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Report

A Local Maximum in Gibberellin Levels Regulates Maize Leaf Growth by Spatial Control of Cell Division

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Summary

Plant growth rate is largely determined by the transition between the successive phases of cell division and expansion [1]. A key role for hormone signaling in determining this transition was inferred from genetic approaches and transcriptome analysis in the *Arabidopsis* root tip [2–5]. We used the developmental gradient at the maize leaf base as a model to study this transition, because it allows a direct comparison between endogenous hormone concentrations and the transitions between dividing, expanding, and mature tissue. Concentrations of auxin and cytokinins are highest in dividing tissues, whereas bioactive gibberellins (GAs) show a peak at the transition zone between the division and expansion zone. Combined metabolic and transcriptomic profiling revealed that this GA maximum is established by GA biosynthesis in the division zone (DZ) and active GA catabolism at the onset of the expansion zone. Mutants defective in GA synthesis and signaling, and transgenic plants overproducing GAs, demonstrate that altering GA levels specifically affects the size of the DZ, resulting in proportional changes in organ growth rates. This work thereby provides a novel molecular mechanism for the regulation of the transition from cell division to expansion that controls organ growth and size.

Results and Discussion

Growth-Related Hormones Are Differentially Distributed within the Growth Zone

The maize leaf is a promising model for investigating the molecular mechanisms controlling growth, because of the linear organization of cell division and expansion along its longitudinal axis. The division zone (DZ) encompasses several centimeters at the leaf base where cell division takes place, whereas the expansion zone (EZ) is located in the adjacent centimeters [6], a separation that allows molecular characterization with a subzonal resolution [7]. The boundary between the DZ and EZ can be localized by the identification of the most distal mitotic events in DAPI stained samples (Figures S1A and S1B available online) [8] and the expression levels of mitosis-specific genes such as B-types cyclin-dependent kinases (CDKs) and B-type cyclins [9]. Plant hormones are proposed to play an important role in the spatial control of the division and expansion process [4], but the experimental evidence has been limited to expression analysis of hormone-responsive genes [10] and the effects of mutations [11].

To gain deeper insight in the role of plant hormones in organ growth control, we aimed to correlate endogenous plant hormone concentrations with the spatial distribution of cell division and expansion in the growing maize leaf (Figure 1A). The concentrations of auxin (IAA), cytokinins (CKs), the brassinosteroid castasterone (CS), and gibberellins (GAs) varied significantly across the developmental gradient ($p < 0.05$, $n = 5$) (Figure 1B–1D; Figure S1C). Endogenous concentrations of IAA and CKs were the highest at the leaf base and decreased to a basal level at the distal boundary of the DZ (Figures 1B and 1C). CS could not be measured at the same resolution, but its concentration was also higher in the DZ compared to the EZ and mature zone (MZ) (Figure S1C). Remarkably, bioactive GAs (GA_1 and GA_4) peaked at the transition between the DZ and the EZ (Figure 1D).

The levels of stress-related hormones [12], abscisic acid, jasmonic acid, and salicylic acid did not differ significantly along the leaf (Figure S1C). These data indicate that the stress-related hormones are unlikely to regulate developmental transitions but reflect a broader role involving the inhibition of cell division [13, 14], cell expansion [12, 15], and stomatal closure in the mature tissue [16].

The Interplay between GA Biosynthesis and Degradation Results in a Narrow Accumulation Peak of GA

