Synthesis of Very-Long-Chain Fatty Acids in the Epidermis Controls Plant Organ Growth by Restricting Cell Proliferation

Takashi Nobusawa¹, Yoko Okushima¹, Noriko Nagata², Mikiko Kojima³, Hitoshi Sakakibara³, Masaaki Umeda¹,4*

¹ Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara, Japan, ² Faculty of Science, Japan Women’s University, Bunkyo-ku, Tokyo, Japan, ³ RIKEN Plant Science Center, Tsurumi, Yokohama, Japan, 4 JST, CREST, Ikoma, Nara, Japan

Introduction

The epidermis is formed from the outermost L1 layer in the shoot apical meristem (SAM) and functions as an important interface with the environment. However, recent studies have shown that it also plays an essential role in the establishment and maintenance of the plant body. Arabidopsis mutants with defects in epidermal cell specification exhibit disorganized morphology [1–3]. Biophysical manipulation of the epidermis revealed that it generates mechanical constraints on inner layers, and therefore that shoot growth is controlled by the interaction between the surface (epidermis) and the axis (vasculature) of the plant body.

Abstract

Plant organ growth is controlled by inter-cell-layer communication, which thus determines the overall size of the organism. The epidermal layer interfaces with the environment and participates in both driving and restricting growth via inter-cell-layer communication. However, it remains unknown whether the epidermis can send signals to internal tissue to limit cell proliferation in determinate growth. Very-long-chain fatty acids (VLCFAs) are synthesized in the epidermis and used in the formation of cuticular wax. Here we found that VLCFA synthesis in the epidermis is essential for proper development of Arabidopsis thaliana. Wild-type plants treated with a VLCFA synthesis inhibitor and pasticcino mutants with defects in VLCFA synthesis exhibited overproliferation of cells in the vasculature or in the rib zone of shoot apices. The decrease of VLCFA content increased the expression of IPT3, a key determinant of cytokinin biosynthesis in the vasculature, and, indeed, elevated cytokinin levels. These phenotypes were suppressed in ipt3;5;7 triple mutants, and also by vasculature-specific expression of cytokinin oxidase, which degrades active forms of cytokinin. Our results imply that VLCFA synthesis in the epidermis is required to suppress cytokinin biosynthesis in the vasculature, thus fine-tuning cell division activity in internal tissue, and therefore that shoot growth is controlled by the interaction between the surface (epidermis) and the axis (vasculature) of the plant body.

A characteristic feature of the epidermis is that it is covered with a hydrophobic barrier, the cuticle, which prevents plants from transpiring and protects tissues from pathogen attack [7]. The cuticle is mainly composed of cutin matrix and cuticular wax; cutin is a plant-specific lipid polymer that consists of long-chain fatty acids (LCFAs) with an acyl chain length of 16 or 18 carbons, whereas cuticular wax contains very-long-chain fatty acids (VLCFAs) with fully saturated unbranched hydrocarbon chains (≥20 carbons). Plant VLCFAs are synthesized in the endoplasmic reticulum by sequential addition of 2-carbon moieties to the 18-carbon LCFAs, which is made in the plastid. The carbon donor malonyl-CoA is synthesized from acetyl-CoA by acetyl-CoA carboxylase and used for each cycle of the elongation reaction. VLCFA synthesis consists of four enzymatic steps: (1) condensation of acetyl-CoA with malonyl-CoA catalyzed by ketoacyl-ACP synthase (KCS), (2) reduction of 3-ketoacyl-CoA by 3-ketoacyl-CoA reductase (KCR), (3) dehydration of 3-hydroxyacyl-CoA by 3-hydroxyacyl-CoA dehydratase (HCD), and (4) reduction of enoyl-CoA by enoyl-CoA reductase (ECR). VLCFAs are also

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Abbreviations: CDKA, cyclin-dependent kinase A; CKX, cytokinin oxidase; GUS, β-glucuronidase; HCD, 3-hydroxyacyl-CoA dehydratase; IP, isopenetylamine; IPT, adenosine phosphate-isopentenyltransferase; KCR, 3-ketoacyl-CoA reductase; KCS, ketoacyl-CoA synthase; RZ, rib zone; SAM, shoot apical meristem; tZ, trans-zeatin; VLCFA, very-long-chain fatty acid.
Author Summary

The epidermis functions as an important interface with the environment, but in plants it is also essential for establishing and maintaining the primary plant body. Recent studies have shown that the epidermis participates in both driving and restricting plant growth via inter-cell-layer communication. However, it remains an open question as to whether the epidermis can send signals to internal plant tissues to control cell proliferation during development. Here we report that the synthesis of very-long-chain fatty acids (VLCFAs) in the epidermis is essential for the proper control of cell proliferation in the plant Arabidopsis thaliana. We find that defects in VLCFA synthesis cause cells in the vasculature or in the rib zone of shoot apices to overproliferate. When VLCFA levels decrease, we observe that the synthesis of the phytohormone cytokinin increases in the vasculature. We also find that when cytokinin is degraded by the expression of cytokinin oxidase in the vasculature, enhanced cell proliferation in internal tissues is suppressed, indicating that VLCFA synthesis in the epidermis is required to suppress cytokinin biosynthesis and thus cell overproliferation. Our results demonstrate that shoot growth is controlled by interactions between the surface (epidermis) and the axis (vasculature) of the plant body, and highlight a role for VLCFAs in this interaction.

Results

VLCFA Synthesis in the Epidermis Is Essential and Sufficient for Proper Development

*pas2-1* mutant seedlings exhibit various morphological defects to a variable extent in individual plants; for example, true leaves are fused (Figure 1A) [14,15,17], and hypocotyls are swollen and possess more cortical cell layers (Figure 1B and 1C) [14]. Previous reports also noted that mutant leaves sometimes produce a callus-like structure as a result of increased cell proliferation [14–17]. We therefore observed the SAM, which gives rise to organs like leaves and flowers. We found that more cells accumulate in the rib zone (RZ)—a region below the self-renewing stem cell pool that contributes to the meristem pith—and that the vasculature was disorganized (Figure 1D).

