Unraveling the molecular mechanisms controlling the transition between cell division and cell expansion during maize leaf development

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轻轻的我走了，正如我轻轻的来；
我轻轻的招手，作别西天的云彩。
那河畔的金柳，是夕阳中的新娘；
波光里的艳影，在我的心头荡漾。
软泥上的青荇，油油的在水底招摇；
在康河的柔波里，我甘心做一条水草！
那榆荫下的一潭，不是清泉，是天上虹；
揉碎在浮藻间，沉淀着彩虹似的梦。
寻梦？撑一支长篙，向青草更青处漫溯；
满载一船星辉，在星辉斑斓里放歌。
但我不能放歌，悄悄是别离的笙箫；
夏虫也为我沉默，沉默是今晚的康桥！
悄悄的我走了，正如我悄悄的来；
我挥一挥衣袖，不带走一片云彩。

--徐志摩
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<tr>
<td>4CL</td>
<td>4-coumarate-CoA ligase</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>ADT</td>
<td>Arogenate dehydratase</td>
</tr>
<tr>
<td>AN3</td>
<td>ANGUSTIFOLIA3</td>
</tr>
<tr>
<td>ANT</td>
<td>Anthranilate</td>
</tr>
<tr>
<td>ARF</td>
<td>Auxin response factor</td>
</tr>
<tr>
<td>ASI</td>
<td>Anthesis-silking interval</td>
</tr>
<tr>
<td>BR</td>
<td>Brassinosteroid</td>
</tr>
<tr>
<td>BR1</td>
<td>Brassinosteroid insensitive1</td>
</tr>
<tr>
<td>C3H</td>
<td>p-coumarate 3'-hydroxylase</td>
</tr>
<tr>
<td>CDK</td>
<td>Cycline-dependent kinase</td>
</tr>
<tr>
<td>CGA</td>
<td>Chlorogenic acid</td>
</tr>
<tr>
<td>CHI</td>
<td>Chalcone isomerase</td>
</tr>
<tr>
<td>CHS</td>
<td>Chalcone synthase</td>
</tr>
<tr>
<td>CM</td>
<td>Chorismate mutase</td>
</tr>
<tr>
<td>CSPPs</td>
<td>Candidate Substrate Product Pairs</td>
</tr>
<tr>
<td>CYCB</td>
<td>Cyclin B</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>D8</td>
<td>Dwarf 8</td>
</tr>
<tr>
<td>D9</td>
<td>Dwarf 9</td>
</tr>
<tr>
<td>DAS</td>
<td>Days after stratification</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DIBOA</td>
<td>2,4-dihydroxy-1,4-benzoxazin-3-one</td>
</tr>
<tr>
<td>DIMBOA</td>
<td>2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one</td>
</tr>
<tr>
<td>DZ</td>
<td>Division zone</td>
</tr>
<tr>
<td>EST</td>
<td>Estradiol</td>
</tr>
<tr>
<td>EZ</td>
<td>Expansion zone</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FC</td>
<td>Fold change</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>FLL</td>
<td>Final leaf length</td>
</tr>
<tr>
<td>GA</td>
<td>Gibberellin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GA20OX</td>
<td>GA20-Oxidase</td>
</tr>
<tr>
<td>GA2ox</td>
<td>GA2-oxidase</td>
</tr>
<tr>
<td>GAI</td>
<td>GA insensitive</td>
</tr>
<tr>
<td>GE</td>
<td>Giant embryo</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRF</td>
<td>Growth-regulating factor</td>
</tr>
<tr>
<td>HCT</td>
<td>Hydroxycinnamoyl-CoA shikimate/quinate hydrxycinnamoyl transferase</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>IAA-Asp</td>
<td>IAA-aspartate</td>
</tr>
<tr>
<td>IAA-Glu</td>
<td>IAA-glutamate</td>
</tr>
<tr>
<td>IAM</td>
<td>Indole-3-acetamide</td>
</tr>
<tr>
<td>IAN</td>
<td>Indole-3-acetonitrile</td>
</tr>
<tr>
<td>IAOX</td>
<td>Indole-3-acetaldoxime</td>
</tr>
<tr>
<td>iP</td>
<td>N⁶-(Δ²-isopentenyl)adenine</td>
</tr>
<tr>
<td>IPyA</td>
<td>Indole-3-pyruvic acid</td>
</tr>
<tr>
<td>JA</td>
<td>Jasmonic acid</td>
</tr>
<tr>
<td>JA-Ile</td>
<td>Jasmonoyl-isoleucine</td>
</tr>
<tr>
<td>KRP</td>
<td>KIP related protein</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LED</td>
<td>Leaf elongation duration</td>
</tr>
<tr>
<td>LER</td>
<td>Leaf elongation rate</td>
</tr>
<tr>
<td>LGV</td>
<td>Leaf Growth Viewer</td>
</tr>
<tr>
<td>MZ</td>
<td>Mature zone</td>
</tr>
<tr>
<td>NPA</td>
<td>1-N-naphthylphthalamic acid</td>
</tr>
<tr>
<td>NT</td>
<td>Non-transgenic</td>
</tr>
<tr>
<td>OxiIAA</td>
<td>2-oxoindole-3-acetic acid</td>
</tr>
<tr>
<td>PAL</td>
<td>Phenylalanine ammonia-lyase</td>
</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PIN</td>
<td>PIN-formed</td>
</tr>
<tr>
<td>PPD</td>
<td>PEAPOD</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative Trait Locus</td>
</tr>
<tr>
<td>RIL</td>
<td>Recombinant inbred line</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>RNAseq</td>
<td>RNA sequencing</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>SAM</td>
<td>Shoot apical meristem</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>T</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TAP</td>
<td>Tag affinity purification</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TRA</td>
<td>Tryptamine</td>
</tr>
<tr>
<td>TRP</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>tZ</td>
<td>Trans-zeatin</td>
</tr>
<tr>
<td>TZ</td>
<td>Transition zone</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
Plant growth is a highly plastic process that can be affected by internal signals and environmental cues. Plants can adjust their growth based on its intrinsic genetic information or as adaptation to environmental changes by coordinating the two cellular processes that drive leaf growth, cell division and cell expansion. So far, knowledge on growth regulation was mainly obtained in the model species *Arabidopsis thaliana*. However, at an evolutionary scale, Arabidopsis as a dicot is relatively distant from important crops, which are mainly monocots. In addition, the size of plant organs in Arabidopsis is relatively small, making it difficult to obtain the necessary sampling resolution to study the coordination between the cellular processes that affect growth.

Recently, the maize leaf has gained importance as a model to study growth, due to its bigger size, allowing a higher sampling resolution and the spatially distribution of cellular processes. In our research team, a kinematic analysis was established that allowed to visualize and quantify the contribution of cell division and cell expansion to changes in growth during steady state growth. Using this method, it was shown that gibberellins (GAs), a class of phytohormones, determine the transition between cell division and cell expansion (TZ1). When the GA biosynthesis gene (*GA20OX1*) is constitutively overexpressed (UBI::GA20OX1), the TZ1 shifted more distally, resulting in an increased division zone size (Nelissen et al., 2012). Next to the GA-dependent regulation of leaf growth, mild drought was found to shift TZ1 towards leaf base and consequently reduce leaf growth. The aim of this thesis is to get a better insight in the molecular mechanisms underlying the two conditions that positively and negatively affect TZ1 (high GA level and mild drought conditions, respectively). Therefore, a transcriptome and a hormone profiling on the growth zone of maize leaf will be performed with high resolution. By comparing the transcriptome and hormone data in the two conditions, we expect to sort out condition specific hormonal and transcriptional changes.

In the second part of my PhD, the growth enhancing effect of a single gene, named KLUH will be examined. KLUH encodes a cytochrome P450 that was identified as a DELLA targets in Arabidopsis (Claeys et al., 2014). Interestingly, it was reported that overexpression of KLUH by its endogenous promoter stimulated growth (Anastasiou et al., 2007), while strong overexpression of KLUH resulted in a growth defect in Arabidopsis (Claeys et al., unpublished data). To examine whether the function of KLUH is conserved in maize, constitutive and mild expression of the maize homolog of KLUH will be assessed for their growth promoting potential. As KLUH has enzymatic activity as a monooxygenase with yet unknown substrate and product we aim to take advantage of the size of the maize leaves to perform a metabolome analysis on dividing leaf tissue to detect the
substrate/product couple of KLUH. The growth enhancing capacity of the product of the reaction catalyzed by KLUH will be assessed by performing maize growth assays.

In addition, we would like to examine whether the KLUH homolog is a direct target of DELLA in maize, as was observed in Arabidopsis. A GR based inducible gene expression system will be constructed by fusing the non-degradable DELLA with GR and the chemical induction will be tested in maize. If the system can be successfully translated to maize, the growth zone specific DELLA targets will be identified by performing a transcriptome study at different time points upon induction.

References


With the increasing population, climate change and the decrease of water availability, raising crop yield (especially in drought conditions) becomes a crucial challenge. Leaf growth is one of the key factors affecting yield and is driven by the combination of cell division and cell expansion. In the maize leaf, the two processes take place simultaneously, but the basal leaf cells keep on dividing and form the division zone (DZ). The cell division ceases and cells continue to grow by expanding their size in the expansion zone (EZ). The transition between cell division and cell expansion is defined as transition zone 1 (TZ1). The rate and duration of the two processes can be quantified through kinematic analysis on the growing maize leaf at the steady-state growth stage. Previously, the transition between cell division and cell expansion was shown to be characterized by a local accumulation of bioactive gibberellin acid (GA). Kinematic analysis reveals that overexpression of the GA biosynthesis gene GA20oxidase (UBIL::GA20OX) shifts the transition zone more distally towards leaf tip, resulting in a bigger division zone (chapter 1).