To elucidate the mechanism that causes the remarkable accumulation of GA_1 at the transition, we measured the endogenous concentrations of GA-biosynthetic and catabolic intermediates [17], as well as transcript levels of GA biosynthesis and catabolism genes along the growth zone in B73 leaves (Figure S2). The earliest intermediate measured, GA_{12}, was constant over the growth zone, whereas the subsequent GA-intermediates, GA_{53}, GA_{44}, and GA_{19}, resulting from GA13- and GA20-oxidation of GA_{12}, showed a pattern similar to that of the downstream GA_1, implying that the high GA_1 levels in the DZ are controlled by these early biosynthesis steps. Consistently, the transcript levels of genes encoding the enzymes responsible for the oxidation of GA20 and GA3 peaked where GA_1 accumulated, in contrast to the genes involved in the earlier biosynthetic steps of GA_1 (*ent*-copalyl diphosphate synthase, *ent*-kaurene synthase, and *ent*-kaurene oxidase). The precursor GA_{20} is detected at low levels and fails to show the same accumulation profile, suggesting that this is a transient intermediate subject to high turnover by GA3-oxidase into the bioactive GA_1. These results indicate that the transcriptional control of GA20-oxidase and GA3-oxidase is the rate-limiting step for the biosynthesis of GA_1 in maize leaves, which is consistent with earlier data in *Arabidopsis* [18].

The narrow profile of the GA peak suggests a strict regulatory mechanism. High levels of GAs, as observed in the DZ

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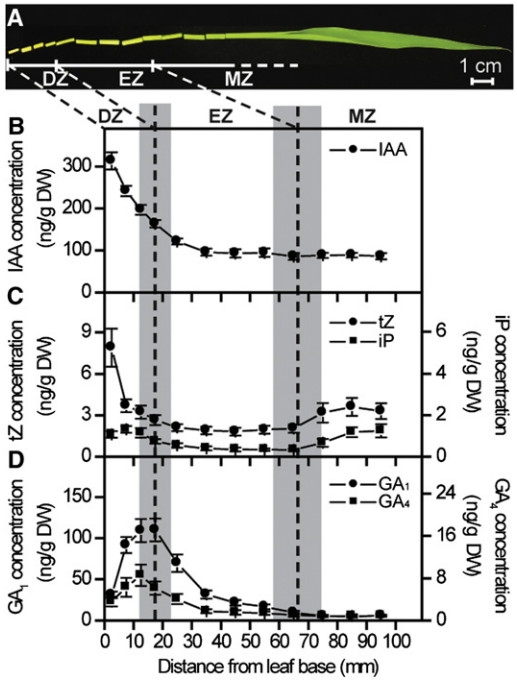

Figure 1. Hormone Concentrations in the Division, Expansion, and Mature Zone of a Maize Leaf

(A) Sampling strategy of the growing fourth leaf.

(B–D) Endogenous concentrations of auxin (indole-3-acetic acid [IAA]), cytokinins (trans-zeatin [tZ] and isopentenyladenine [iP]), and gibberellins ([GA_1] and [GA_4]). Symbols are averages ± SE (n = 5). Dashed lines (average) and gray shading (SE) indicate the transitions between the DZ, EZ, and MZ determined by kinematic analysis. See also Figure S1.

(Figure 2D; Figure S2), have been shown to inhibit their biosynthesis and activate their catabolism [17]. Therefore, we examined whether GA was actively catabolized near the boundary between the DZ and EZ. GA_8, the main catabolic product of GA_1 [17], and the expression levels of GA2-oxidases responsible for this conversion showed a peak distribution similar to GA_1, but displaced by 1 cm toward the tip of the leaf (Figure S2). The accumulation of GA_8 at the position when the levels of GA_1 are declining suggests that GA catabolism plays a role in the maintenance of the localized GA accumulation at the transition from division to expansion. Hormone concentrations can vary due to combinations of dilution by cell expansion [19] and net production and catalysis. To confirm the role of active local GA catabolism we calculated the GA_1 accumulation rate, based on the GA_1 levels along the leaf and the velocity profile derived from the cell length distribution and leaf elongation rate [8, 20]. This shows that the accumulation rate becomes significantly negative immediately distal to the boundary between cell division and expansion (Figure 2D; Figure S2), confirming that in addition to dilution by cell growth, catabolic activity and/or active export of GA_1 takes place. Together, these mechanisms cause rapid GA_1 decline and limit high GA_1 to a narrow peak around the boundary between the DZ and EZ.