![Image](image.png)

Figure 1. Overproliferation phenotypes of *pas2-1* mutants. (A) True leaf of a 2-wk-old *pas2-1* mutant seedling. (B) 5-d-old wild-type and *pas2-1* seedlings. (C) Cross sections of 5-d-old hypocotyls. (D) Transverse sections of shoot apices of 7-d-old seedlings. Bars, 1 mm (A), 500 µm (B), and 100 µm (C, D).

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We monitored the expression pattern of *PAS2* using the *ProPAS2:β-glucuronidase* (*GUS*) reporter gene (a fusion of the ~2.0-kb *PAS2* promoter and the *GUS* gene). GUS signal was detected in mature embryos, cotyledons and true leaves of seedlings, the inflorescence stem, and pistils and anthers of flowers (Figure S1). In tissue sections, *GUS* expression was observed only in the L1 layer of the SAM and in the epidermis of young leaves and the inflorescence stem (Figure 2A–2C). In situ RNA hybridization also indicated L1-specific expression (Figure 2D). To examine protein-level expression, we generated the *ProPAS2::PAS2-GUS* reporter gene (the same promoter and the full-length *PAS2* coding region fused in-frame to *GUS*); the functionality of the *PAS2-GUS* fusion protein was tested as described below. *GUS* expression was again detected in the L1 layer, and faint expression was noted in the vasculature (Figure 2E).

To test whether *PAS2* expression in the epidermis is necessary and sufficient for normal plant development, we downregulated *PAS2* by RNAi using the *AtML1* promoter, which drives L1-specific expression [18]. The expression level of *PAS2* was reduced in the transgenic plants compared to wild-type (Figure 3A). Although the phenotypes were highly variable, we could find *pas2-l* like phenotypes in eight of the 44 transgenic lines, such as
swollen hypocotyls, fused leaves, and retarded growth (Figure 3B). Moreover, in transgenic lines showing no macroscopic phenotype, we observed overproliferation of vasculature cells and enlargement of the RZ, the latter of which appeared to be mainly due to enhanced cell expansion (Figure 3C). As mentioned above, the ProPAS2:PAS2–GUS reporter gene showed faint expression in the vasculature; thus, to verify that PAS2 expression in the epidermis is sufficient for proper development, we introduced the RNAi construct under the procambial ATHB8 promoter [19]. As a result, no pas2-1-like phenotype was found among 79 transgenic lines, supporting the epidermis-specific role of PAS2 in plant development. We then introduced ProATML1:PAS2–GUS into the pas2-1 mutant. GUS expression was specifically observed in the L1 layer (Figure 3E), and five of the six transgenic lines displayed fully rescued phenotypes (Figure 3D), indicating the functionality of PAS2–GUS. On the other hand, when ProATHB8:PAS2–GUS was introduced into pas2-1, none of the 19 homozygous mutants were rescued. These results demonstrate that VLCFA synthesis in the epidermis is essential and sufficient for proper plant development.

Mild Reduction of VLCFA Content Enhances Cell Proliferation and Promotes Shoot Growth

In pas2-1, the cuticular wax content is severely reduced [13]; it is thus difficult to distinguish the outcome of defective cuticular formation from other effects arising from low VLCFA content. Therefore, we examined dose-dependent phenotypes in wild-type seedlings using the synthetic inhibitor cafenstrole, which blocks the first step of VLCFA elongation reactions by targeting KCS [20]. Our recent study showed that cafenstrole treatment of Arabidopsis seedlings reduced the content of C22 and C24 fatty acids, although our experimental conditions did not allow us to detect C26 or longer fatty acids [21].

Seedlings treated with 3 μM cafenstrole displayed severe growth retardation with swollen hypocotyls and fused leaves, as observed in pas2-1 (Figure 4A and 4B). Those treated with 30 nM cafenstrole did not show growth inhibition, but instead produced larger leaves with thicker hypocotyls (Figure 4A). Measurements of leaf area and cell size showed that, in 12-d-old seedlings, leaf blade...
Figure 4. Phenotypes of cafenstrole-treated seedlings. (A) 12-d-old wild-type seedlings grown in the absence (w/o) or presence of cafenstrole (30 nM or 3 μM). pas2-1 grown in the absence of cafenstrole is shown for comparison. Lower images show the upper region of hypocotyls. (B) Magnified view of a true leaf of 2-wk-old wild-type is shown for comparison. Lower images show the upper region of hypocotyls. (C) Cross sections of 7-d-old hypocotyls grown in the absence (w/o) or presence of 30 nM cafenstrole. (D) Transmission electron microscopy analysis of the L1 layer of the SAM. 3-d-old wild-type seedlings grown in the absence (w/o) or presence of 30 nM or 3 μM cafenstrole were observed. Note that, in pas2-1, the L1 layer is not covered with cuticular wax. Arrows indicate the electron-dense cuticular layer. Bars, 2 mm (A, upper panel), 500 μm (A, lower panel), 1 mm (B), 100 μm (C), and 2 μm (D).