In this thesis, mild drought stress was identified to reduce leaf growth by decreasing the division zone size, which is opposite to the effect observed with high GA levels. Strikingly, mild drought is associated with lower levels of GA, but higher levels of GA in the UBI::GA20OX plants were unable to overcome the effects of mild drought, as the growth reduction of the UBI::GA20OX plants under mild drought is comparable to that of the non-transgenic siblings. Thus, another mechanism, besides lowered GA levels, is instrumental for the drought response. The analysis of the transcriptome data, comparing the leaf gradient under the two conditions that affect the position of the transition zone, showed that mild drought specifically induced a reprogramming of transcriptional regulation in the division zone. A user-friendly bioinformatic tool, called Leaf Growth Viewer (LGV), is developed to allow users to visualize and select our transcriptome data. Moreover, with a link to PLAZA3.0, LGV can integrate the expression pattern and the functional enrichment for selected genes, making it a powerful tool for maize leaf research (chapter 2).

Besides the spatial control during the steady-state growth stage, another mechanism, leaf elongation duration (LED), was shown to affect growth when maize plants were subjected to drought stress. Transcriptome data showed that many genes enriched in the GO categories cell cycle and DNA replication, were significantly upregulated around the TZ1 under drought stress, suggesting that the prolonged duration of growth is associated with a prolonged potential in cell division (chapter 2). In addition to the mild drought condition which causes extended LED, perturbation of the maize KLUH gene also affects LED. KLUH belongs to the CYP78A family of the cytochrome P450s and homologs are
known to stimulate cell division during leaf, flower and seed development in several species. Through detailed analysis of growth over time (from when leaf 4 was still growing in the pseudo stem formed by the older leaves until the end of leaf growth), we identified that KLUH stimulated the duration of leaf growth by maintaining the dividing cells for a longer period in a proliferative, undifferentiated state. Moreover, KLUH seemed to reinforce the prolonged growth duration in mild drought conditions (chapter 3).

The growth stimulating feature of KLUH is further analyzed by its overexpression lines. Strong overexpression KLUH (UBIL::KLUH) generated infertile plants with altered plant stature in maize. In contrast, mild overexpression KLUH by GA2-oxidase (GA2ox) promoter (GA2ox::KLUH) resulted in increased biomass accumulation, as was exemplified by increased leaf size and plant height in greenhouse conditions. Longer growth period in GA2ox::KLUH postponed the reproductive growth, including the time of flowering and silking, but the interval of two time points (ASI, the anthesis-silking interval) was shortened in GA2ox::KLUH in the greenhouse. GA2ox::KLUH was grown in field conditions to examine the growth enhancement effect. The biomass was consistently enhanced by the GA2ox::KLUH transgene, in both inbred and hybrid background, in two consecutive years in the field. The longer vegetative growth and shorter ASI was also observed in field conditions. Moreover, KLUH stimulated ear growth in field conditions, resulting in significantly longer ears and slightly increased seeds weight (chapter 3).

Transcriptome studies and hormone measurements show that KLUH functions through an increase of auxin, of which the accumulation pattern in the leaf growth zone is highly similar to the KLUH expression pattern (chapter 3). Metabolome profiling at the basal division zone of maize leaf, where KLUH is expressed, was performed to identify the so far enigmatic product ad substrate of KLUH. Chlorogenic acid (CGA) was found to be highly abundant in the KLUH overexpression line but decreased in kluh mutants. Moreover, similar to the KLUH function that also seemed conserved between Arabidopsis and maize, CGA treated Arabidopsis seedlings resulted in bigger rosette leaves due to stimulated cell division, making it a candidate product of KLUH. However, as Arabidopsis with CGA treatment did not completely resemble the transgenic plants with strong KLUH overexpression, we cannot exclude other molecules to be the product of KLUH. Further experiments are needed to evaluate the timing effect of CGA on leaf growth and to confirm the link between KLUH and CGA (chapter 4).

Additionally, as KLUH is one of the strongest and fastest genes responding to DELLA protein stabilization in Arabidopsis, the glucocorticoid receptor was fused to the DELLA dominant negative mutation (d8). This way, it was tested whether the induction of the dominant negative version of DELLA
can cause a growth reduction. In the next step, the direct target of DELLA in maize could be determined and the link between KLUH and DELLA in maize could be examined (chapter 5).
Chapter 1—Introduction

The maize leaf as a model to understand organ growth
The maize leaf as a model to understand organ growth

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AUTHOR CONTRIBUTION

X.S. was the main author of this chapter. D.I. and H.N. contributed to the writing of this chapter.
The shape, form and organization of leaves greatly determines plant architecture and was used in taxonomy (Bell and Bryan, 2008). Besides the effect on plant appearance, leaves are important plant organs to drive plant growth as they convert light energy to chemical energy. The way carbon is being fixed by the plants is reflected by the leaf anatomy, as C4 plants often display specialized structures, referred to as Kranz structure that is absent in C3 plants. Maize (Zea mays) is one of the most important C4 crops and literature on research performed on the maize leaf goes back as early as 1945, and covers a wide range of physiological, cytological and pathological studies. To date, many biological questions are still addressed using the maize leaf, but the technologies and approaches evolved.

Over the past years, many different aspects of the C4 metabolism were studied at a molecular level using the maize leaf, ranging from the sink-source transition (Li et al., 2010; Tausta et al., 2014) over C4 differentiation (Majeran et al., 2010) to chloroplast biogenesis (Belcher et al., 2015). Besides studying the C4 metabolism, the maize leaf was also used extensively to examine patterning. Several genes were identified that determine the patterning of maize leaves on the stem, also referred to as phyllotaxy (Giulini et al., 2004; Yang et al., 2015). The maize leaf contains structures, such as the ligule and auricle, that make it an interesting organ to study proximo-distal patterning (Figure 1) (Foster et al., 2004; Alexander et al., 2005; Moon et al., 2013; Lewis et al., 2014), but also genes involved in mediolateral (Foster et al., 2004; Alexander et al., 2005; Douglas et al., 2010) and dorso-ventral patterning (Marja et al., 1999; Tsiantis et al., 1999; Zhang et al., 2012b; Dotto et al., 2014) were first identified in maize. Even at the cellular level the patterning was studies for asymmetric cell divisions and stomata development (Cartwright et al., 2009; Humphries et al., 2011; Facette and Smith, 2012; Zhang et al., 2012a).

These studies illustrate the power of maize as a model system to isolate mutations that lead to the identification of important developmental regulators. But besides the gene-centered approach, the size of the maize leaf also facilitated the use of a range of analytical technologies to increase our understanding of the molecular processes at the level of the transcriptome (Li et al., 2010; Yu et al., 2015), proteome (Majeran et al., 2010; Facette et al., 2013), phosphoproteome (Bonhomme et al., 2012), metabolome (Pick et al., 2011), chromatin modifications (Perduns et al., 2015) and DNA methylation (Candaele et al., 2014). The linear organization of the growth processes cell division and cell expansion offers another advantage of the maize leaf, that makes it particularly suited to study genetic variability of growth regulation or how plants adapt their growth to altered environmental conditions (Nelissen et al., 2013). However, not only cellular analyses but also many forward genetic screens using natural variation, as well as the rapidly evolving field of phenotyping (Welcker et al.,
Maize leaf as a model to study growth

2011; Baute et al., 2015) were instrumental to understand how the growth processes contribute to final leaf size in maize. Therefore, the focus of this review is on the current view on growth regulation in the maize leaf.

Figure 1. The tissues and major growth axes of the maize leaf. (A) image of the maize plant illustrating the axes of the maize leaf relative to the main axis of the plant. (B) image of the adaxial (upper) surface of the maize leaf showing distal blade and proximal sheath, separated by the ligule/auricle region. This figure is changed based on (Foster and Timmermans, 2009).

Maize leaf growth

In maize, leaf initiation cells are recruited from the peripheral zone of the shoot apical meristem (SAM) to form leaf primordia. Leaves are continuously initiated from the SAM and the plastochron defines the time interval between successive initiated leaves. A leaf that has just emerged from the meristem is referred to as plastochron 1 (P1), while a P2 leaf is one plastochron older, and the position of the P0 leaf can be predicted as part of the SAM. Recently, the architecture of the SAM has been shown to be correlated with adult plant traits. A small but significant negative correlation was found between SAM architecture and leaf length and width, meaning that the undifferentiated cells affect differentiated organs to some extent, albeit negatively (Thompson et al., 2015). After leaf initiation, leaf growth can be divided into three dimensions (Figure 1): the proxio-distal development is from the leaf base towards the leaf tip; the abaxial-adaxial axis marks the leaf thickness; and the medio-lateral axis defines the leaf width. The fully grown maize leaf is a flat organ that is typically longer than wider, and the thickness is difficult to observe. Phenotypic data of a maize B73xH99
recombinant inbred line (RIL) population showed that leaf area significantly and very highly correlated to leaf weight, indicating that leaf thickness does not contribute much to leaf biomass (Baute et al., 2015). On the other hand, leaf length and width are both equally highly correlated with leaf area, so the review focusses on growth along the leaf length and width direction.

*Leaf length growth*

Maize leaf growth was first monitored by using the pinning method or leaf segment growth rate (Bernstein et al., 1993; Neves-Piestun and Bernstein, 2001) to determine the leaf elongation rate, by marking the basal part of the plant with fine needles. As the leaf grows the puncture holes will move apart and by measuring the distances between the puncture holes allowed to calculate the relative expansion rate (RER) (Figure 2A-B) (Muller et al., 2001). This method severely affects growth and becomes destructive and as such only provides a view on leaf growth in a short period and it is impossible to follow the leaf during its complete growth. Alternatively, the growth of the maize leaf can be monitored when it is still in the whorl of older leaves by dissecting the plants and collecting quantitative measurements. This method does not allow to follow one plant over time, but rather takes the average of substantial number of individuals that were carefully selected to be in the same developmental stage (Fournier et al., 2005; Andrieu et al., 2006).