GA Is Necessary for the Position of the Transition from Cell Division to Cell Expansion

The strictly controlled accumulation of bioactive GAs at the transition zone suggests an important role for GA in the spatial control of the cell division and expansion. This was also inferred by recent genetic studies showing an accumulation of GA responsive transcripts at the transition zone of *Arabidopsis* roots [10] and in the DZ of maize leaves [7] and by evidence that GA controls the size of the *Arabidopsis* root meristem [21].

To investigate the consequence of reduced GA levels on GA accumulation and the transition between cell division and expansion in the maize leaf, we analyzed the *dwarf3* mutant, defective in the conversion of *ent*-kaurenoic acid to GA_{12} early in GA biosynthesis [22] (Figure S2). This mutation blocked the biosynthesis of GA_1 almost completely [22] and strongly reduced its concentration throughout the growth zone (Figure 2A). Curiously, the distributions of remaining GA_1 and GA_8 still showed a distinct maximum that was located closer to the leaf base than the substantially higher peak in wild-type (WT) plants (Figure 2A). Consistent with its dwarf phenotype [22] (Figure 3A), mutant leaf growth, measured as leaf elongation rate, only reached one-third of the rate of the WT (Figure 3C). Kinematic analysis revealed that the growth inhibition in *dwarf3* was primarily due to a smaller size of the DZ (Figure 3E; Table S1A), which consequently contained fewer dividing cells (Table S1A; Figures S3A and S3B). Lowered GA levels result in a smaller peak, which is shifted toward the base of the leaf (Figure 2A) coinciding with the location of the transition from division to expansion, showing that GAs play a role in positioning the division-to-expansion transition. In addition, we also analyzed the *dwarf8* mutant, defective in GA signaling by DELLA stabilization [23]. This mutant phenocopied the *dwarf3*, with reduced leaf growth caused by a reduced DZ (Table S1B), indicating that the positioning of the transition is accomplished through a DELLA-dependent GA signaling.

Increased GA Levels Can Increase Cell Division and Leaf Elongation Rates

The results of the mutants predicted that an increased production of GAs would drive additional cell divisions and increase the size of the DZ thereby enhancing leaf growth. To investigate this, we constructed maize plants overproducing the rate-limiting enzyme AtGA20-oxidase1 (UBI::GA20-OX), which was previously shown to enhance GA production and growth in *Arabidopsis* [24, 25]. Across independent transformants, the transcript level of AtGA20-oxidase1 correlated positively with growth (Figures S4A–S4F) and plant height (Figures 2B and 3B). The strongest overexpression line, *UBI::GA20-OX-1*, showed elevated GA biosynthesis along the entire growth zone as evidenced by the GA20-oxidation metabolites, GA_{44}, GA_{19}, and GA_{20}, resulting in a 3-fold higher concentration of bioactive GA_1 at the transition (Figure 2E). The elevated GA levels in the *UBI::GA20-OX-1* resulted in a 40% increase in leaf elongation rate (Figure 3D) and microscopic analysis of mitotic cells indicated an increased size of the DZ (Figure 3F). Kinematic analysis showed that the increased size of the DZ and thereby the larger number of dividing cells (Table S1C) fully accounted for the enhanced growth (Figure 3F; Table S1C). The enlarged DZ was independently validated by the expression profiles of the mitosis markers *CDKB1-1* and *CyclinB2-2* [9] (Figures S4C and S4D). Together, these data demonstrate that elevated levels of GAs are able to drive additional cell divisions at the boundary between the DZ and EZ. The mechanism by which GA regulates maize leaf growth differs from the published role of GAs in

