *FaNAC2*-TRV2 and *FaHAN*-TRV2 were sampled at 4 weeks after planting.

DATA AVAILABILITY STATEMENT

The raw data of RNA-seq can be obtained from Sequence Read Archive of National Center for Biotechnology Information (accession number: PRJNA632583).

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AUTHOR CONTRIBUTIONS

HW, JL and ZW conceived and designed the research. JL, HW, ZW, JZ, LF, GF, DG, ZD, SH, ZF and FW performed the experiments. EK and TH provided the FIN56 seedlings. HW, JL and ZW drafted the manuscript. TH and EK revised the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Figure S1. Localization of *FaHAN* expression by *in situ* hybridization.

Figure S2. Relative expression of *FaHAN* in the AXBs of control plants and *FaHAN*-OE#18, *FaHAN*-OE#21, and *FaHAN*-OE#24 lines.

Figure S3. Ectopic expression of *FaHAN* in *N. benthamiana*.

Figure S4. Overexpression of *FaHAN* promotes the outgrowth of the first runner AXB.

Figure S5. Leaf phenotypes of *FaHAN*-OE strawberry transgenic lines and *35S-FaHAN N. benthamiana* transgenic lines.

Figure S6. Trichome phenotypes of *FaHAN*-OE and control plants.

Figure S7. Heatmap showing the value of FPKM of genes involved in initiation and outgrowth of AXBs.

Figure S8. KEGG pathway enrichment and GO term enrichment of DEGs in *FaHAN*-OE lines.

Figure S9. Heat map of genes involved in different hormone pathways, including auxin response, cytokinin response, and gibberellin response, biosynthesis and catabolism.

Figure S10. Differential accumulation of endogenous auxin, cytokinin, gibberellin in AXBs of *FaHAN*-OE#21 and control plants.

Figure S11. Amino acid sequence alignment, phylogenetic analysis of FaNAC2.

Figure S12. The distribution of four predicted *cis*-elements in the *FaHAN* promoter.

Figure S13. Localization of *FaNAC2* expression by *in situ* hybridization.

Figure S14. Ectopic expression of *FaNAC2* in *N. benthamiana*.

Figure S15. Relative expression of *FaNAC2* and *FaHAN* after exogenous hormone treatment.

Figure S16. The relative expression of *FaNAC2* and *FaHAN* in the AXBs of control and *FaHAN*-OE lines.

Figure S17. Heat map of *GA20ox4* and *GAIP* genes in two *FaHAN*-OE strawberry lines and the control plants.

Table S1. Candidate upstream regulators of *FaHAN* screened by yeast one-hybrid analysis.

Table S2. Oligonucleotide primers used in this study.

Table S3. NCBI accession number of proteins used in the study.

Table S4. The genomic position of strawberry genes used in this study.

Table S5. The number of reads in RNA-seq data.

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FIGURE LEGENDS

Figure 1. Conceptual model showing the steps of axillary bud development. AXM initiates at the leaf axils, and is characterized by the similar developmental potential as the SAM. AXM then produces its own leaf primordium changing its identity to AXB. Finally, AXB grows out to form a branch. Abbreviation: SAM, shoot apical meristem; LP, leaf primordia; YL, young leaf; AXM, axillary meristem; AXB, axillary bud.

Figure 2. Amino acid sequence alignment, phylogenetic analysis, and subcellular

localization of FaHAN. (a) Protein sequence alignment of HAN proteins from cucumber, Arabidopsis, rice, maize and strawberry. The blue boxes enclose the conserved HAN motif and GATA zinc finger motif. **(b)** Phylogenetic analysis of *FaHAN* and related plant proteins. The red circles refer to the *HAN* homologous proteins in Rosaceous species, the dicotyledonous *HAN* homologs are in the purple rectangle, and the monocotyledonous *HAN* homologs are in the green rectangle. **(c)** Subcellular localization of FaHAN protein determined by transient expression of the FaHAN-GFP fusion protein in *N. benthamiana* leaves. Confocal microscopy of *N. benthamiana* leaf epidermal cells following agroinfiltration with the *FaHAN*-C-terminal-*GFP* construct under control of the CaMV *35S* promoter. The *35S*-GFP vector served as a negative control.