When the leaf grows out of the whorl, it becomes much easier to follow growth. Leaf segment growth is calculated by tracking the initially marked ink spots on the leaf surface over time (Erickson and Silk, 1980). The growth of the leaf after appearance can also be followed by daily measuring the leaf length from leaf emergence until the leaf stops growing. By dividing the increase in leaf length by the time interval between the measurements allows to calculate the leaf growth speed or leaf elongation rate (LER in mm/h) (Figure 2C). Typically, the LER remains consistently high in the first few days after leaf emergence and declines gradually, until it stops growing. During the first days of maximal growth, leaf growth is stable and therefore this period is also considered as steady-state growth (Figure 2C) (Ben-Haj-Salah and Tardieu, 1995; Muller et al., 2001; Bouchabke et al., 2006; Rymen et al., 2010).

At the cellular level, maize leaf length growth is driven by cell division and cell expansion. Cell division occurs on the proximal leaf base called the division zone (DZ). The newly produced cells are pushed up whorl, until a point (the transition zone 1 (TZ1)) where the cells stop dividing and continue to grow by extending their size, giving rise to the expansion zone (EZ). When cells reach their maximal size after expansion, they pass through a second transition zone (TZ2) and are considered part of the mature zone (MZ). During the steady state growth phase, the three cell processes occur
Maize leaf as a model to study growth

simultaneously and linearly arranged along maize leaf, which is similar to the arrangement of growth processes in roots, allowing for the same basic approach to analyze growth (Avramova et al., 2015a). At the cellular level the growth analysis can be performed by what is called a kinematic analysis that is performed approximately two days after leaf emergence, which is in the middle of the steady state growth phase, when LER is stable and the leaf contains all growth processes (cell division, cell expansion and mature cells) (Ben-Haj-Salah and Tardieu, 1995). Kinematic analysis is performed using microscopy based technology. DAPI (4’,6-diamidino-2-phenylindole) as a fluorochrome is used to determine the nuclear DNA content of the epidermal cells of the leaf and the DZ is therefore defined as the region containing mitotically dividing cells. The epidermal cell length prolife along the axis of the leaf is measured by a microscope fitted with differential interference contrast (DIC) optics and the size of the growth zone (combination of DZ and EZ) is estimated as the distance from the leaf base to the position where the cells reach 95% of their final length on the cell length profile (Nelissen et al., 2013). Through combining the LER, division zone size, and the cell length data, the cellular parameters affecting cell production (number of dividing cells, cell division rate and cell cycle duration) and cell expansion (the number of expanding cells, expansion rate and duration) in leaf development can be determined (Figure 2D) (Rymen et al., 2010; Nelissen et al., 2013).

Combining these kinematic analyses with genetics and molecular approaches revealed the importance of the transition between cell division and cell expansion for final leaf length. Bioactive gibberellins (GAs; GA1 and GA4) resulting from the increased expressions of GA biosynthesis enzymes around the transition, specifically peak at the TZ1 between cell division and cell expansion. As these data suggested the importance of the GA peak in determining the transition from cell division to cell expansion, mutants defective in GA biosynthesis and perception as well as transgenic lines overexpressing the rate-limiting enzyme GA20-Oxidase (GA20OX) were analyzed by kinematic analysis (Nelissen et al., 2012). The leaf growth was highly promoted by an elevated GA content in the GA20OX transgenic plants, driven by an increased size of the DZ and a consequently increased maximal LER. In contrast, leaf growth was reduced in GA defective mutants due to a decreased DZ size and LER (Nelissen et al., 2012; Voorend et al., 2014). Interestingly, the GA specific peak was shifted more distal along the leaf axis in GA20OX overexpression line and more basal in GA defective mutant, suggesting that GA accumulation determines the spatial boundary of dividing and expanding cells (Nelissen et al., 2012). Besides GA, also brassinosteroids (BRs) play a role in leaf growth. The steady state LER is decreased in plants with reduced levels of the BR receptor BRASSINOSTEROID INSENSITIVE1 (BRI1). This growth reduction results from a decrease in cell number and to a lesser extent in cell size (Kir et al., 2015). The growth regulating SWI/SNF chromatin remodeling complex associated with ANGUSTIFOLIA3 (AN3) is also involved in controlling the balance between cell division
and cell expansion by dynamic binding to different GROWTH-REGULATING FACTORs (GRFs) in the growth zone (Vercruyssen et al., 2014; Nelissen et al., 2015). When breaking the balance by altering the expression levels of different DZ or EZ specific GRFs, the final leaf length can be modified (Candaele et al., 2014; Wu et al., 2014; Nelissen et al., 2015).

Figure 2. Schematic diagram of the arrangement of developing maize leaf and the methods to study maize leaf growth.

Relative growth rate (A) is calculated based on the pinning method (B). As a destructive method, the pinning method severely affects leaf growth and therefore can only determine the leaf segment growth rate within 12 hours following labeling the leaves. (C) Leaf length is measured daily to calculated leaf elongation rate. (D) Kinematic analysis identifies cellular processes at the steady state growth. DZ: division zone; EZ, expansion zone; MZ, mature zone.

The A and B panels are adapted from Muller et al., 2001. The C and D panels are adapted from Nelissen et al., 2013.

The majority of the maize leaf growth genes identified to date affect the spatial organization of the growth processes and so far there is very little information on the timing of the different processes. In Arabidopsis, however, leaf growth is mainly characterized by the timing of the different processes. At first, all cells are actively dividing but, later in development, cell division ceases from the tip to the
base of the leaf, resulting in a gradual transition to cell expansion over time (Kazama et al., 2010; Andriankaja et al., 2012). Overexpression of GA20OX and AN3 in Arabidopsis increases leaf size due to an increase in cell division, which is reminiscent of phenotypes in maize, suggesting similar organ growth controls between species (Gonzalez et al., 2010; Vercruyssen et al., 2014). The Arabidopsis growth regulators which related for time related growth processes including the timing of cell division and the extent of cell expansion are an important source of novel growth regulators in maize (Gonzalez et al., 2012).

Leaf width growth

Leaf width is determined by the distance from the midrib to leaf margins. Leaf primordia are formed at the SAM, and expand while the leaf grows (Scanlon and Freeling, 1997; Scanlon, 2000). To date, it is not possible to follow the growth of a single leaf from the SAM, but by imaging the incipient leaves at different plastochron stages (P) some information on the development of the leaf margin was obtained (Johnston et al., 2015). The leaf primordium starts to emerge at the P0 stage, later at the P2 stage, the entirety of the as yet elaborated emerged primordium is suggested to comprise blade tissue (Scanlon and Freeling, 1997; Johnston et al., 2015). The initial margins of leaf blade primordium do not overlap the SAM at the P2 stage, but with continuous growth, the leaf primordium enclose the SAM at the P3 stage (Scanlon and Freeling, 1997). Later, the leaf is still expanding in the width direction during steady-state growth stage (Muller et al., 2007). When following the growth of a leaf segment for 12 hours, the relative rate of leaf widening increased at the early DZ and reached its maximal in the EZ, then decreased gradually slightly towards the distal end of growth zone, suggesting that both cell division and cell expansion contribute to the increase in leaf width in early leaf development (Muller et al., 2007).

So far, many identified mutants with a narrow blade or bladeless phenotype were showed to have a deficit in leaf primodium development. Mutations of leafbladeless1 (lbl1) modify the dorsoventrality of lateral organs and produce radially symmetrical, threadlike leaves. LBL1 is also required for the lateral propagation of the recruitment of leaf founder cells (Timmermans et al., 1998). Ragged seedling2 (rgd2) mutants have leaf phenotypes that are morphologically similar to lbl1, including the formation of narrow, split, or half leaves. But unlike the lbl1 loss of adaxial cell identity, rgd2 exhibits patches of epidermal swapping. Scanning electron microscopy of young primordia and analysis of the expression of genes for leaf development in mutant apices reveal that RGD2 functions during the recruitment of leaf founder cells and during expansive growth of leaf primordia (Henderson et al., 2005). LBL1 is identified to function downstream of RGD2 in the biogenesis of ta-siARF, a trans-acting
small interfering RNAs that targets transcripts of AUXIN RESPONSE FACTOR3a for degradation, which is required to spatial control adaxial-abaxial patterning (Nogueira et al., 2007; Douglas et al., 2010). But how all this regulates leaf founder cells recruitment is still unclear. The maize gene PUNCTATE VASCULAR EXPRESSION1 (PVE1) is downregulated in rgd2 apices (Zhang et al., 2012b). pve1 mutants also exhibit narrow leaves, and frequently associated with outgrowth of abnormal abaxially derived ectopic leaf. Genetic analysis suggests that PVE1 functions in a separate pathway downstream of tasiARF biogenesis pathway (Zhang et al., 2012b), suggesting a more complex network controlling leaf initiation and patterning establishment. Additionally, mutation in ROUGH SHEATH2 (RS2), encoding a Myb protein that functions as a transcriptional regulator, has a reduced number of leaf founder cells in SAM and consequently semi-bladeless leaves (Schneeberger et al., 1998). RS2 negatively regulates KNOTTED1-LIKE HOMEBOX1 (KN1) genes (Marja et al., 1999), which needs to be absent in the leaf primordia in order to develop a new leaf (Jackson et al., 1994). The incomplete downregulation of KN1 in leaf primordia in rs2 mutants reduces the number of leaf founder cells in the mutant SAM, resulting in narrower or semi-bladeless leaves (Marja et al., 1999; Tsiantis et al., 1999). Another mutant narrow sheath (ns) displays a phenotype with narrow leaves and short, curved stems, conditioned upon homozygosity for each of the unlinked mutations ns1 and ns2 (Scanlon et al., 1996; Scanlon and Freeling, 1997). But different to the other mutants with narrow leaves, ns mediated narrow leaves phenotype does not extend into the upper portion of the leaf, which is derived from the central meristematic domain during leaf initiation, as postulated in the P2 stage (Scanlon and Freeling, 1997; Nardmann et al., 2004; Johnston et al., 2015). NS1 and NS2 are homologues of Arabidopsis PRESSED FLOWER (PRS). Also in the Arabidopsis prs mutants, the leaf margins are deleted in the lower leaves, suggesting that NS/PRS function similarly during the recruitment of founder cells from the lateral meristem domain during leaf primordia initiation (Nardmann et al., 2004).