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area increased 1.7-fold after 30 nM cafenstrole treatment (14.4±2.5 mm² for the control and 24.0±5.0 mm² for the cafenstrole treatment; mean ± standard deviation [SD], n=11), whereas cell size did not change significantly (830±55 μm² for the control and 852±158 μm² for the cafenstrole treatment) (Figure S2). Cell number also increased 1.7-fold in cafenstrole-treated leaves (17,265±2,467 for the control and 29,297±6,765 for the cafenstrole treatment) (Figure S2), accounting for the 1.7-fold enlargement of leaf blades. In shoot apices, 30 nM cafenstrole caused more cells to accumulate in the vasculature (Figure 5A), and, as a result, cell number in the vasculature of the hypocotyl dramatically increased (Figure 4C). To examine cell division activity, we used the ProCDKB2;1:NT–GUS reporter (comprising the CDKB2;1 promoter and the first CDKB2;1 exon [NT] fused in-frame to GUS), which monitors mitotic cells during the G2 and M phases [22]. The number of GUS-stained cells increased when the cafenstrole concentration was elevated, especially in the region along the vasculature (Figure 5A). These results demonstrate that a mild reduction of VLCFA content (with 30 nM cafenstrole or in PAS2 RNAi plants) enhances proliferation of vasculature cells, while a severe reduction (with 3 μM cafenstrole or in pas2-1) causes overall growth retardation with impaired cuticular formation, as described below.

Transmission electron microscopy revealed that an electron-dense cuticular layer disappeared in pas2-1, and that only a trace of cuticular layer was formed in wild-type seedlings treated with 3 μM cafenstrole (Figure 4D). On the other hand, a thicker cuticle was formed in the presence of 30 nM cafenstrole (Figure 4D); thus, it is unlikely that cell proliferation was enhanced as a consequence of reduced cuticle synthesis. This idea is supported by the observation that Arabidopsis mutants specifically impaired in cuticular formation did not display enhanced cell proliferation, as described later. Moreover, the expression of the L1-specific reporter ProPDF1:GUS [23] retained its L1 specificity in pas2-1 (Figure S3A), indicating that epidermal identity is maintained under low-VLCFA conditions.

Low VLCFA Content Increases Cytokinin Level and Enhances Cell Proliferation

We quantified phytohormone content, and found that levels of the cytokinins isopentenyladenine (iP) and trans-zeatin (zI), and of their ribosylated and phosphorylated precursors (iPR, iPRPs, zZR, and zZRPs), increased in pas2-1 and in wild-type treated with 30 nM or 3 μM cafenstrole (Table 1). This indicates that active cytokinins are highly synthesized in pas2-1 and after cafenstrole treatment. Indeed, expression of the primary cytokinin response marker ARABIDOPSIS RESPONSE REGULATOR 6 (ARR6) [24] was stimulated by cafenstrole treatment in vascular bundles (Figure 5B and 5C). Moreover, 30 nM cafenstrole did not enlarge leaves, but instead slightly reduced the cell number and the leaf size, in ipf5;5;7 triple mutants, in which cytokinin levels are severely decreased because of defects in cytokinin biosynthetic isopentenyltransferases (Figure 6) [25]. This finding suggests that cytokinin is associated with the cafenstrole-induced activation of cell division. A higher level of cytokinin would also explain the previously observed hypersensitivity of pas2-1 to cytokinin treatment [14,17]. On the other hand, the content of indoleacetic acid (IAA) and gibberellins (GA1 and GA4) did not increase, except that IAA became elevated in the presence of 3 μM (but not 30 nM) cafenstrole (Table 1).

To further examine whether low VLCFA content is responsible for higher cytokinin level and enhanced cell proliferation, we next used Arabidopsis mutants with defects in LCFA and VLCFA synthesis (Figure 7A). In mutants of PAS3 and PAS1, which encode acetyl-CoA carboxylase and a scaffold protein for the elongase complex, respectively, VLCFA content is dramatically reduced and, as a result, organ growth is severely inhibited [14,26–28]. As observed in pas2-1, these mutants contained higher amounts of iZ and iP compared to wild-type, and more cells accumulated in the RZ (Figure 7B and 7C), indicating an enhancement of cell division. A recent report demonstrated that glossyhead1 (gsd1), another mutant allele for PAS3, did not show severe growth inhibition [29]. However, overproliferation of vasculature cells was observed in the shoot apex of gsd1, as in the case of 30 nM

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cafenstrole treatment and PAS2 RNAi plants (Figure S4). FIDDLEHEAD (FDH)/KCS10 encodes one of the 21 KCSs in *Arabidopsis*, and is thus associated with VLCFA synthesis; however, in the *fdh-13* mutant [2], only a mild leaf phenotype and a small reduction in C24 fatty acids were reported, probably due to redundancy in KCS genes [30]. Correspondingly, we detected a small increase of tZ level in seedlings, and a mild enhancement of cell proliferation in the RZ, but these phenotypes were less

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**Figure 5. Reduced VLCFA synthesis increases the expression of CDKB2;1 and ARR6.** (A) Transverse sections of shoot apices of 5-d-old seedlings expressing ProCDKB2;1:NT–GUS. pas2-1 grown in the absence of cafenstrole is shown for comparison. (B, C) Expression patterns of ProARR6:GUS. Aerial parts of 5-d-old seedlings grown in the absence (w/o) or presence of cafenstrole (30 nM or 3 μM) (B) and transverse sections of shoot apices (C). Bars, 100 μm (A, C), and 1 mm (B).

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**Table 1. Quantification of phytohormones.**

