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Brief Communication

Tissue-specific expression of *barnase* in tobacco delays axillary shoot development after topping

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Shoot branching is an important agronomic trait that determines plant architecture and affects crop productivity (Shen *et al.*, 2019). Molecular signals from the shoot apical meristem (SAM) create a hormonal environment that integrates with the expression of axillary bud-specific repressors such as BRANCHED1 (BRC1) to inhibit axillary shoot formation (Wang *et al.*, 2019 and references therein). The signal is eliminated by topping (SAM removal), enabling the formation of new shoots (suckers) from axillary buds (Figure 1a). In tobacco (*Nicotiana tabacum*), topping is necessary to enhance leaf development/maturation before harvesting, but sucker growth after topping is undesirable because it reallocates resources to axillary buds, reducing yield and quality of the main leaves. Sucker growth can be inhibited by fatty alcohols, flumetralin or maleic hydrazide, but chemical control is time-consuming and expensive, and the chemicals may persist after leaf processing due to environmental variability (Bailey *et al.*, 2019). Tobacco plants with delayed axillary bud initiation or shoot growth would therefore significantly improve harvest and/or product quality, as previously shown for other species (e.g., Groot *et al.*, 1994).

To analyse the transcriptomes of axillary meristems/buds from tobacco before and after topping, we grew plants in the greenhouse for 8 weeks and took seven samples (n = 3) including young leaf, SAM and axillary meristems/buds before and 2, 6, 24 and 72 h after topping, from plants with 8–10 fully expanded leaves. RNA was extracted for RNA-Seq analysis on an Illumina HiSeq 2000 device (100-bp single reads, at least 30 million reads per sample), and we identified 17 candidate genes that were deregulated in axillary buds post-topping (Figure 1b). Expression was validated by qPCR, and the six most promising genes were selected for further analysis. The corresponding promoters were analysed in the axillary meristem of a commercial dark tobacco before and after topping using a GUS reporter assay. Four of the promoters showed nonspecific activity, but *gusA* expression driven by promoters P#$1_{2.5kb}$ and P#$15_{2.5kb}$ was limited to the axillary meristem, with P#15 showing the more restricted spatial domain (Figure 1c). P#$15_{2.5kb}$::GUS activity was stable even 7 days post-topping, but GUS activity in the P#$1_{2.5kb}$::*GUS* transgenic lines declined shortly after topping. P#$1_{2.5kb}$ sequence analysis revealed the presence of the sugar-repressible element TTATCCA (Tatematsu *et al.*, 2005) at positions –2401 to –2407. Shortening P#$1_{2.5kb}$ to 2.4 kb (P#$1_{2.4kb}$) did not change its axillary meristem specificity but prolonged its activity, so that GUS staining was still detected 10 days post-topping in P#$1_{2.4kb}$::*GUS* transgenic plants (Figure 1c).

Gene#1 encoded a BRC1 homolog, and silencing enhanced sucker growth even before topping, as reported in other species, whereas strong overexpression driven by the constitutive CaMV35S promoter was lethal, allowing the regeneration of only one transgenic line with severely stunted growth (data not shown). Transgenic lines with weak gene#1 expression showed sucker development comparable to wild-type controls, and the expression of gene#1 driven by P#$1_{2.4kb}$ only slightly reduced sucker growth (data not shown), probably reflecting endogenous regulation and/or a positive regulator of bud formation such as NtBRC2 (Ding *et al.*, 2020). Gene#15 encoded a vicilin-like protein, and neither RNAi nor constitutive overexpression generated a notable phenotype (data not shown).

To selectively inhibit axillary bud initiation and subsequent sucker growth, we expressed the cytotoxic ribonuclease barnase from the bacterium *Bacillus amyloliquefaciens* under the control of the axillary bud-specific promoters P#$15_{2.5kb}$ and P#$1_{2.4kb}$ in order to ablate the cells responsible for sucker formation. We initially generated 11 P#$15_{2.5kb}$::*barnase* transgenic plants, seven of which did not develop axillary bud primordia during vegetative growth. Next, we topped two lines (L10 and L11) and no axillary bud primordia were visible even 1 week post-topping (Figure 1d). These plants showed a normal phenotype, but axillary bud initiation was delayed by at least 3 weeks, resulting in fewer and shorter suckers with a weight reduction of 50%–79% even 4 weeks post-topping compared with wild-type controls (Figure 1d). Seeds from the remaining lines failed to germinate on selective medium, indicating that the P#$15_{2.5kb}$ promoter (and thus barnase) is also active during seed formation/germination. We analysed the P#$15_{2.5kb}$::*GUS* transgenic lines again and confirmed weak GUS staining in the seeds (Figure 1d).