Besides the genetic mutations in above discussed growth regulators, treatment with the exogenous auxin transport inhibitor 1-N-Naphthylphthalamic acid (NPA) also causes incomplete lateral leaf development (Scanlon, 2003). Through monitoring the localization of the polar auxin transporter PIN1 by a PIN1-YFP fusion, it was shown that PIN1 localization is associated with the recruitment of founder cells that give rise to the sheath margins (Johnston et al., 2015). These data indicate that founder cells are recruited from the central and lateral meristematic domains through different gene functions, and proper leaf initiation and formation of leaf margins in SAM are very important for leaf width determination.

Maize expansins are likely to be involved in determining the leaf width at the SAM and in leaf widening after leaf initiation. Especially three expansins, ZmEXPA4, ZmEXPA9 and ZmEXPB2, show
similar changes in expression and consistent association with the relative leaf widening rate (Muller et al., 2007). Evidence also suggests that these three expansins are implicated in integrating both environmental cues and developmental signals in the process of leaf widening (Muller et al., 2007).

The relation between leaf length and leaf width is revealed by classical genetics approaches

Leaf length and width seem to be complex traits, and the sources of genetic variation in leaf have been studied by using quantitative trait locus (QTL) mapping in many maize mapping populations (Reymond et al., 2004; Pelleschi et al., 2006; Tian et al., 2011; Ku et al., 2012; Guo et al., 2015). QTLs controlling leaf length and leaf width are clearly distinct in a RIL population derived from two parental lines F-2 and Io (Reymond et al., 2004). Similarly, QTLs for leaf length and leaf width in the population Yu82×Yu87-1 are located on different chromosomes (Ku et al., 2010). Another study using a B73×H99 RIL population showed that leaf width is not correlated with LER (Baute et al., 2015), suggesting that the two directions of growth may be controlled by different genetic processes. By using meta-analysis to integrate detected QTLs across several independent QTL studies, nine QTLs in total were identified for controlling leaf length and leaf width, but only two QTLs (predicted as candidate genes LIGULELESS2 and CYCD3;2) were responsible for both traits (Ku et al., 2012). Although these studies provide opportunities to reveal the genetic architecture for leaf growth in the length and width direction, few causal genes for the QTLs have been identified, leaving the molecular basis for regulating leaf length and leaf width and their interactions still ambiguous.

Leaf growth in stress conditions

Low temperature, drought, and high salinity are common stress conditions that adversely affect plant growth and crop production. In general, low temperature mainly results in mechanical constraint, whereas salinity and drought disrupt the ionic and osmotic equilibrium of the cell (Mahajan and Tuteja, 2005). However, one of the common symptoms of plants suffering from these stresses is growth inhibition, although the mechanisms leading to the growth inhibition are different.

Chilling stress

Chilling stress in maize affects photosynthesis by inhibiting the activity of certain enzymes of the C4 and the Calvin cycle (Kingston-Smith et al., 1997; Leipner and Stamp, 2009). The decreased
photosynthesis and low temperature induced accumulation of redox oxygen species (ROS) may cause a growth arrest and eventually result in cell death (Gómez et al., 2004; Suzuki and Mittler, 2006; Leipner and Stamp, 2009). However, even before visible chilling effects occur, suboptimal temperature inhibit leaf growth (Ben-Haj-Salah and Tardieu, 1995).

When maize was subjected to a cold night treatment without causing significant chilling symptoms, a decrease in leaf growth resulted in reduction of final leaf length (Rymen et al., 2007). The cell cycle duration was specifically prolonged by this cold stress, leading to a significant decrease of cell production. The delayed cell cycle progression was explained by the disturbed expression level of cell cycle and cell cycle related genes in the leaf division zone under cold treatment. Especially genes that control cell cycle entry were most significantly downregulated. Also the cell expansion rate was reduced in cold-treated leaves but the duration of cell expansion was prolonged, resulting in unaffected mature cell size (Figure 3) (Rymen et al., 2007). Transient exposure to chilling stress also affects cell division and cell expansion, but the effect is dependent on the development stage when the stress is applied: leaves that mainly contain dividing cells exposed to chilling temperatures showed a decreased cell number, but longer cell size due to a longer duration of cell expansion; while leaves exposed to cold during late growth stage produced shorter but equal amounts of cells compared to those in control leaves (Figure 3) (Louarn et al., 2010). Noticeably, a longer cell expansion period was observed when cell division was affected by cold, no matter in long term or short term stress, indicating that the compensation from cell expansion is independent of the length of the chilling stress period but associated with defected cell division as a result of chilling stress.

**Drought stress**

In response to drought stress, plants attempt to avoid detrimental dehydration by stomatal closure and growth inhibition under the regulation of abscisic acid (ABA) (Pei et al., 2000; Finkelstein, 2013). At the whole plant level, slightly elevated ABA levels (characteristic of mild drought stress conditions) promote root growth but inhibit shoot growth (Finkelstein, 2013). In maize, leaf growth reduction is one of the most sensitive processes to drought, as leaf growth rate decreases immediately when soil water content is lowered and it is correlated to the severity of drought (Acevedo et al., 1971; Tardieu et al., 2000; Nayyar and Gupta, 2006; Avramova et al., 2015b). At low transpiration rates the effect of the soil water status on LER was related to the concentration of ABA in the xylem sap and could be mimicked by feeding exogenous ABA. Conversely, ABA could not account for LER reduction for plants at high evaporative demand. Therefore, at least two effects cause the growth reduction in early drought response: ABA signaling and probably a hydraulic signal (Salah and Tardieu, 1997). A model
based on the rhythmic leaf growth and the root aquaporin transcripts abundance in roots suggests that growth can be stimulated in water deficit conditions by improving the plant hydraulic conductance, through high circadian oscillation amplitudes of root hydraulic conductance to increase root water uptake (Caldeira et al., 2014a). Moreover, ABA was further shown to rescue the reduced LER by improving plant hydraulic properties via aquaporin activity, indicating a crosstalk with ABA and plant hydraulic signals in the maintenance of a favorable plant water status for leaf growth (Parent et al., 2009; Caldeira et al., 2014b).

Recently, leaf growth under drought condition was characterized at the cellular level using kinematic analysis. Both mild and severe drought reduced LER and thereby final leaf length by altering the growth processes: a significant reduction of the number of dividing cells was observed in both drought treatments, but the cell division rate was only affected by severe drought; cell expansion was not significantly affected in mild drought stress, but cell expansion rate was strongly inhibited while the time in expansion zone was significantly prolonged in severe drought stress (Figure 3) (Avramova et al., 2015b).

Transcriptome analysis on three samples, representative for the DZ, EZ and MZ, revealed that cell cycle-related genes, including cyclin dependent kinases, cyclins, minichromosome maintenance transcripts, and subunits of Anaphase Promoting Complex were significantly downregulated in the division zone by drought. These data indicate that the reduced cell division activity by drought can be explained by the transcriptional downregulation of all stages of the cell cycle (Avramova et al., 2015b). The lowered cell cycle activity under drought was also reflected at protein level, as the phosphorylation status of many proteins involved in chromatin remodeling and cell division changed when exposed to severe drought (Bonhomme et al., 2012). Instead of completely terminating the cell cycle, about one third of the phosphorylation status of chromatin or cell cycle related proteins can be recovered by rehydration of maize leaves. Kinases or phosphatases rapidly (within half an hour) react to the rehydration, and hormone-related and signaling proteins, transcription regulators, and chromatin-related or cell cycle-related proteins gradually recovered as the time after the rehydration increased (Bonhomme et al., 2012).

For cell expansion, increasing drought levels progressively increased hydrogen peroxide (H$_2$O$_2$) levels and the transcriptional level of two peroxidases throughout the growth zone of maize leaf (Avramova et al., 2015b). Peroxidases control the availability of H$_2$O$_2$ in the cell wall and therefore directly the expansion process (Passardi et al., 2005). When the increase in peroxidase activity and the formation of ROS occur in the same tissue, cross-linking of cell wall components might strengthen the mechanical properties of the wall (Passardi et al., 2005; Wakabayashi et al., 2012; Tenhaken, 2014).
Accordingly, many studies have showed that plants under drought stresses increase the transcript level of peroxidases (Kim et al., 2008; Sečenji et al., 2010; Maia et al., 2013), suggesting that increased peroxidase and H$_2$O$_2$ participate in reducing cell expansion under drought stress in maize.

**Salt stress**

Salt in soil water reduces the plant’s ability to take up water and leads to slower growth. Next the salt may enter in the transpiration stream and eventually injure cells in the transpiring leaves, even further reducing growth (Munns, 2005). The primary response of reduced leaf growth is thus mainly due to water deficit and salt uptake, while the subsequent step may cause leaf death from the ion toxicity in transpiring leaves (Lazof and Bernstein, 1998; Munns, 2005).