| Hormone | 3 DAG (pmol/g Fresh Weight) | 5 DAG (pmol/g Fresh Weight) |
|---------|-----------------------------|-----------------------------|
|         | w/o | 30 nM | 3 μM | pas2-1 | w/o | 30 nM | 3 μM | pas2-1 |
| tZ      | 1.08±0.22 | 2.22±0.07 | 2.31±0.25 | 6.36±0.85 | 0.81±0.22 | 0.95±0.10 | 1.44±0.24 | 4.49±0.24 |
| tZR     | 2.50±0.36 | 36.69±2.68 | 29.17±0.97 | 118.64±18.09 | 1.63±0.38 | 6.49±1.17 | 19.66±2.07 | 108.23±15.53 |
| tZRP    | 33.64±4.72 | 221.40±11.07 | 189.47±28.32 | 245.46±17.34 | 19.33±1.81 | 71.06±9.71 | 143.57±7.89 | 363.67±31.70 |
| iP      | 0.79±0.18 | 2.38±0.15 | 2.47±0.20 | 1.76±0.31 | 0.57±0.09 | 1.44±0.23 | 2.85±0.34 | 1.70±0.03 |
| iPR     | 0.25±0.03 | 1.76±0.11 | 1.45±0.06 | 2.14±0.24 | 0.17±0.01 | 0.74±0.08 | 1.78±0.15 | 3.13±0.59 |
| iPRP    | 41.81±5.18 | 353.11±25.27 | 275.74±35.66 | 74.99±4.83 | 24.33±1.22 | 147.64±11.40 | 390.17±49.42 | 155.17±14.60 |
| IAA     | 612.0±104.3 | 741.7±100.0 | 909.9±89.2 | 481.6±283.8 | 731.3±72.1 | 780.0±94.6 | 1,546.4±273.0 | 624.9±115.5 |
| GA1     | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| GA4     | 0.79±0.14 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

Amounts of phytohormones were measured in 3- and 5-d-old wild-type seedlings grown in the absence (w/o) or presence of cafenstrole (30 nM or 3 μM). *pas2-1* was grown without cafenstrole. Data are presented as mean ± SD (n=3).

DAG, days after germination; IAA, indoleacetic acid; n.d., not detected.
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Figure 6. Cafenstrole-induced leaf expansion is suppressed in the \textit{ipt3;5;7} mutant. (A) 11-d-old seedlings of wild-type and the \textit{ipt3;5;7} triple mutant grown in the absence (−) or presence (+) of 30 nM cafenstrole. Bar, 5 mm. (B) First leaves of 11-d-old seedlings grown in the absence (white bars) or presence (green bars) of 30 nM cafenstrole were measured for leaf blade area, cell area, and cell number. Data are presented as mean ± SD (n≥13). Significant differences between non-treatment and 30 nM cafenstrole treatment were determined by Student’s t-tests: ***, p<0.001; the other differences are not significant (p>0.05).

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prominent than those in \textit{pas} mutants (Figure 7B and 7C). Note that the RZ in the control (\textit{Le}) was already larger than that in Col-0 (Figure 7C). We also found a similar trend in the leaky mosaic death\textit{1} (\textit{mod1-1}) mutant, in which the activity of the LCFA-synthesizing enzyme enoyl-ACP reductase was reduced by half (Figure 7A) [31]. Although LCFA and VLCFA content in \textit{mod1-1} have not been reported so far, we noticed that tZ and iP levels increased slightly and that cell proliferation was enhanced in the RZ (Figure 7B and 7C). This suggests that VLCFA content might be reduced as a result of decreased LCFA synthesis, but not as severely as in \textit{pas} mutants, leading to modest effects on cytokinin level and cell division. The above results indicate that VLCFA synthesis in the epidermis is responsible for suppressing cytokinin biosynthesis and cell proliferation. We also observed three mutants with defects in cuticular wax formation from VLCFAs, \textit{cer4-1}, \textit{wax2}, and \textit{mah1-3}, which display impaired synthesis of primary alcohols, aldehydes, and secondary alcohols and ketones, respectively (Figure 7A) [11,32,33]. However, we found neither an increase of cytokinin level nor an enhancement of cell proliferation in these mutants (Figure 7B and 7C).

VLCFA Synthesis Is Required for Suppression of Overproliferation by Repressing Cytokinin Biosynthesis in the Vasculature

To identify the cause of higher cytokinin production under low-VLCFA conditions, we conducted microarray analyses and examined the expression levels of cytokinin biosynthesis genes. (Microarray data have been deposited in the ArrayExpress database under accession number E-MEXP-3315.) In \textit{pas2-1}, the mRNA levels of \textit{IPT3} and \textit{CYP735A2} were 3.9- and 6.6-fold higher, respectively, than in wild-type (Table S1). \textit{IPT3} encodes one of the nine adenosine phosphate-isopentenyltransferases (IPTs), which catalyze the first and rate-limiting step of cytokinin biosynthesis to produce isopentenyladenine riboside phosphates (iPRPs) [34]. CYP735A2 converts iPRPs to \textit{trans}-zeatin riboside phosphates (tZRPs) [35].

We examined cytokinin levels and \textit{IPT3} expression in the \textit{pas2-1} mutant carrying \textit{ProATML1:PAS2–GUS}, which rescued \textit{pas2-1} phenotypes (Figure 3D). As described above, levels of tZ and iP were highly elevated in \textit{pas2-1} compared to those in wild-type (Table 1), but in \textit{pas2-1} carrying \textit{ProATML1:PAS2–GUS}, no such increase of cytokinin content was detected (tZ, 0.57±0.06 pmol/g fresh weight for Col-0 and 0.71±0.13 pmol/g for the transgenic line; iP, 0.49±0.03 pmol/g for Col-0 and 0.51±0.03 pmol/g for the transgenic line; mean ± SD, 7-d-old seedlings [n=3]).

The elevated level of \textit{IPT3} transcripts in \textit{pas2-1} was also reduced to the wild-type level by \textit{PAS2–GUS} expression in the epidermis (the relative mRNA level, with that for wild-type set to 1, was 3.77±0.15 for \textit{pas2-1} and 0.81±0.09 for \textit{pas2-1} carrying \textit{ProATML1:PAS2–GUS}; mean ± SD, 7-d-old seedlings [n=3]). These results indicate that VLCFA synthesis in the epidermis is required to suppress not only cytokinin biosynthesis but also \textit{IPT3} expression.