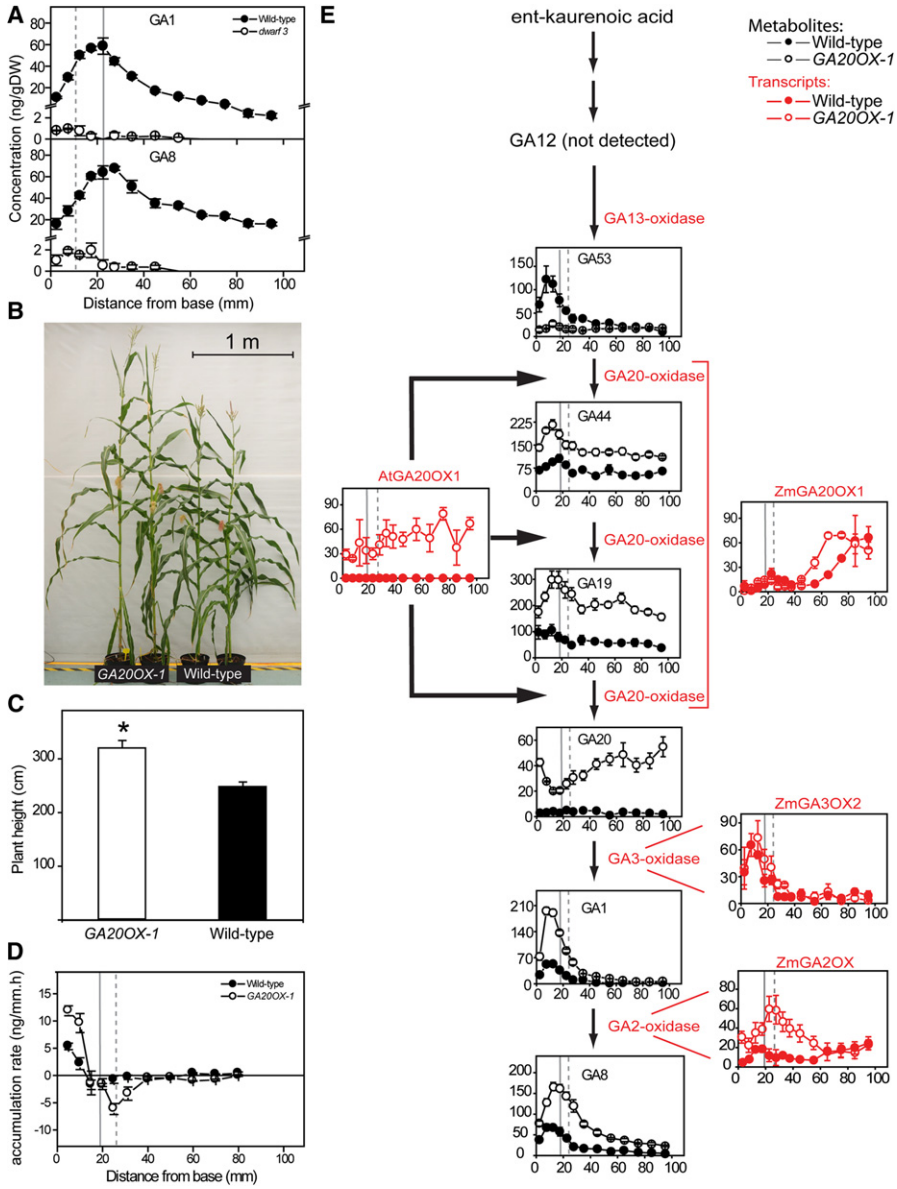

Figure 2. Localization of the GA Maximum and Molecular Kinetics of Its Establishment

(A) Endogenous concentration of GA_1 and GA_8 in the fourth leaf of the *dwarf3* mutants and segregating WTs.
(B and C) Phenotype (B) and average plant height (C) of mature *UBI::GA20OX-1* plants compared to segregating WTs.
(D) Accumulation rate of GA_1.
(E) Concentrations of metabolites (black) and transcript levels (red) in the GA metabolic pathway in *UBI::GA20OX-1* and its WT. Vertical lines indicate the distal boundary of DZ in WT (solid) and *UBI::GA20OX-1* (dashed). Values are averages ± SE (n = 3). See also Figure S2 and Table S2.

internode and hypocotyl elongation, where GA mainly stimulates cell expansion [26]. We also found a strong effect on cell expansion in the internodes of our *UBI::GA20-OX-1* line (Figures 2B and 2C; Figures S4G and S4H), demonstrating GA performs distinct functions in the different organs of the same plant.