The maximal LER of maize leaves was decreased by salt stress, and the relative growth rate decreased dramatically at the distal elongation zone indicating that salinity significant affects cell expansion (Figure 3) (Neves-Piestun and Bernstein, 2001). The increase of the apoplastic pH may reduce the activation of wall-loosening enzymes such as expansins, and as such cause shoot-growth reduction (Pitann et al., 2009). By using two maize varieties with different degrees of salt tolerance, it was shown that the epidermal cell wall loosening was associated with leaf size, a process that was impaired under salt stress (Zorb et al., 2015). The more sensitive variety exhibited a reduced abundance of the cell wall-loosening $\beta$-expansin protein following a high salinity treatment, while the $\beta$-expansin protein abundance was not affected in the more tolerant variety. The reduction of the $\beta$-expansin protein content contributed to the stiff epidermal cell walls, and consequently resulting in a significant leaf growth reduction (Zorb et al., 2015). Although salt stress has been reported to inhibit cell division through an osmotic stress (Zhu, 2001; Mahajan and Tuteja, 2005), the relation between salt and cell division is not clear yet in maize.
Gibberellins in leaf growth

Bioactive gibberellin (GA) accumulation specially determines the transition between cell division and cell expansion during maize leaf growth (Nelissen et al., 2012). In addition, GAs play crucial roles in many other plant developmental stages including germination, stem elongation, flower and fruit development and affect both cell division and expansion (Olszewski et al., 2002; Hedden, 2003; Ueguchi-Tanaka et al., 2007; Schwechheimer and Willige, 2009). GAs played a pivotal role in the “Green Revolution”, resulting in dramatic increases in crop productivity by introducing semi-dwarf phenotype (Evenson and Gollin, 2003), that decreases lodging and increases the partitioning of assimilates into the grain rather than into the stem or leaves and as such increasing the harvest index (Hedden, 2003). The genetic basis for these high-yielding semi-dwarf varieties can be traced back to the mutation of a small number of genes that are mostly involved in the biosynthesis and signaling pathways of GAs (Peng et al., 1999; Monna et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002).
GA metabolism

The major bioactive GAs (including GA1, GA3, GA4 and GA7) were derived from geranylgeranyl diphosphate (GGDP) and synthesized through a complex pathway (Figure 4) (Yamaguchi, 2008). Three classes of enzymes are required for the biosynthesis of bioactive GAs in plants: terpene synthases (TPSs), cytochrome P450 monooxygenases (P450s), and 2-oxoglutarate-dependent dioxygenases (2ODDs). Two TPSs, ent-copalyl diphosphate synthase (CPS) and ent-kaurene synthase (KS) are involved in the conversion of GGDP to ent-kaurene. Both CPS and KS are located in the plastids (Sun and Kamiya, 1997). Two P450s, ent-Kaurene oxidase (KO) and ent-Kaurenoic acid oxidase (KAO), convert ent-Kaurene into ent-kaurenoic acid and then into GA12 and are located in the outer membrane of the plastids and the endoplasmic reticulum, respectively (Helliwell et al., 2001). GA12 is next converted into bioactive GAs by GA20-Oxidase (GA20OX) and GA3-Oxidase (GA3OX), both of which are soluble 2ODDs. There are two different pathways to synthesize the bioactive GA: i) the non-13-hydroxyl pathway forms from GA12, ii) the 13-hydroxyl pathway starts from GA53 formed by GA13OXIDASE (GA13ox) using GA12 as substrate. The bioactive GA4 and GA7 are synthesized through the non-13-hydroxyl pathway, while bioactive GA3 and GA3 are derived from the 13-hydroxyl pathway.

Deactivation is important for effective regulation of the concentrations of bioactive hormones and for GAs, the deactivation is accomplished in several different ways. The best known deactivation reaction is catalyzed by GA2-oxidases (GA2oxs), which are a class of 2ODDs. Class I and II GA2oxs are reported to hydroxylase bioactive GAs and their immediate precursors, GA9 and GA20 (Lee and Zeevaart, 2005), while class III GA2oxs are likely to play a role in depleting pools of precursor GAs (such as GA12 and GA53) (Schomburg et al., 2003). Besides GA2oxs, other enzymes have been shown to deactivate GAs in some plant species: a P450 enzyme EU1/CYP714D1 catalyzes 16α,17-epoxidation of GAs in rice, GAMT1 and GAMT2 encode enzymes (gibberellin methyltransferase) catalyze methylation of C-6 carboxyl group of GAs in Arabidopsis seeds (Varbanova et al., 2007). Whether those GA deactivation ways are common among different plant species has yet to be investigated.
Figure 4. The gibberellins (GAs) biosynthesis pathways from GGPD and deactivation by GA2-oxidase in plants. Bioactive GAs found in a wide variety of plant species (highlighted grey) are shown. In each metabolic reaction, the modification is highlighted in color. GA7 (13-nonhydroxy GA3) is biosynthesized from GA9 in a similar pathway to the synthesis of GA3 from GA20, but is not shown in this figure. 2ox, GA2-oxidase (Class I and II); 2ox*, GA2-oxidase (Class III); 3ox, GA3-oxidase; 13ox, GA13-oxidase; 20ox, GA20-oxidase; GGDP, geranylgeranyl diphosphate; ent-CDP, ent-copalydiphosphate; CPS, ent-copalydiphosphate synthase; KS, ent-kaurene synthase; KO, ent-kaurene oxidase; KAO, ent-kaurenoic acid oxidase. The figure is adapted from (Yamaguchi, 2008).

The GA-GID1-DELLA signaling pathway

During the past decade, most of the components of the GA signaling pathway have been identified through genetic approaches (Murase et al., 2008) (Figure 5). Key components of GA signaling pathway include the GA receptor Gibberellin Insensitive Dwarf1 (GID1), the DELLA growth inhibitors (DELLAs) and the F-box proteins. Initially, GID1 binds with high affinity to bioactive GAs, inducing a
conformational switch stabilizing the GA-GID1-DELLA complex. This enables the recognition of the SCF (SKP1, CULLIN, F-box) E3 ubiquitin-ligase complex and subsequent degradation of DELLAs by the proteasome. Absence of DELLAs allows activity of transcription factors that start expression of the GA response genes (Figure 5) (Davière and Achard, 2013; Claeys et al., 2014).

Through interaction of DELLAs with transcription factors and transcriptional regulators, GA signaling controls the expression of a multitude of target genes functioning in distinct pathways. The GA-DELLA signaling pathway controls seed germination by targeting SCARECROW-LIKE 3 (SCL3) (Zhang et al., 2011). Decreased GA level by red light stabilizes DELLAs, and further inhibits cell expansion during Arabidopsis hypocotyl growth by interacting with PHYTOCHROME INTERACTING FACTOR 3 (PIF3) and PIF4, central transcription factors that activate the transcription of cell expansion-promoting genes (de Lucas et al., 2008; Feng et al., 2008). Moreover, the interaction between DELLAs and PIF3 can be inhibited by JASMONATE-ZIM-DOMAIN PROTEIN 9 (JAZ9) for growth promotion (Yang et al., 2012). DELLAs are also suggested to be involved in floral organ development by targeting phytohormones and stress signaling pathways, such as genes responsive to ABA and ethylene-related genes (Hou et al., 2008). So far, many DELLA target genes have been identified by transcriptome analysis using inducible gene expression system in Arabidopsis, which allows to detect the molecular events controlled by DELLAs over time (Zentella et al., 2007; Hou et al., 2008; Claeys et al., 2014).

Figure 5: Gibberellin signaling. In the absence of GAs, DELLAs repress GA responses by forming DNA-binding complexes to affect gene expression, and by sequestering TFs that promote GA responses, such as PIF4. When GAs bind their receptor GID1, this triggers the formation of an SCF complex that ubiquitinates DELLAs, causing their proteasomal degradation. This allows GA response-promoting TFs to become active. This figure is adapted from (Claeys et al., 2014).
**GA mediate cell division and cell expansion**

GA levels appear to be required to promote and maintain the increase in root growth rate through control of root meristem size (Ubeda-Tomás et al., 2009). Reduction of endogenous GA levels by treating wild-type seedlings with Paclobutrazol (PAC, an inhibitor of GA biosynthesis) or in GA biosynthesis deficit mutants result in a reduced root meristem size and as a consequence reduced growth rate. This meristem size reduction can be rescued by external application of bioactive GA. The role of GA in determining the transition between in cell division and cell expansion in the Arabidopsis root reminisces the function of GAs during maize leaf growth. Overexpression of GAs biosynthetic enzyme, GA20OX promotes maize leaf growth by delaying the transition from cell division to expansion (Nelissen et al., 2012). In contrast, the growth promoting effect of GA seems to be organ and/or species dependent, given that in Arabidopsis, overexpression of the same gene results in increased leaf size from both increases in cell number and cell size (Gonzalez et al., 2010). GA effects on cell expansion have been shown during Arabidopsis hypocotyl and root growth as well. During hypocotyl elongation, DELLAs interact with the prefoldin (PFD) complex in the absence of GAs, and retain it in the nucleus. PFD is required for proper folding of tubulin in the cytosol and without it the crucial arrangement of cortical microtubule, anisotropic cell expansion cannot be formed, affecting both expansion rate and direction (Locascio et al., 2013). By ectopically expressing the non-degraded DELLLA protein in Arabidopsis root, the GA-mediated removal of DELLA in the endodermis was found to be crucial for proper cell expansion (Ubeda-Tomás et al., 2008). Although many studies reveal growth regulators involved in GA-DELLLA pathway controlling cell division and expansion, a meta-analysis using available transcriptome data from Arabidopsis showed that growth-regulating genes in response to GA-DELLLA appear to be tissue- and condition-specific (Claeys et al., 2014), indicative for a large unknown network. With the requirement for dissecting tissue-, condition- and/or developmental-specific samples, larger model plants than Arabidopsis hold great promise. Thus, it is interesting to explore the growth-regulating network in which GA-DELLLA functions by using maize leaf as a model.