Figure 3. *FaHAN/FvHAN* expression pattern. (a) *FaHAN* expression in different organs and tissues of 'Benihoppe' strawberry. Data are represented as the mean ± SD. n = 3. Scale bar = 1 cm. **(b-c)** *FaHAN* expression in the axils of leaf primordium (arrow) of the shoot apex as detected by *in situ* hybridization. **(e)** *FaHAN* localized in the AXM (arrow), the morphologically detectable bump, that hasn't formed its own leaf primordia to become an AXB. Sense probe of *FaHAN* was used in **(d, f)**. Scale bar = 50 μm. **(g-h)** *FvHAN* expression in the meristematic dome and in the axils of leaf primordium (arrows) of developing AXBs of diploid strawberry FIN56. **(i)** No signal was found on hybridization with the *FvHAN* sense probe. Abbreviation: ML, mature leaf; YL, young leaf; SA, shoot apex; AXB, axillary bud; FlO, flower; RF, red fruit; LP, leaf primordia; SAM: shoot apical meristem; AXM: axillary meristem; MD, meristematic dome. Scale bar = 50 μm.

Figure 4. The phenotype analysis of runner production and anatomical structures of *FaHAN*-overexpression and control lines. The number of runners **(a)** and plant height **(b)** per plant of *FaHAN*-overexpression (OE) and control ('Benihoppe') lines about one month after subculturing *in vitro*. The arrows indicate young runners from the main crown of *FaHAN*-OE lines. Data are represented as the mean ± SD. n = 10 (*t* test, ** $p<0.01$). **(c)** *FaHAN*-OE and control lines 50 days after planting in soil (DAP). The arrows indicate runners arising from the main crown of *FaHAN*-OE and control lines. **(d)** Number of runners from the main crown of the control plants and *FaHAN*-OE#18, *FaHAN*-OE#21, *FaHAN*-OE#24 lines at 80 DAP. Data are represented as the mean ± SD. n = 10 (*t* test, ** $p<0.01$). Scale bar = 10 cm (a, c). **(e)** The proportion of dormant AXBs in the axils of the first to sixth expanded leaves of *FaHAN*-OE#21 and control plants at 50 DAP. Data are represented as the mean ± SD. n = 11 (*t* test, ** $p<0.01$). **(f)** Longitudinal sections of the shoot apex and young AXB in control plants grown on tissue culture. 0/5 refers to 0 elongated AXBs in the 5 sections observed. **(g)** Longitudinal sections of the shoot apex and young AXB in *FaHAN*-OE#21 plants grown on tissue culture. 4/5 refers to 4 elongated AXBs in the 5 sections observed. **(h-i)** The shoot apices of control (h) and *FaHAN*-OE#21 plants (i) under scanning electron microscope. The young leaf enclosed the shoot meristematic dome, and trichomes protruded from between young leaf and meristematic dome. **(j-k)** The anatomical structure of shoot apices of control (j) and *FaHAN*-OE line#21 plants (k). **(l-m)** Longitudinal sections of the shoot apices from control and *FaHAN*-OE line#21 plants.

The black boxes refer to the enlarged view of the AXMs, the width means the boundary width between shoot apex and the young leaf. Scale bars = 100 μm (f-m). Measuring method was shown in (l-m), and the measuring results were shown in **(n)**. **(o)** Cell density of AXMs with similar plastochrons across the *FaHAN*-OE#21 and control lines. Abbreviation: MD: meristematic dome; YL: young leaf; TR: trichome; S: stipule. SA: shoot apex. Data are represented as the mean ± SD. n = 3 (*t* test, * $p<0.05$). The growth condition is 23±2□ and long day (16 h/8 h light/dark cycle).

Figure 5. Relative expression of *STM*, *BRC1* and several hormone action genes in the AXBs of *FaHAN*-OE#18, *FaHAN*-OE#21 and control lines grown in the plant culture room. (a) Relative expression of *STM* and *BRC1* in the AXBs of *FaHAN*-OE and control lines verified by qRT-PCR. **(b)** Relative expression of GA biosynthetic and catabolic pathway genes in the AXBs of *FaHAN*-OE and control lines verified by qRT-PCR. Relative expression of several auxin responsive genes **(c)** and cytokinin responsive genes **(d)** in the AXBs of *FaHAN*-OE and control lines verified by qRT-PCR. Gene IDs of all the verified genes are shown in Table S4. Bars are means ± SD of two independent experiments (*t* test, * $p<0.05$; ** $p<0.01$).