Remarkably, in the *UBI::GA20-OX-1* line, the amplitude but not the localization of the GA_1 peak was altered although the GA precursors levels were induced across the entire leaf (Figure 2E), suggesting a regulation downstream of GA biosynthesis. To determine the role of GA catabolism in this restriction of GA accumulation, we measured the catabolite levels, expression levels of the catabolic genes, and GA_1 accumulation rates in the *UBI::GA20-OX-1* line. The main GA_1 catabolic product, GA_8, accumulated at much higher levels but at the same position as in the corresponding WT, ~1 cm more distal than the GA_1 peak (Figure 2E). Also the levels of the GA2-oxidases are much more elevated at the boundary between the dividing and the expanding cells in the *UBI::GA20-OX-1* line, as compared to WT (Figure 2E). In addition, the enhanced GA catabolism in the *UBI::GA20-OX-1* line was reflected by more negative GA_1 accumulation rates (Figure 2D). Taken together, we conclude that the levels of bioactive GA_1 determine cell-cycle exit and onset of cell expansion. The 3-fold higher accumulation of GA_1 in the *UBI::GA20-OX-1* line, despite increased degradation results in a delay to reach the threshold level of GA_1 below which cell division stops.

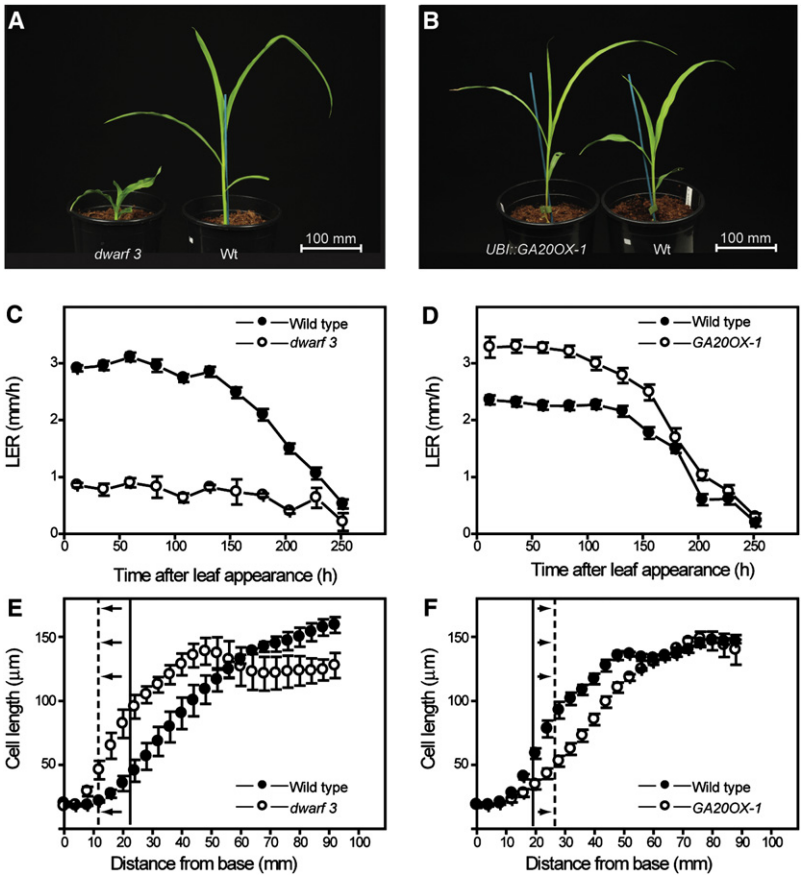

Figure 3. Effects of Altered GA Biosynthesis on Cell Length and Leaf Growth

Leaf growth phenotypes (A and B), leaf elongation rate (C and D), and cell length profiles (E and F) of the *dwarf3* mutant, the *UBI::GA20OX-1* line and their WTs. The difference in final cell length in (E) is not significant ($p = 0.08$). Vertical lines indicate the distal boundary of DZ in WT (solid), *UBI::GA20OX1*, and *dwarf3* (dashed). Arrows mark the changes compared to WT. Symbols are averages ± SE ($n = 3$). See also Figure S3 and Table S1.