**Conserved mechanisms in regulating leaf and ear development**

As a source organ to gather energy by photosynthesis, leaves make a major contribution to the grain yield in crops. This perspective is studied on the effect of defoliation on grain yield, showing that defoliation during ear and kernel development severely affects kernel initiation and grain dry matter accumulation (Egharevba et al., 1976; Tollenaar and Daynard, 1978). By contrast, the extent of leaves
contributing to yield was evaluated using a maize Leafy hybrid, which containing higher green leaf area than the conventional hybrid at silking stage (Subedi and Ma, 2005). Although the maize Leafy hybrid contains more leaves above the ear, it produces a comparable grain yield than conventional hybrid. However, a more pronounced reduction in grain yield (both kernel number and kernel dry weight) was observed in the Leafy hybrid compared with conventional hybrid when all leaves above the ear-leaf were removed. These data indicated that leaves provide significant contribution to grain yield, but there is probably a threshold of this contribution (Subedi and Ma, 2005).

In addition to the fact that leaves act as an energy source to support reproductive growth, research in Arabidopsis and rice shows that many growth regulators, especially cell division regulators, have a conserved function in regulating vegetative and reproductive growth (Mizukami and Fischer, 2000; Hu et al., 2003; Disch et al., 2006; Anastasiou et al., 2007; Li et al., 2008). Therefore, we evaluated whether the same growth regulating pathways are conserved in leaf and ear development in maize. Maize ear forms laterally in axils of leaves, similarly, leaf and ear are derived from meristems, structures that harbor pluripotent stem cells: leaves are created at the SAM, while axillary meristems (AM) gives rise to ears. Many proteins controlling stem cells maintenance affect leaf and ear development in maize. Mutants of the maize CLV receptors, FASCIATED EAR2 (FEA2, maize ortholog of CLV2) and THICK TASSEL DWARF1 (TD1, maize ortholog of CLV1) showed similar fasciated ears (Taguchi-Shiobara et al., 2001; Bommert et al., 2005). Mutants of the maize COMPACT PLANT2 (CT2), which encodes a predicted GTP binding protein, resembles fea2 and td1 ear phenotype, and also exhibit a shorter stature, and shorter and wider leaves (Bommert et al., 2013). Genetic interactions suggest TD1 and CT2 act in different pathway, while FEA2 is epistatic to CT2 to control meristem size (Bommert et al., 2013). Another newly discovered pathway, which is distinct from the CLV-WUS signaling cascade in maize, consists of two genes that antagonistically regulate SAM size: FASCIATED EAR4 (FEA4) encoding a bZIP transcription factor negatively regulating SAM size (Pautler et al., 2015), and MALE STERILE CONVERTED ANther 1 (MSCA1) encoding a glutaredoxin gene specifically expressed at the P0 domain and positively regulating SAM size (Yang et al., 2015). Besides the similar fascination in the ear, fea4 shows a semi-dwarf stature, while aberrant phyllotaxy2 (abph2, the mutation caused by transposition of MSCA1) has decussate phyllotaxy (Pautler et al., 2015; Yang et al., 2015). These observations demonstrate that stem cell maintenance is crucial for organ development, although several mutations in different genes result in similar defects in ear development, but the more diverse leaf growth phenotype may provide cues for their actual functions.

After the initiation at the axillary meristem (AM), the inflorescence meristem (IM) grows
longitudinally from tip to base. Later during ear development more meristems initiate from the developing IM, including spikelet pair meristems (SPMs), spikelet meristems (SMs) and floret meristems (FM) (Figure 6) (Gallavotti et al., 2008; Eveland et al., 2014). As a more complex developmental process, the study on ear development has recently advanced by the ability to study auxin related signal and transport at cellular level using reporter constructs. Auxin response maxima was detected on the flanks of IM to direct SPMs formation, and then auxin is translocated in the subsequent emerging lateral SMs and glume primordia (Gallavotti et al., 2008; Eveland et al., 2014). Therefore, similar to auxin function in leaf development, polar auxin transport directs auxin maxima in the developing ear is also involved in primordia initiation (Figure 6). Additionally, several genes have been shown that control cell differentiation during both leaf and ear development. SQUAMOSA PROMOTER BINDING (SBP)-box transcription factors TASSELSHEATH4 (TSH4) and UNBRANCHED2 (UB2) and UB3, which are targeted by miRNA156, share redundant function in regulating lateral organ growth. Mutants with elevated expression level of miR156 genes (a dominant mutant Corngrass1) or decreased SBP-box transcription factors expressions (ub2 ub3 tsh4) produce more tillers and leaves (Chuck et al., 2007; Chuck et al., 2014). Strikingly, an increased kernel row number was also observed in triple mutant ub2 ub3 tsh4. It is hypothesized that the SBP-box transcription factors limit the rate of cell differentiation to the lateral domains of meristems. These findings also suggest that at least in some range the leaf and spikelet meristem initiation both are controlled by the SBP-box transcription factors (Figure 6) (Chuck et al., 2014). After stem cells initiation, a growth regulating complex ANGUSTIFOLIA3 (AN3) associated the SWI/SNF chromatin remodeling complex is highly active during leaf development and is also present in developing ear (Figure 6) (Nelissen et al., 2015). Overexpression the miRNA396a-resistant GROWTH-REGULATING FACTOR1 (GRF1), one of the components of the SWI/SNF protein complex directing cell division in the growing leaf, has a severely disturbed female fertility (Nelissen et al., 2015). Moreover, the maize ARGOS1 (ZAR1) enhances maize organ growth, causing the formation of larger leaves and longer ears with increased kernel numbers per ear (Guo et al., 2014; Shi et al., 2015).

Taken together, many genes involved in controlling ear growth from the axillary meristem seem to be present in SAM, indicating a similarity in growth initiation from meristematic cells. Besides, although the studies on mechanism involved in maize ear development, especially in ear length and width are still limited, it is becoming increasingly clear that some growth mechanisms are conserved in different organs.
Figure 6. Schematic figure showing the structures and the controlling molecular regulators of the developing maize leaf and ear. The different events and genetic pathways that could influence the leaf (green) and ear (orange) growth are shown. The auxin maxima determines leaf initiation at shoot apical meristem and spikelet meristem formation in developing ear. SBP-box transcription factors were repressed by miR156, and negatively control leaf and spikelet meristem initiation. The ANGUSTIFOLIA3-SWI/SNF complex regulate leaf growth including cell division and cell expansion, and ear development by targeting different growth-regulating factors in different growth stages and/or organs. DZ: division zone; EZ: expansion zone; MZ: mature zone; TZ: transition zone; IM, inflorescence meristem; SPM, spikelet pair meristem; SM, spikelet meristem; gl, glume.
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Maize leaf as a model to study growth


Maize leaf as a model to study growth


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Maize leaf as a model to study growth


Maize leaf as a model to study growth


Chapter 2

The interplay between mild drought and
Gibberellic Acid metabolism
in the growing maize leaf
The interplay between mild drought and Gibberellic Acid metabolism in the growing maize leaf

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AUTHOR CONTRIBUTION


Manuscript is in preparation and will be submitted for publication after minor changes to text.
Abstract
Growth is characterized by the interplay between cell division and cell expansion. In the maize leaf, the two processes occur very localized, but spatially separated along the growth zone which is located at the base. To further gain insight into the transition between cell division and cell expansion, conditions were selected in which the position of this transition zone was positively and negatively affected by overexpression of the rate limiting gibberellic acid (GA) biosynthesis gene GA20-Oxidase (GA20OX-1) and mild drought, respectively. High levels of GA in GA20OX-1OE plants shift the transition more distally while mild drought, that is associated with lowered GA biosynthesis, resulted in a more basal positioning of the transition. However, the increased levels of GA in the GA20OX-1OE line are insufficient to convey tolerance to the mild drought treatment, indicating that another mechanism besides lowered GA levels is restricting growth during the drought response. Next, transcriptome analysis with high spatial resolution was performed showing that mild drought specifically induces a reprogramming of transcriptional regulation in the division zone. “Leaf Growth Viewer” was developed as an online searchable tool containing the high resolution transcriptome data along the growth zone of the two conditions in which the transition zone was oppositely affected. In addition, the combination of the cellular and transcriptomics data showed that the growth reduction caused by mild drought is counteracted by a prolonged duration of growth by postponing the cell cycle arrest at the transition zone.
Introduction

Plants are continuously producing organs that grow to fulfill specific roles for plant development and reproduction. In addition, the sessile nature of plants urges them to adjust their growth and development when changes take place in the environment. Therefore, it is pivotal to gain a better insight in the coordination of the growth processes of cell division and cell expansion in a growing organ and to understand how both intrinsic signals and environmental cues impinge on these. Depending on the organ, the processes of cell division and cell expansion are strictly spatially and/or temporally regulated (Gonzalez et al., 2012; Nelissen et al., 2012; Sozzani and Iyer-Pascuzzi, 2014). The identification of distinct molecular mechanisms involved in this decision (Breuninger and Lenhard, 2010; Gonzalez et al., 2012; Sozzani and Iyer-Pascuzzi, 2014; Avramova et al., 2015a) further consolidates the concept that transitioning from cell division to cell expansion is an important developmental switch.