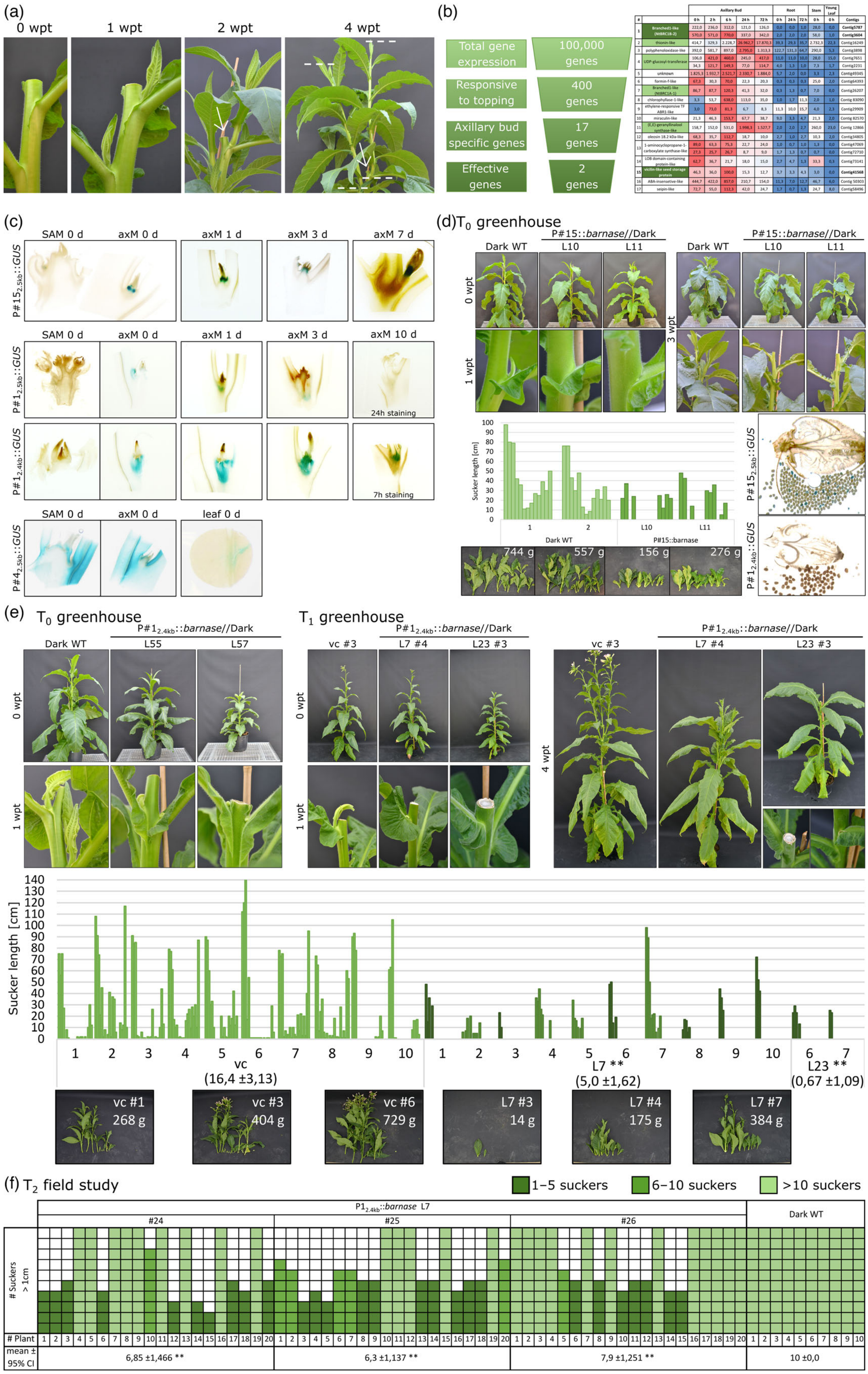
(a)
0 wpt
1 wpt
2 wpt
4 wpt
(b)
Total gene expression
100,000 genes
Responsive to topping
400 genes
Axillary bud specific genes
17 genes
Effective genes
2 genes
(c)
SAM 0 d
axM 0 d
axM 1 d
axM 3 d
axM 7 d
axM 10 d
24h staining
7h staining
leaf 0 d
P#15 2,5kb::GUS
P#1 2,5kb::GUS
P#1 2,4kb::GUS
P#4 2,5kb::GUS
(d) T0 greenhouse
P#15::barnase//Dark
Dark WT
L10
L11
0 wpt
1 wpt
3 wpt
Sucker length [cm]
Dark WT
P#15::barnase
744 g
557 g
156 g
276 g
(e) T0 greenhouse
P#1 2.4kb::barnase//Dark
Dark WT
L55
L57
T1 greenhouse
vc #3
L7 #4
L23 #3
4 wpt
Sucker length [cm]
vc
(16,4 ±3,13)
L7 **
(5,0 ±1,62)
L23 **
(0,67 ±1,09)
vc #1 268 g
vc #3 404 g
vc #6 729 g
L7 #3 14 g
L7 #4 175 g
L7 #7 384 g
(f) T2 field study
1–5 suckers
6–10 suckers
>10 suckers
P1 2.4kb::barnase L7
#24
#25
#26
Dark WT
Suckers > 1cm
Plant
mean ± 95% CI
6,85 ±1,466 **
6,3 ±1,137 **
7,9 ±1,251 **
10 ±0,0