GA Drives Cell Division at the Distal End of the Division Zone

The occurrence of cell division in the basal region of the DZ in the *dwarf3* mutant, with low levels of remaining bioactive GAs, suggests that cell division in this region is independent of GA. This implies that another factor drives cell divisions at the base and that the GA maximum is necessary to maintain cell division at the distal end of the DZ. Our direct quantification of hormone levels along the developmental gradient in the maize leaf implicates auxin, cytokinin, and the brassinosteroid CS as plausible candidates to promote cell division in the basal region (Figures 1B and 1C; Figure S1C). Brassinosteroids were shown to control the size of the *Arabidopsis* root meristem [27], and high auxin and cytokinin levels, as observed in the basal part of the DZ, are known to promote cell division [28, 29]. Interestingly, high auxin levels induce GA biosynthesis [30], whereas subsequent high GA levels induce GA catabolism [17], providing a mechanism whereby both auxin and GA levels rapidly decrease toward the transition, promoting cell differentiation over cell division. In the *dwarf3* mutant, high levels of auxin at the leaf base are unable to stimulate GA biosynthesis, leading to maintained auxin-driven cell division in the basal part and less GA accumulation and cell divisions in the distal part of the DZ. In this way, the combination of auxin-GA crosstalk and GA-self-regulating properties dictates the size of the DZ and thereby controls the growth of the maize leaf as a whole.

Conclusions

The observation that GA accumulation is restricted to a spatially localized "hot spot" at the boundary of the DZ and EZ provides an important growth control mechanism and stresses the relevance of precision sampling. Moreover, our spatial data provide a detailed mechanistic insight into a molecular mechanism of a previously hypothesized function of GA in controlling organ growth and size (Figure 4).

Supplemental Information

Supplemental Information includes four figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2012.04.065.

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References

1. Beemster, G.T.S., and Baskin, T.I. (1998). Analysis of cell division and elongation underlying the developmental acceleration of root growth in *Arabidopsis thaliana*. Plant Physiol. *116*, 1515–1526.
2. Ubeda-Tomás, S., Swarup, R., Coates, J., Swarup, K., Laplaze, L., Beemster, G.T.S., Hedden, P., Bhalerao, R., and Bennett, M.J. (2008). Root growth in *Arabidopsis* requires gibberellin/DELLA signalling in the endodermis. Nat. Cell Biol. *10*, 625–628.
3. Achard, P., Gusti, A., Cheminant, S., Alioua, M., Dhondt, S., Coppens, F., Beemster, G.T.S., and Genschik, P. (2009). Gibberellin signaling controls cell proliferation rate in *Arabidopsis*. Curr. Biol. *19*, 1188–1193.
4. Perilli, S., Di Mambro, R., and Sabatini, S. (2012). Growth and development of the root apical meristem. Curr. Opin. Plant Biol. *15*, 17–23.
5. Grieneisen, V.A., Xu, J., Marée, A.F.M., Hogeweg, P., and Scheres, B. (2007). Auxin transport is sufficient to generate a maximum and gradient guiding root growth. Nature *449*, 1008–1013.
6. Rymen, B., Fiorani, F., Kartal, F., Vandepoele, K., Inzé, D., and Beemster, G.T.S. (2007). Cold nights impair leaf growth and cell cycle progression in maize through transcriptional changes of cell cycle genes. Plant Physiol. *143*, 1429–1438.
7. Li, P., Ponnala, L., Gandotra, N., Wang, L., Si, Y., Tausta, S.L., Kebrom, T.H., Provart, N., Patel, R., Myers, C.R., et al. (2010). The