We then monitored \textit{IPT3} expression in 3-d-old seedlings using the \textit{promoter:GUS} reporter. Consistent with a previous observation of \textit{IPT3} expression in the phloem [19], we detected the GUS signal in vascular bundles (Figure 8). Cafenstrole treatment increased the intensity of the GUS signal and extended the expression domain in shoot apices and leaves; a similar expression pattern was also observed in \textit{pas2-1} (Figure 8A and 8B). In cafenstrole-treated leaves, expression of the procambial marker \textit{ProATHB8:GUS} [21] was restricted to vascular bundles (Figure 8C). This indicates that low-VLCFA conditions increase \textit{IPT3} expression in the vasculature and cause ectopic expression in non-vascular cells.

To examine whether increased cytokinin synthesis is a cause or a consequence of the overproliferation phenotype, we monitored \textit{IPT3} expression...
expression after transfer of 3-d-old seedlings to a medium containing 30 nM or 3 μM cafenstrole. We also observed the ProCYCB1;2:NT–GUS reporter (comprising the CYCB1;2 promoter and the N-terminal region of CYCB1;2 [VT fused in-frame to GUS]), which monitors G2/M phase cells [36]. As shown in Figure S5, higher IPT3 expression was noted after 6 h and 12 h for 3 μM and 30 nM cafenstrole, respectively, compared with the non-treated control. By contrast, CYCB1;2 expression increased from 12 to 24 h in the SAM and in young true leaves regardless of cafenstrole treatment (Figure S5), suggesting a general activation of cell division at this developmental stage. Expression was even higher after 48 h for both 30 nM and 3 μM cafenstrole, but not for the control (Figure S5), demonstrating that cafenstrole-induced overproliferation occurred later than 24 h. Measurement of cytokinin content revealed that cytokinin precursors, especially iPR and iPRPs, increased after 6 to 12 h of 3 μM cafenstrole treatment, and that iP and tZ increased after 24 h (Table S2). These results indicate that cafenstrole induces cytokinin synthesis, which is then followed by activation of cell division, implying that enhanced cytokinin synthesis is the cause of overproliferation triggered by low-VLCFA conditions.
The above results suggested an interesting hypothesis, namely, that VLCFA synthesis in the epidermis is required to confine cytokinin biosynthesis to the vasculature and prevent cells from overproliferating. To test this hypothesis, we examined whether the effect of cafenstrole is suppressed by reducing cytokinin levels. We expressed the gene for Venus-fused cytokinin oxidase 1 (CKX1), which degrades active forms of cytokinins [37], under the control of ATML1 and ATHB8 promoters. Venus fluorescence showed that the ATML1 and ATHB8 promoters conferred epidermis- and vasculature-specific expression, respectively (Figure S6A and S6B). We measured leaf area in four independent lines for each promoter construct, and found that 30 nM cafenstrole enlarged leaves in wild-type and ProATML1:CKX1–Venus, but that no such enlargement occurred in ProATHB8:CKX1–Venus (Figures 9A, 9B, and S6C). The latter effect was due to the suppression of cafenstrole-induced enhancement of cell proliferation in leaves (Figure 9B). Enhanced cell accumulation in the vasculature, expansion of hypocotyl width at the base of cotyledons, and an increase in vascular cell number in hypocotyls were also suppressed by CKX1–Venus expression in vascular bundles (Figures 9C, S6D, and S6E). We also expressed CKX1–Venus in pas2-1, but the macroscopic phenotype of the mutant was not suppressed with either promoter, probably owing to severely impaired cuticular formation. However, when shoot apices were observed microscopically, we found that the enhanced cell accumulation in the RZ and disorganization of the vasculature were partially suppressed with ProATHB8:CKX1–Venus, but not with ProATML1:CKX1–Venus in pas2-1 (Figure 9D). These results indicate that, under low-VLCFA conditions, an increase of cytokinin biosynthesis in the vasculature is the major cause of overproliferation. The inability of ProATML1:CKX1–Venus to suppress overproliferation suggests that non-cell-autonomous factors (other than cytokinins) act from mesophyll cells to the epidermis to promote cell division, as reported previously [38].

Discussion

In this study, we showed that a higher concentration of cafenstrole (3 μM) caused severe growth defects, notably swollen hypocotyls and fused leaves, which are similar to those observed in the leaky pas2-1 mutant. More cells accumulated in the RZ of pas2-1, and the number of cortical cell layers increased in the hypocotyl. In contrast, at a lower concentration (30 nM), seedlings showed neither overall growth inhibition nor organ fusions; rather, the leaves were enlarged due to increased cell number. Enhanced cell proliferation was also observed in the vasculature of shoot apices, resulting in a dramatic increase of cell number in the vasculature of hypocotyls. When PAS2 expression was specifically downregulated in the epidermis, we could again observe disorganized vasculature due to enhanced cell proliferation. It is likely that, in pas2-1 and in wild-type plants treated with 3 μM cafenstrole, the stimulatory effect on cell division might be difficult to observe macroscopically, except for the swollen hypocotyl, due to impaired cuticular formation and consequent growth defects. However, a common feature was observed following mild or severe inhibition of VLCFA synthesis, namely, enhanced cell proliferation in the vasculature or in the RZ, respectively. It is noteworthy that, in pas2-1, cell accumulation was prominent in the RZ but not in the vasculature. One possible explanation for this observation is that the amount of cytokinin in the vasculature may be so high that cell division is actually inhibited. The lower level of cytokinin in the RZ than in the vasculature may efficiently enhance cell proliferation. It is also probable that faster accumulation of RZ cells in pas2-1 suppresses cell division in the
Figure 9. Vasculature-specific expression of CKX1 suppresses cafenstrole-induced enhancement of plant growth. (A) 8-d-old seedlings of wild-type, ProATML1:CKX1–Venus and ProATHB8:CKX1–Venus grown in the absence (−) or presence (+) of 30 nM cafenstrole. (B) First leaves of 12-d-old seedlings of wild-type, ProATML1:CKX1–Venus and ProATHB8:CKX1–Venus grown in the absence (white bars) or presence (green bars) of 30 nM cafenstrole were measured for leaf blade area, cell area and cell number. Data are presented as mean ± SD (n=11). Significant differences between non-treatment and 30 nM cafenstrole treatment were determined by Student’s t-tests: ***, p<0.001; the other differences are not significant (p>0.05). (C, D) Transverse sections of shoot apices. 5-d-old seedlings of wild-type, ProATML1:CKX1–Venus and ProATHB8:CKX1–Venus grown in the absence (−) or presence (+) of 30 nM cafenstrole (C), and 7-d-old seedlings of wild-type, pas2-1, and pas2-1 expressing ProATML1:CKX1–Venus or ProATHB8:CKX1–Venus (D). CKX1–Venus was introduced into the heterozygous pas2-1 mutant, and homozygous plants were isolated for observation of shoot apices. Bars, 5 mm (A) and 100 μm (C, D).