The importance of the transition between cell division and cell expansion is also reflected by the relatively large number of genes and molecular pathways involved in this mechanism which only represents one of the five known mechanisms determining dicot leaf size (Gonzalez et al., 2012). The genes involved in the transition from cell division to cell expansion function in transcriptional regulation (Mizukami and Fischer, 2000; Nath et al., 2003; Horiguchi et al., 2005; Gonzalez et al., 2010; Vercruyssen et al., 2014), protein degradation (Disch et al., 2006; Li et al., 2008), hormone metabolism and signaling (Hu et al., 2003; Achard et al., 2009) and even the production of a non-cell-autonomous growth promoting signal (Kazama et al., 2010; Czesnick and Lenhard, 2015). In Arabidopsis, the cell cycle arrest front, visualized by a CYC LINB1;1 reporter gene (Donnelly et al., 1999) is often used together with the analysis of growth over time (kinematic analysis) (Andrianakaja et al., 2012; Nelissen et al., 2013), as a tool to show alterations in the transition from cell division to cell expansion. Differences in the cell cycle arrest front were observed in genetic (e.g. ectopic expression of ANTEGUMENTA (Mizukami and Fisher, 2000) or ANGUSTIFOLIA3 (Vercruyssen et al., 2014)) and environmental (e.g. osmotic stress; (Skirycz et al., 2011b)) perturbations. In addition, in Arabidopsis, mild osmotic stress resulted in a pausing of the cell cycle progression that allowed for a quick recovery when the stress was alleviated (Skirycz et al., 2011b).

Indeed, plants adjust their growth in order to cope with abiotic stresses as they have no possibility to escape changes in the environment. One of the most economically relevant abiotic stresses is drought, as globally agriculture is practiced in areas where the water supplies are often insufficient to compensate for evapotranspiration. Therefore, crops cannot realize their full yield potential. Long
term drought is a severe problem but even short term drought decreases crop productivity (Kostandini et al., 2009). Despite the economic impact of drought, the exact molecular networks that function to adjust plant growth to water deficit still remain elusive. It is however clear that water deficit only marginally affects photosynthesis mechanism or even results in the activation of the carbon metabolism (Skirycz et al., 2011a; Tardieu et al., 2011). The resulting increased carbohydrate concentrations in plant tissues are thought to serve in osmotic adjustment and are incorporated in structures such as cell walls (Tardieu et al., 2011).

In the maize leaf, the growth processes are spatially separated: at the base of the leaf, cells are actively dividing. As the cells move upwards in the leaf, they transition from dividing to expanding and later from expanding to mature (Nelissen et al., 2013). As the regions in the maize growth zone in which these processes occur are quite large, encompassing more than 10 mm, they are referred to as division zone (DZ), expansion zone (EZ) and mature zone (MZ). The respective transitions are defined here as transition zone 1 (TZ1) and transition zone 2 (TZ2). In the maize leaf, the TZ1 was shown to be characterized by a local accumulation of bio-active gibberellic acid (GA) (Nelissen et al., 2012). This GA peak was instrumental to determine the position of TZ1, and thus in defining the position where cells decide to exit cell division and to enter cell expansion (Nelissen et al., 2012). Also in roots, another system in which growth processes are linearly, spatially organized, genetic evidence suggested a role of GA in the transition between cell division and cell expansion (Ubeda-Tomás et al.; Birnbaum et al., 2003). Alternatively, it was suggested that water deficit affects the growth processes depending on the position along the growth zone: in the division zone, a reduced cell division rate that stopped at a closer distance from the leaf insertion point was associated with water deficit. A lowered expansion rate in the expansion zone resulted in a decrease in final cell size under water deficit (Tardieu et al., 2000; Tardieu et al., 2011). At the molecular level, mild drought resulted in a downregulation of the cell cycle in the division zone and an upregulation of photosynthesis and oxidative stress in both division and expansion zone (Avramova et al., 2015b).

The aim of this study is to gain further insight in the molecular process that dynamically regulated the transition from cell division to cell expansion in growing maize leaves. A high resolution transcriptomics study throughout the growth zone showed that previously described division and expansion zone, based on microscopical analyses, can be further subdivided in a basal and distal zone, based on molecular profiles. Furthermore, many processes are transcriptionally regulated exactly at the TZ1, reinforcing the major developmental switch that occurs when cells stop to divide and enter the expansion zone. In order to further functionally characterize this TZ1 in more detail, we examined the effects of perturbations that positively (overexpression of GA biosynthetic enzyme GA20OX-1 (Nelissen et al., 2012) or negatively affect (mild drought stress) the position of the TZ1.
Strikingly, a reduced level of bioactive GA (GA$_1$ and GA$_4$) and its biosynthetic precursors was observed at TZ1 under mild drought conditions. However, merely increasing the GA levels was insufficient to overcome this mild drought phenotype as the GA20OX-1$^{OE}$ line was not more drought tolerant, compared to its non-transgenic siblings. Next, we analyzed the high resolution transcriptome data of mild drought treated plants and GA20OX-1$^{OE}$ plants by aligning the samples according to the relative position of the transition zone, rather than merely comparing samples at the absolute same distance from the base of the leaf. In this manner, numerous genes were identified that are specifically in the transition zone affected by either mild drought or high GA levels. Our data reveal growth-zone specific, mild drought induced changes in transcription factors, E2F/DP target genes, aquaporins and photosynthesis. In addition, in combination with the cellular analysis, these data provide the basis for a compensatory mechanism that allows for maize leaves to cope with the reduced growth rate, by maintaining the capacity to resume growth for a longer period. To visualize these high resolution data and to provide the research community access in a user-friendly format, we present “Leaf Growth Viewer” (LGV) as an online, searchable tool.

Results

*Mild drought affects the position of the transition between the division zone and expansion zone*

To further functionally characterize the transition zone between cell division and cell expansion, we aimed to look for conditions that positively and negatively perturbed the size of the transition zone. Previously, we showed that overexpression of the rate limiting GA biosynthetic enzyme GA20-Oxidase (UBIL::GA20OX, the line containing the highest expression level is referred to as GA20OX-1$^{OE}$) has an enhancing effect on the size of the DZ and thus results in a more distal position of the TZ1 (Nelissen et al., 2012). In addition, it was previously reported that drought could negatively affect cell division and thus positioned the transition zone more basally in the fifth leaf of B73 (Avramova et al., 2015b). Here, we determined the drought conditions that affect the position of the transition zone, without the unwanted physiological effects of leaf rolling and leaf wilting in B104, an inbred that is closely related to B73 (Liu et al., 2003) and that routinely can be transformed (Frame et al., 2006; Coussens et al., 2012).

A mild drought stress, consisting of watering the plants only with 70% of the soil water content of the controls reduced the leaf elongation rate with 28.8%, without causing visual physiological effects.
Drought and GA affect maize leaf growth

such as leaf rolling or wilting. A kinematic analysis (Nelissen et al., 2013) performed during the steady-state growth of the maize leaf (Table 1; Supplemental Table 1) under control and mild drought revealed that this growth reduction was in part caused by a reduction of the size of the DZ (22%, \(p=0.039\)) resulting in a significant reduction in the number of cells that are part of the DZ (14 \%, \(p=0.005\)), and thus a more basal position of TZ1. Oppositely to what was described for mild drought in Arabidopsis (Skirycz et al., 2011b) and severe drought stress in maize (Avramova et al., 2015b), this mild drought did not pause or delay the progression through the cell cycle as the duration of one cell cycle and hence the rate of cell division was not significantly altered (\(p=0.454\) and 0.439, respectively). Besides an effect on cell division, also a reduction in cell expansion was observed, as the mature cell size was significantly lowered by 15 \% (\(P=0.036\)), showing that mild drought conditions affected both growth processes. While both cell division and cell expansion were negatively affected by mild drought (22% and 15%, respectively), the final reduction in leaf length was less pronounced. To investigate the reason for this compensation, we used LEAF-E (Voorend et al., 2014) to calculate the duration of growth, also referred to as leaf elongation duration (LED). The LED was significantly increased by 14 \% (\(p=0.0025\)), indicating that under mild drought the growth rate or LER was reduced but growth was maintained for a longer period of time (increased LED). The same mild drought treatment resulted in a similar repositioning of the transition zone between cell division and cell expansion and an increase in LED for B73 (Table 1), the reference inbred with a fully sequenced genome (Liu et al., 2003).

To visualize the effect of prolonged LED at the cellular level, the size of the division zone was monitored over time under mild drought and well-watered conditions. During early leaf growth the size of the division zone remains more or less constant and referred to as steady state growth (Figure 1) and six days after the appearance of leaf four, the LER profile of the well-watered plant declined (Figure 1A), a process that takes place in parallel to the decrease in the size of the DZ (Figure 1B). At the same time point, the size of the division zone (Figure 1B) and the LER profile (Figure 1A) are not yet decreasing in the mild drought treated plants, a process that starts at least one day later. These data show that the mild drought induced reduction in LER during steady state growth might at least in part be compensated by maintaining steady state growth and cell division active for a longer period of time.

Taken together, mild drought and GA20OX-1\(^{OE}\) plants both affect the number of dividing cells and in the linear organization of the growth zone, this is reflected in a more basally and more distally positioned transition between cell division and cell expansion (TZ1), respectively. The change in size of the division zone also resulted in shift in the position of the transition zone between cell expansion and mature cells (TZ2) (Table 1; Supplemental Table1; Figure 2).
Drought and GA affect maize leaf growth

Figure 1. Effects of mild drought on LER and LED (A) and the size of the division zone over time (B). The blue arrows indicate LED and the green arrow indicates the difference in LER. A subset of the plants grown for (A) were sampled at different time points during leaf four appearance to determine the size of the DZ (B). *** and * indicate significant differences using a Student T-Test with p-value 0.01 and 0.05, respectively. The error bars represent standard error. For at least 17 plants leaf length was monitored until leaf four stopped growing (A) and per time point five plant were sampled for each condition to determine the size of the DZ (B).