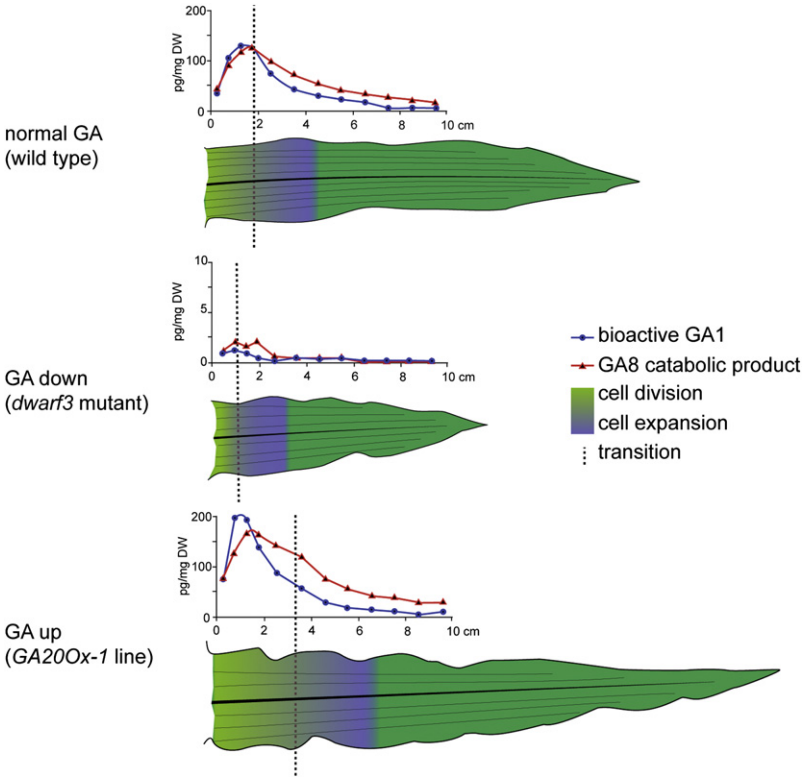

Figure 4. Schematic Overview of the Cellular Effects and GA_1 and GA_8 Levels in the Maize Leaf Due to Altered GA Levels

In plants with normal, low, and elevated GA levels, the bioactive GA1 (blue) and catabolic GA8 (red) levels were measured (pg per mg of dry weight), the position of the transition (dashed line) between the DZ (yellow) and the EZ (purple) was determined microscopically, and the final leaf size was recorded. See also Figure S4.

developmental dynamics of the maize leaf transcriptome. Nat. Genet. *42*, 1060–1067.