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vasculature by intertissue communication. Further studies are needed to examine such possibilities.

PAS2 has been identified as an antiphosphatase, which interacts with tyrosine-phosphorylated cyclin-dependent kinase A (CDKA) and prevents it from being dephosphorylated and activated [39]. However, in a pas2 mutant carrying phosphomimic mutations in CDKA, the phenotype of phosphomimic CDKA plants was not epistatic to the pas2 phenotype; rather, the two phenotypes were additive, indicating that PAS2 functions in parallel to CDKA [40]. This is supported by the fact that PAS2 is exclusively localized to the endoplasmic reticulum, whereas CDKA is distributed in both the nucleus and the cytoplasm [13,41]. Here, we observed enhanced cell proliferation in the RZ or in the vasculature not only in pas2, but also in other VLCFA-related mutants, such as pas1, pas3, gdh1, and fdlh, as well as by treatment with the KCS inhibitor cafenstrole. Therefore, the overproliferation phenotype is not specifically linked to PAS2 functions, but instead is caused by inhibition of VLCFA synthesis.

We revealed that, in pas2-1 and under cafenstrole treatment, IPT3 expression in the vasculature was elevated and its domain expanded to the spongy mesophyll cells. Indeed, the content of active cytokinins increased prior to the activation of cell division, whereas the overproliferation phenotype was suppressed in ipt3;5;7 mutants and by vasculature-specific degradation of cytokinins. These results demonstrate that VLCFA synthesis in the epidermis confines cytokinin biosynthesis to the vasculature and restricts cell proliferation. The idea that IPT3 is a possible target of epidermis-derived signals is supported by a previous report that overexpression of IPT3 in Arabidopsis resulted in a 3.4-fold increase of cytokinin content, and enlarged leaves with increased cell number [42]. However, it is also likely that CYP733A2, whose expression increased 6.6-fold in pas2-1, is another target. In Arabidopsis and rice plants, impairment of VLCFA synthesis elevates the expression of KNOTTED-like homeobox (KNOX) genes [17,43]. Moreover, overexpression of class I KNOX (KNOXI) genes is known to promote cytokinin synthesis in Arabidopsis [44]. However, it is unlikely that VLCFA synthesis in the epidermis restricts the cytokinin level by controlling KNOXI expression, because we observed overproliferation not only in the SAM but also in leaves, where KNOXI genes are not expressed. It is known that cytokinin induces expression of the KNOXI genes KNAT1/BP and STM [45], suggesting that KNOX upregulation under low-VLCFA conditions results from increased cytokinin synthesis in the shoot apex. Our results indicate that epidermis-derived signals fine-tune cell division activity in internal tissue, suggesting that shoot growth is controlled by the interaction between the surface (epidermis) and the axis (vasculature) of the plant body (Figure 10). Indeed, perturbing this regulation by lowering VLCFA synthesis increased leaf size, demonstrating that non-autonomous signals are essential to restrict organ size.

Arabidopsis mutants with defects in cuticular wax formation from VLCFAs (cer4, mah1, wax2) did not exhibit phenotypes similar to those observed in pas mutants or cafenstrole-treated wild-type plants. Although we cannot exclude the possibility that some level of wax classes synthesized in these mutants suppresses the overproliferation phenotype, it is more likely that VLCFA derivatives function as signaling molecules to control cytokinin biosynthesis and cell division (Figure 7A). In yeast and animals, sphingolipids made from VLCFAs act as signaling molecules controlling cell proliferation, cell death and stress responses [8]. Although Arabidopsis mutants defective in sphingolipid biosynthesis are impaired in cell growth, and in severe cases die [46,47], some types of sphingolipids may control cell division by affecting cytokinin synthesis. It is also possible that VLCFA-containing lipids may function as mediators or ligands that control gene transcription, as suggested in mammals, yeast, and bacteria [48]. Indeed, arachidonic acid is known to induce stress-related gene expression and elicit defense signaling in Arabidopsis [49]. It is also likely that some metabolites, whose levels change depending on VLCFA synthesis, confine cytokinin biosynthesis to the vasculature.

In Arabidopsis, 21 genes have been identified for KCS, which catalyzes the first step of VLCFA elongation reactions. Some of them are expressed predominantly in the epidermis, as observed for PAS2 [50]. Indeed, PAS2, KCS11, KCS16, and KCS20 have one L1-box, and KCS6, KCS9, KCS10/FDH, and KCS18 have two L1-boxes, in their promoter regions, at which transcription factors ATML1 and PDF2 bind and control the L1/epidermis-specific gene expression [1]. Four kcs mutants, kcs6, kcs10/fdh, kcs2, and kcs20, exhibit a glossy appearance and/or organ fusion, but no overproliferation phenotype was described [51–54]. However, we observed mildly enhanced cell proliferation in the RZ of fdlh-13,
suggested that other kcs mutants may also accumulate more cells in the RZ (or in the vasculature) than wild-type plants. Alternatively, specific VLCFAs may be required to suppress cell proliferation, because the distribution of various VLCFA species may depend on the substrate preference of each KCS (e.g., for a particular carbon chain length). HCD is unlikely to have such substrate preference, as it is encoded by the single-copy gene PAS2 in Arabidopsis; thus, the pas2 mutation reduces the overall level of VLCFAs including those that are responsible for suppressing cell proliferation. Further studies will reveal which VLCFA species or VLCFA-containing lipids are associated with non-autonomous signals to suppress cell proliferation in tissues.