Figure 6. The FaNAC2 protein activates *FaHAN* by binding to its promoter. (a) The interaction between FaNAC2 and the *FaHAN* promoter was determined in yeast one-hybrid assays by yeast growth on synthetic dropout (SD) nutrient medium lacking Trp, Leu and His and containing 0 or 20 mM 3-Amino-1,2,4-triazole (3-AT). **(b)** Subcellular localization of the FaNAC2 protein in *N. benthamiana* leaves. Confocal microscopy of *N. benthamiana* leaf cells transiently expressing the FaNAC2-GFP fusion protein following agroinfiltration. The *35S*-GFP served as the negative control. **(c)** Transactivation activity assay in yeast cells. SD-Trp medium was used to detect transformation, SD-Trp/-His medium and SD-Trp/-His medium containing 10 mM 3-AT was used to examine the transformants' growth. At least three yeast colonies were tested for each construct, and one representative is shown. GAL4: positive control; BD: negative control. **(d)** β-galactosidase activity of the yeast colonies grown on SD/-Trp medium in (c). The higher the value of enzyme activity, the higher the transcriptional activity. Error bars = ± SD (n = 3, *t* test, ** $p<0.01$). **(e)** Transient luciferase (LUC) reporter assay of the FaNAC2 and FaHAN protein and schematic diagram of injection of *N. benthamiana* leaves. Plasmid combinations of the *LUC* gene driven by the 5×*UAS GAL4* DNA-binding domain as a reporter and effectors consisting of pEAQ-FaNAC2, pEAQ-FaHAN or pEAQ-BD were co-transformed into *N. benthamiana* leaves. Images were observed 48h later. **(f)** Quantification of trans-activity of FaNAC2 and FaHAN by LUC/REN assay in *N. benthamiana* leaves. Error bars = ± SD (n = 8, *t* test, ** $p<0.01$). **(g)** The interaction between FaNAC2 and the *FaHAN* promoter using a dual-luciferase reporter assay in *N. benthamiana* leaves. FaNAC2-SKII means the effector, Pro*FaHAN*-LUC means the reporter. **(h)** The ratio of LUC/REN of the empty vector (SKII) co-transformed with Pro*FaHAN*-LUC vector was used

as a calibrator (set as 1). Error bars = ± SD (n = 6, *t* test, * p<0.05). **(i)** FaNAC2 activates *FaHAN* promoter in Pro*FaHAN*-GUS *N. benthamiana*. Seedlings of three Pro*FaHAN*-GUS *N. benthamiana* lines were infiltrated by *Agrobacterium* containing *35S-FaNAC2*-1300 and the empty vector *35S*-1300. **(k)** FaNAC2 activates transcription from the *FaHAN* promoter in strawberry fruits. *35S-FaNAC2*-1300 and *35S*-1300 were agroinfiltrated separately along with the Pro*FaHAN*-GUS construct into degreening stage strawberry fruits. Scale bar = 1 cm. **(j, l)** Relative expression of *GUS* of the Pro*FaHAN*-GUS *N. benthamiana* lines and strawberry fruits 48 h after infiltration. Error bars = ± SD (n = 3, *t* test, * p<0.05; ** p<0.01).

Figure 7. FaNAC2 activates *FaHAN* and runner formation in strawberry. (a) The expression of *FaNAC2* in different organs and tissues of strawberry. Data are represented as the mean ± SD. n = 3. **(b)** *FaNAC2* expression in the shoot apex as detected by *in situ* hybridization. The arrows mean the axils of leaf primordia. **(c)** *FaNAC2* expression in the AXMs (the detectable bump). Sense probe of *FaNAC2* was used in **(d-e)**. Scale bar = 50 μm. Abbreviation: ML, mature leaf; YL, young leaf; SA, shoot apex; AXB, axillary bud; FLO, flower; RF, red fruit; MD, meristematic dome; LP, leaf primordia. **(f)** The number of runners per plant of *FaNAC2*-overexpression (OE) and control lines about one month after subculturing *in vitro*. The arrows indicate young runners from the main crown of *FaNAC2*-OE lines. Data are represented as the mean ± SD. n = 10 (*t* test, ** p<0.01). Scale bar = 10 cm. **(g)** The relative expression of *FaNAC2* and *FaHAN* in the AXBs of control and *FaNAC2*-OE#6, #11 and #20. Error bars = ± SD (n = 3, *t* test, * p<0.05; ** p<0.01). **(h)** Schematic representation of AXB formation in nodes of main crown of TRV2 control, *FaNAC2*-TRV2, and *FaHAN*-TRV2 lines at 8 weeks after planting in the VIGS experiment. Each column represents one main crown with each square depicting the fate of an individual leaf axil. The node 2 means the location of the second new leaf after infection. **(i)** The relative expression of *FaNAC2* and *FaHAN* in the AXBs of three independent lines of TRV2 control, *FaNAC2*-TRV2, and *FaHAN*-TRV2, respectively, at 4 weeks after planting. The data shown are the average of three technical replicates with the SD.

Figure 8. Conceptual model showing how the FaNAC2-FaHAN module regulates runner growth in strawberry. FaNAC2 activates *FaHAN* by directly binding to its promoter. FaHAN, in turn, may regulate AXB initiation by affecting the expression of *STM*, and influences *BRC1* expression through precise regulation of the expression of gibberellin biosynthesis and auxin and cytokinin responsive genes, thus regulating AXB outgrowth. The dotted green boxes represent the development of a runner. Abbreviation: SAM, shoot apical meristem.