8. Rymen, B., Coppens, F., Dhondt, S., Fiorani, F., and Beemster, G.T.S. (2010). Kinematic analysis of cell division and expansion. In Plant Developmental Biology, *Volume 655*, L. Hennig and C. Köhler, eds. (New York: Humana Press), pp. 203–227.
9. De Veylder, L., Beeckman, T., and Inzé, D. (2007). The ins and outs of the plant cell cycle. Nat. Rev. Mol. Cell Biol. *8*, 655–665.
10. Birnbaum, K., Shasha, D.E., Wang, J.Y., Jung, J.W., Lambert, G.M., Galbraith, D.W., and Benfey, P.N. (2003). A gene expression map of the *Arabidopsis* root. Science *302*, 1956–1960.
11. Ubeda-Tomas, S., and Bennett, M.J. (2010). Plant development: size matters, and it's all down to hormones. Curr. Biol. *20*, R511–R513.
12. Bari, R., and Jones, J.D.G. (2009). Role of plant hormones in plant defence responses. Plant Mol. Biol. *69*, 473–488.
13. Świątek, A., Lenjou, M., Van Bockstaele, D., Inzé, D., and Van Onckelen, H. (2002). Differential effect of jasmonic acid and abscisic acid on cell cycle progression in tobacco BY-2 cells. Plant Physiol. *128*, 201–211.
14. Miura, K., Lee, J., Miura, T., and Hasegawa, P.M. (2010). *SIZ1* controls cell growth and plant development in Arabidopsis through salicylic acid. Plant Cell Physiol. *51*, 103–113.
15. Clouse, S.D. (2011). Brassinosteroid signal transduction: from receptor kinase activation to transcriptional networks regulating plant development. Plant Cell *23*, 1219–1230.
16. Seo, M., and Koshiba, T. (2011). Transport of ABA from the site of biosynthesis to the site of action. J. Plant Res. *124*, 501–507.
17. Yamaguchi, S. (2008). Gibberellin metabolism and its regulation. Annu. Rev. Plant Biol. *59*, 225–251.
18. Fleet, C.M., Yamaguchi, S., Hanada, A., Kawaide, H., David, C.J., Kamiya, Y., and Sun, T.P. (2003). Overexpression of *AtCPS* and *AtKS* in Arabidopsis confers increased *ent*-kaurene production but no increase in bioactive gibberellins. Plant Physiol. *132*, 830–839.
19. Band, L.R., Wells, D.M., Larrieu, A., Sun, J., Middleton, A.M., French, A.P., Brunoud, G., Sato, E.M., Wilson, M.H., Péret, B., et al. (2012). Root gravitropism is regulated by a transient lateral auxin gradient controlled by a tipping-point mechanism. Proc. Natl. Acad. Sci. USA *109*, 4668–4673.
20. Silk, W.K., Walker, R.C., and Labavitch, J. (1984). Uronide deposition rates in the primary root of Zea mays. Plant Physiol. *74*, 721–726.
21. Ubeda-Tomás, S., Beemster, G.T.S., and Bennett, M.J. (2012). Hormonal regulation of root growth: integrating local activities into global behaviour. Trends Plant Sci. Published online March 6, 2012. 10.1016/j.tplants.2012.02.002.
22. Fujioka, S., Yamane, H., Spray, C.R., Gaskin, P., Macmillan, J., Phinney, B.O., and Takahashi, N. (1988). Qualitative and quantitative analyses of gibberellins in vegetative shoots of normal, *dwarf-1, dwarf-2, dwarf-3*, and *dwarf-5* seedlings of Zea mays L. Plant Physiol. *88*, 1367–1372.
23. Lawit, S.J., Wych, H.M., Xu, D., Kundu, S., and Tomes, D.T. (2010). Maize DELLA proteins dwarf plant8 and dwarf plant9 as modulators of plant development. Plant Cell Physiol. *51*, 1854–1868.
24. Gonzalez, N., De Bodt, S., Sulpice, R., Jikumaru, Y., Chae, E., Dhondt, S., Van Daele, T., De Milde, L., Weigel, D., Kamiya, Y., et al. (2010). Increased leaf size: different means to an end. Plant Physiol. *153*, 1261–1279.
25. Coles, J.P., Phillips, A.L., Croker, S.J., García-Lepe, R., Lewis, M.J., and Hedden, P. (1999). Modification of gibberellin production and plant development in *Arabidopsis* by sense and antisense expression of gibberellin 20-oxidase genes. Plant J. *17*, 547–556.
26. Peng, J., Richards, D.E., Hartley, N.M., Murphy, G.P., Devos, K.M., Flintham, J.E., Beales, J., Fish, L.J., Worland, A.J., Pelica, F., et al. (1999). 'Green revolution' genes encode mutant gibberellin response modulators. Nature *400*, 256–261.
27. González-García, M.P., Vilarrasa-Blasi, J., Zhiponova, M., Divol, F., Mora-García, S., Russinova, E., and Caño-Delgado, A.I. (2011). Brassinosteroids control meristem size by promoting cell cycle progression in *Arabidopsis* roots. Development *138*, 849–859.
28. Depuydt, S., and Hardtke, C.S. (2011). Hormone signalling crosstalk in plant growth regulation. Curr. Biol. *21*, R365–R373.
29. Dewitte, W., Scofield, S., Alcasabas, A.A., Maughan, S.C., Menges, M., Braun, N., Collins, C., Nieuwland, J., Prinsen, E., Sundaresan, V., and Murray, J.A. (2007). *Arabidopsis* CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses. Proc. Natl. Acad. Sci. USA *104*, 14537–14542.
30. Fu, X., and Harberd, N.P. (2003). Auxin promotes *Arabidopsis* root growth by modulating gibberellin response. Nature *421*, 740–743.