Previously, cyclin-dependent kinase inhibitor genes were ectopically expressed in the L1 layer, and meristem organization was investigated [55]. Cell number in the epidermis was reduced, while that in the cortex and mesophyll was the same as in wild-type. These and our present observations indicate that cell proliferation in shoot growth is not coordinated between the L1 and the inner layers; rather, it is controlled by VLCFA-derived signals that act on cytokinin biosynthesis in the vasculature. A likely benefit of this system is that plants can coordinate cuticular wax formation and organ growth, and can thus maintain proper development under various environmental conditions. Several KCS genes are induced by abiotic stress, such as salt, dehydration, and osmotic stress [50]; the transcription factor MYB30, which activates expression of VLCFA biosynthesis genes, is also induced by pathogen infection [56]. Therefore, plants may deploy mechanisms to actively form cuticular wax and suppress cell proliferation to minimize energy consumption under stressful conditions. It is also known that BES1, a downstream transcription factor in the brassinosteroid signaling pathway, directly interacts with and activates MYB30 [56,57]. While BES1 is involved in proliferation to minimize energy consumption under stressful conditions at 23°C, 2% (w/v) sucrose, and 0.8% agar (pH 6.3) under continuous light conditions, and diluted 1,000-fold into the media.

Histological Analysis

GUS staining and tissue sectioning were performed as described previously [62]. For counter-staining, sections were incubated with 0.05% (w/v) toluidine blue O. In the case of GUS-stained samples, sections were incubated with 0.05% (w/v) ruthenium red.

In Situ RNA Hybridization

Arabidopsis tissues were fixed in FAA (50% [v/v] ethanol, 5% [v/v] acetic acid, and 3.7% [v/v] formaldehyde), and 8-μm paraffin sections were hybridized with digoxigenin-labeled probes according to the protocol from the manufacturer (Roche). The PAS2 probe was the antisense strand corresponding to the region 6 to 506 of the PAS2 ORF.

Microscopy Observation

For measurements of leaf blade area, healthy first leaves were harvested and fixed in a solution of 2.5% glutaraldehyde, and stored at 4°C. The area of edited microscopic images was measured using the image analysis program NIH ImageJ 1.3u (http://rsb.info.nih.gov/nih-image/). To measure cell size and cell number, data were collected by scanning images of the abaxial epidermis located at 50% of the distance between the tip and the base of the leaf blade, halfway between the midrib and the leaf margin. Images that included at least 40 cells in focus were edited

Plasmid Construction for Plant Transformation

The 2-kb promoter fragment of PAS2 was PCR-amplified and cloned into the SfI-BamHI site of the pBI101.2 binary vector (Clontech Laboratories) to generate a fusion construct with GUS (ProPAS2:GUS). The promoter and the coding region of PAS2 were PCR-amplified from genomic DNA and cloned into the Gateway entry vector pDONR221 (Invitrogen) by a BP reaction. An LR reaction was performed with the destination vector pGWB3 [58] to generate a binary vector carrying the fusion construct with GUS (ProPAS2:GUS). The 3.4-kb promoter fragment of ATML1 and the coding region of PAS2 were PCR-amplified from genomic DNA and cloned into the EcoRI and Smal sites, respectively, of the pBluescript II KS(−) vector (Stratagene). The resultant plasmid was digested with BamHI and HindIII, and the fragment was cloned into the HindIII-BamHI site of the pBl101 binary vector (Clontech) to generate ProATML1:GUS. To make the PAS2 RNAi construct, the region encompassing nucleotides 6 to 641 of the PAS2 ORF was cloned into the EcoRI-KpnI and BamHI-HindIII sites of the pHANNIBAL vector [59]. The 358 promoter region in the vector was then replaced by the 3.4-kb ATML1 promoter, and the resultant ProATML1:GUS RNAi construct was cloned into the NotI site of the pART27 binary vector [60]. To express the CKX1–Venus fusion protein, the ORF of Venus and the genomic fragment comprising the coding region of CKX1 were PCR-amplified and tandemly cloned into the Smal site of pAN19, a derivative of the pUC19 vector (Invitrogen), to be in-frame with each other. The resultant CKX1–Venus construct was then PCR-amplified and cloned into pDONR221 by a BP reaction. The 3.4-kb and 1.7-kb promoter fragments of ATML1 and ATBB8, respectively, were PCR-amplified and cloned into the Gateway entry vector pDONR4-P1R (Invitrogen) by a BP reaction. An LR reaction was conducted with the destination vector pGWB501 [61] and the above-mentioned entry vectors to generate a binary vector carrying each promoter fragment fused to CKX1–Venus. To express ProPAS2:GUS and the PAS2 RNAi construct under the ATBB8 promoter, the fragments of ProPAS2:GUS and the PAS2 RNAi construct were PCR-amplified using the above-mentioned binary vectors with the ATML1 promoter, and cloned into pDONR221 by a BP reaction. These entry clones were used for an LR reaction with the destination vector pGWB501 and the entry vector pDONR4-P1R carrying the ATBB8 promoter fragment. Primers used for plasmid constructions are listed in Table S3.
using Photoshop Elements 6 [Adobe, http://www.adobe.com/]. Epidermal cells in the edited image were counted, and the area of the edited image was measured with ImageJ. The average cell area was determined on the basis of these measurements. The total number of epidermal cells on the abaxial side was estimated on the basis of the average cell area and leaf blade area. For detection of Venus fluorescence, plant seedlings were embedded in 7% agarose and sliced manually with a razor, and sections were observed with a confocal laser scanning microscope (LSM710; Carl Zeiss).