Figure 1 Regulation of tobacco sucker formation. (a) After SAM removal, suckers form a bushy growth phenotype 4 weeks post-topping (wpt). Arrows show topping site. Dashed lines indicate the top suckers. (b) Identification of candidate genes by RNA-Seq. (c) GUS activity (staining for 4 h if not stated otherwise) in P#$15_{2.5kb}$::*GUS*, P#$1_{2.5kb}$::*GUS*, P#$1_{2.4kb}$::*GUS* and P#$4_{2.5kb}$::*GUS* transgenic lines at various times before and after topping (SAM = shoot apical meristem and axM = axillary meristems). (d) Barnase expression driven by P#$15_{2.5kb}$ and (e) P#$1_{2.4kb}$ in transgenic lines delays sucker growth and reduces their number and length post-topping. All suckers were removed 4 wpt and the length (chart) and total fresh weight (lower images) were determined. Weak GUS activity was also detected in P#$15_{2.5kb}$::*GUS* transgenic seeds, but no GUS activity was detected in P#$1_{2.4kb}$::*GUS* transgenic seeds (lower right-hand images). (f) Field study of P#$1_{2.4kb}$::*barnase* transgenic plants. We analysed 60 L7 plants (T_2 generation, representing three T_1 parents) for sucker number and length (lighter colour = more suckers > 1 cm per plant). In (e) and (f), mean values for sucker number are shown with $\pm$ 95% confidence intervals (CI). Statistical significance was determined using a pairwise Welch's *t*-test with Bonferroni–Holm correction (***P* < 0.01).

Barnase expression driven by the P#$1_{2.4kb}$ promoter also strongly inhibited sucker growth in T_0 plants, and seven of an initial population of 21 plants produced no or only a few axillary bud primordia during vegetative growth (Figure 1e). Again, two lines (L55 and L57) were topped, revealing that sucker growth was delayed by at least 1 week. In contrast to the P#$15_{2.5kb}$::*barnase* plants, seeds from the P#$1_{2.4kb}$::*barnase* plants germinated, allowing comprehensive analysis of the T_1 generation. Ten L7 and nine L23 plants were compared to 10 vector control plants, and again, no axillary bud primordia formed during vegetative growth in the transgenic plants (Figure 1e). After topping, axillary bud initiation in L7 plants was delayed by 2 weeks. Four weeks post-topping, all L7 plants formed fewer than eight, short suckers (vector control average ~16) with an average weight reduction of ~65% (Figure 1e). Axillary bud initiation was completely abolished in seven L23 plants even 4 weeks post-topping, and the other two plants produced fewer and shorter suckers with a reduced weight (Figure 1e). However, L23 plants were also smaller with thinner leaves than vector controls.

Finally, we conducted a field study (Southern Piedmont AREC, Blackstone, Virginia) with 20 offspring each from three T_1 parents of line L7. The T_2 plants were cultivated from April to September 2019, and a comparative analysis of suckers 2 weeks post-topping revealed that 43% of the transgenic plants and the 10 wild-type controls formed >10 suckers (Figure 1f). However, 45% of the transgenic plants (27 of 60) formed ≤5 suckers and ~12% (7 of 60) formed 6–10 suckers, confirming the stability of the trait even under field conditions.

In summary, we found that the axillary meristem-specific expression of barnase significantly delays and reduces sucker number, length and weight in tobacco after topping, adding to the body of knowledge on the control of axillary branching in plants. For tobacco field cultivation, our results may help to reduce the use of chemicals and the laborious work required for sucker control.

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Conflict of interest

The authors are/were employees or contracted workers of Altria Client Services LLC, which funded the work.

Author contributions

L.G., A.K., C.K., D.X., D.P. and G.N. designed the experiments L.G., J.M., F.B., M.J., Y.S., C.K. and J.Y. conducted the experiments. L.G., A.K., Y.S., C.K. and J.Y.: analysed the data. Y.S., C.K., D.X., J.Y., U.W. and J.S. contributed to reagents, materials and analysis tools. L.G., A.K. and G.N. wrote the manuscript. All authors revised the manuscript and approved the submitted version.

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