Transmission Electron Microscopy

Samples were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.0) at 4°C overnight, and then postfixed with 1% osmium tetroxide in the same buffer at 4°C for 1 h. Fixed samples were dehydrated in an ethanol series and embedded in Spurr resin, and polymerized at 73°C. Ultrathin sections were prepared with a diamond knife, stained with uranyl acetate and lead citrate, and observed with a JEOL 1200EX microscope.

Quantification of Phytohormones

Sampling of about 100 mg of fresh whole seedlings was repeated three times. Extraction and determination of hormones were performed as described previously [63]. Data were processed by MassLynx software with QuanLynx (version 4.0, Waters).

Microarray Analysis

Total RNA was extracted from 3-d-old whole seedlings using TRIzol (Invitrogen) and purified with an RNeasy microkit (Qiagen) as described in the manufacturer’s instructions. GeneChip analyses were independently performed twice with the Arabidopsis ATH1 Genome Array (Affymetrix) as described in the GeneChip Expression Analysis Technical Manual (Affymetrix). Probe synthesis was performed with the GeneChip 3' IVT Express kit (Affymetrix) following the manufacturer’s protocol. Hybridization and washes were performed as described in the GeneChip Expression Analysis Technical Manual. Signal detection and global normalization were performed using GeneChip Operating Software (Affymetrix; version 1.4) with standard parameters.

Supporting Information

Figure S1 Expression pattern of Pas2. GUS staining of transgenic plants carrying ProPas2:GUS. Mature embryo (A), 5-d-old seedling (B), 8-d-old seedling (C), inflorescence (D), and flowers and anthers (E). Bars, 100 μm (A), 1 mm (B, E), and 2 mm (C, D). (TIF)

Figure S2 Kinematic analysis of leaf growth. First leaves of wild-type seedlings grown in the absence (w/o) or presence of 30 nM cafenstrole were measured for leaf blade area, cell area, and cell number per leaf. Data are presented as mean ± SD (n=10). DAG, days after germination. (TIF)

Figure S3 Reduced VLCFA synthesis does not affect the expression patterns of PDF1 and ATTHB8. (A) Expression pattern of ProPDF1:GUS in wild-type and pas2-1. Transverse sections of shoot apices of 5-d-old seedlings. (B, C) Expression pattern of ProATTHB8:GUS. 5-d-old wild-type seedlings grown in the absence (w/o) or presence of cafenstrole (30 nM or 3 μM) (B) and cross sections of cotyledons (C). pas2-1 grown in the absence of cafenstrole is shown for comparison. Bars, 50 μm (A), 1 mm (B), and 100 μm (C). (TIF)

Figure S4 Enhanced cell proliferation in the gsd1 mutant. Transverse sections of shoot apices of 7-d-old Col-0 and gsd1 seedlings. Bar, 100 μm. (TIF)

Figure S5 IPT3 and CYCB1;2 expression pattern after cafenstrole treatment. 3-d-old seedlings carrying ProIPT3:GUS or ProCYCB1;2:NT–GUS were transferred onto a medium without cafenstrole (w/o), or containing 30 nM or 3 μM cafenstrole, and GUS expression was observed at the indicated time points thereafter. Enlarged images of ProCYCB1;2:NT–GUS seedlings after 48 h are shown below (a–c). Bars, 1 mm and 500 μm (a–c). (TIF)

Figure S6 Vasculature-specific expression of CKXI suppresses the increase of hypocotyl width caused by cafenstrole treatment. (A, B) Expression patterns of CKXI–Venus controlled by the ATML1 (A) and ATTHB8 (B) promoters. Transverse sections of shoot apices (left image of A) and B cross section of a first leaf (right image of A). Venus fluorescence was merged with autofluorescence. Asterisks indicate the SAM. (C) First leaves of 10-d-old seedlings of wild-type, ProATML1: CKXI–Venus and ProATTHB8:CKXI–Venus grown in the absence (white bars) or presence (green bars) of 30 nM cafenstrole were measured for leaf blade area. For each promoter construct, three independent lines, which are different from those shown in Figure 9, were used for measurement. Data are presented as mean ± SD (n=20). (D) Measurement of hypocotyl width. Hypocotyls of 8-d-old seedlings grown in the absence (−) or presence (+) of 30 nM cafenstrole were measured. Data are presented as mean ± SD (n=20). Significant differences between wild-type and CKXI–Venus transgenic seedlings were determined by Student’s t-test: ***, p<0.001; the other differences are not significant (p>0.05). (E) Cross sections of 5-d-old hypocotyls grown in the absence (−) or presence (+) of 30 nM cafenstrole. Bars, 50 μm (A), 20 μm (B), and 100 μm (E). (TIF)

Table S1 Expression levels of cytokinin biosynthesis genes in the pas2-1 mutant. Average values of biological duplicates in microarray analysis are shown as relative values, with those for wild-type set to 1. (DOCX)

Table S2 Cytokinin contents after cafenstrole treatment. 3-d-old wild-type seedlings were transferred onto a medium without cafenstrole (w/o), or containing 30 nM or 3 μM cafenstrole, and measured for cytokinin contents after indicated time points. Data are presented as mean (pmol/g fresh weight) ± SD (n=3). (DOCX)

Table S3 Primers used for plasmid constructions. CDS (coding sequence) was PCR-amplified from the genomic DNA. (DOCX)

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Author Contributions

The author(s) have made the following declarations about their contributions: Prepared data for submission to databases and did the submission: TN. Conceived and designed the experiments: TN YO HS MU. Performed the experiments: TN NN MK. Analyzed the data: TN HS. Contributed reagents/materials/analysis tools: NN MK HS. Wrote the paper: TN YO MU.
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