Table 1. Overview of the effects of mild drought on leaf growth and cellular parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>B104</th>
<th>B73</th>
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<tbody>
<tr>
<td>LER (mm/h)</td>
<td>-28 (8.10^-5)</td>
<td>-30 (0.008)</td>
</tr>
<tr>
<td>P (Cell/h)</td>
<td>-16 (7.10^-4)</td>
<td>-18 (0.05)</td>
</tr>
<tr>
<td>Ldi (mm)</td>
<td>-22 (0.04)</td>
<td>-34 (0.03)</td>
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<tr>
<td>Ndi (Cells)</td>
<td>-14 (0.005)</td>
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<td>D (Cells/cell.h)</td>
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<td>-4 (0.8)</td>
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<tr>
<td>Ima (µm)</td>
<td>-15 (0.04)</td>
<td>-15 (0.07)</td>
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<tr>
<td>Lez (mm)</td>
<td>-7 (0.6)</td>
<td>-7 (0.3)</td>
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Percentages of growth related parameters of drought relative to the well-watered plants for the inbred lines B104 and B73. P-values as obtained by Student-test are indicated between parentheses (n=3). For the absolute numeric values see Supplementary Table1.

FLL: final leaf length; LER: leaf elongation rate; P: cell proliferation rate; Ldi: division zone size; Ndi: number of dividing cells; D: cell division rate; Ima: mature cell size; Lez: expansion zone size.
Mild drought lowered levels of auxin and cytokinin in the division zone and of gibberellic acid at the transition zone

Previously, it was shown that the auxin indole-3-acetic acid (IAA), the cytokinins trans-zeatin (tZ) and N6-(α2-isopentenyl)adenine (iP) and gibberellic acid (GA₁ and GA₄) had pronounced accumulation patterns within the maize leaf growth zone, while the levels of salicylic acid (SA) and abscisic acid (ABA) did not change significantly over the maize growth zone (Nelissen et al., 2012). Here, the effect of the repositioning of the transition zone by elevated GA levels and mild drought was assessed at the hormone level. Both mild drought and GA20OX-1⁰E plants were grown with their respective controls (well-watered and non-transgenic segregating siblings, respectively). The growth zone was sampled every 0.5 cm, while the remainder of the leaf until cm ten was sampled in one cm pieces (Figure 2). As the TZ1 in B104 is positioned around 1.3 cm, the most basal half cm reflects the basal division zone, while the second half cm represents the distal division zone. The third half cm coincides with the location of the transition between cell division and cell expansion, and thus contains cells that are both dividing and expanding. The fourth half cm contains cells in early cell expansion, while later phases of cell expansion and cell maturity are present in the successive 1 cm long leaf samples taken along the leaf gradient (Figure 2). The TZ2 is located between 4-5 cm in B104.

We previously showed that there was a large increase in bioactive GAs in GA20OX-1⁰E plants (Nelissen et al., 2012) (Figure 3A). In addition, the higher GA levels in GA20OX-1⁰E resulted in elevated levels of IAA (Figure 3B), ABA (Figure 3C), jasmonoyl-isoleucine (JA-Ile) (Figure 3D). Cytokinin levels (iP and tZ) are unaffected in the division zone of the GA20OX-1⁰E line, but showed a delayed rise towards the end of the expansion zone (Figure 3E). This rise in iP corresponds to the position of TZ2 which is shifted to a more distal position as compared to the non-transgenic siblings (Figure 2), indicating that iP played an important role at the transition between cell expansion and cell maturity. SA levels are not significantly affected in the GA20OX-1⁰E plants (Figure 3F).
Drought and GA affect maize leaf growth

**hormone analysis**

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**transcript analysis**

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<td>control GA20OX1OE</td>
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Figure 2. Schematic overview of the sampling strategy of leaf four samples for hormone and transcriptome analysis. The transition between cell division and cell expansion (TZ1) is indicated by a red line and the transition between cell expansion and mature cells (TZ2) is indicated by a black line. Although the boundaries between different zones as shown fixed, the transition between different zones is a gradual process. To investigate the robust transcriptional changes with high resolution in the growth zone, the WT samples from the mild drought and GA20OX-1OE experiment were analyzed together (bottom panel). DZ: division zone; TZ: transition zone; EZ: expansion zone; MZ: mature zone.

Under the mild drought conditions, the levels of ABA and SA (Figure 4 C, F) showed a strong increase, indicating that although no leaf rolling or leaf wilting was observed, the observed growth reduction due to mild drought was reflected by a molecular stress response. Strikingly, the levels of SA under mild drought significantly increased between the basal and distal division zone to stay maximally throughout the expansion and mature zone (Figure 4 F). No significant differences between mild drought and well-watered plants are found for JA and JA-Ile levels (Figure 4 D).
Drought and GA affect maize leaf growth

Figure 3. Hormone metabolites along the growth zone of GA20-Oxidase overexpressing plants and their non-transgenic siblings. Symbols are averages ±SE (n=5). ABA=abscisic acid; iP=N6-(△2-isopentenyl) adenine; tZ=trans-zeatin; GA=gibberellic acid; IAA=Indole-3-acetic acid; JA=jasmonic acid; JA-Ile=jasmonic acid-isoleucine; SA=salicylic acid.
Drought and GA affect maize leaf growth

Figure 4. Hormone levels along the growth zone under drought and control conditions. Symbols are averages ±SE (n=5). ABA=abscisic acid; iP=N6-(Δ2-isopentenyl) adenine; tZ=trans-zeatin; GA=gibberellic acid; IAA=Indole-3-acetic acid; JA=jasmonic acid; JA-Ile=jasmonic acid-isoleucine; SA=salicylic acid.

The levels of IAA and tZ (Figure 4 B, E) are lower in plants exposed to mild drought stress but the accumulation profile of these hormones is essentially not affected. Both IAA and tZ were significantly downregulated at those positions in the growth zone where their levels were highest, at the base of the leaf. When steady state levels of IAA and tZ were reached, no significant difference between control and mild drought stress treated samples could be observed anymore. Opposite to what is observed in the GA20OX-1OE plants, a rise in iP was observed under mild drought closer to the leaf basis, corresponding to the position of TZ2 which is shifted to a more basal position as compared to the non-transgenic siblings (Figure 2). The levels of the bioactive GA1 (and GA4) (Figure 4A) were downregulated by mild drought and maximal levels were reached approximately 5 mm closer to the leaf basis as compared to the maximal levels reached in the control leaves. This shift in GA maxima towards the leaf base corresponds to the shift in TZ1 caused by the mild drought condition (Figure 2, Supplemental Table S1).
GA thus showed both a difference in the level and its distribution profile in the growth zone under mild drought. As the interplay between GA biosynthesis and degradation was shown to play an important role in determining the position of the TZ1 (Nelissen et al., 2012), the reason for the shift and reduction in the GA profile was examined by measuring the levels of the biosynthetic and catabolic GA products under mild drought.

**GA biosynthesis is lowered by the mild drought stress**

All GA biosynthetic intermediates from GA12 onwards as well as two major GA inactivation products (GA29 and GA3) were measured in plants grown under mild drought and control conditions (Figure 5). The levels of the first-formed GA, GA12, were increased by the drought treatment, but the levels of the subsequent metabolites (GA15; GA24) resulting from the soluble dioxygenases in the cytosol (GA13-oxidation, GA20-oxidation and GA3-oxidation) are significantly lowered by the drought treatment. The GA biosynthetic intermediates, GA9, GA15 and GA34 were hardly detectable, and differences in their levels were not significant. For most metabolite precursors of GA4 and GA1 (GA15, GA44, GA24), a significant interaction between the drought treatment and the position along the growth zone was shown by ANOVA, showing that the accumulation pattern of the GA metabolites was shifted towards the base of the leaf. The lower levels of bioactive GAs (GA1 and GA4) also resulted in a significant reduction in the levels of the GA2-oxidase (GA2-ox) mediated degradation products (GA29 and GA8) (Figure 5).

Next, we analyzed the expression levels of several GA biosynthetic genes, as well the GA inactivation gene, GA2-ox, by qRT-PCR. No significant downregulation of the expression levels of CPS (ent-copalyl diphosphate synthase), KS (ent-kaurene synthase), KO (ent-kaurene oxidase), KAO (ent-kaurenoic acid oxidase) at the DZ and EZ was observed, despite the downregulation of KS and KAO expressions at the MZ. However, all tested genes encoding GA metabolic enzymes from the GA13 oxidation step onward were significantly downregulated by the drought treatment. The gene encoding the rate-limiting biosynthetic enzyme, GA20-Oxidase (GA20OX-1) (Nelissen et al., 2012), seemed to be the earliest GA biosynthetic enzyme, known so far, to show a lowered and shifted expression pattern. Since mild drought lowered the level of GA20OX-1, we tested whether a boost in GA levels by overexpressing the GA20OX-1 alters the drought response and renders plants more tolerant to drought.
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Figure 5. Gibberellin (GA) biosynthesis and catabolism in the growth zone of B104 leaves. GA metabolites along the growth zone under mild drought and well-watered conditions (grey and black, respectively). Values are average ± SE (n=5). Transcript levels of enzymes in the GA metabolic pathway under mild drought and well-watered conditions are indicated in red (dark and light red, respectively). Expression values are represented as relative values to the expression value at the leaf base in well-watered plants. Values are average ± SE (n=3). CPS = ent-copalyl diphosphate synthase; KS = ent-kaurene synthase; KO = ent-kaurene oxidase; KAO = ent-kaurenoic acid oxidase.
High GA levels in the GA20OX-overexpressing plants did not affect the response to mild drought

If the shift in TZ1 caused by the mild drought condition was the result of lowered levels of bio-active GA, we hypothesize that a boost in GA biosynthesis would counteract the effect of drought and allow for plants to grow better under mild drought conditions. To test this hypothesis, we grew GA20OX-1\textsuperscript{OE} plants under mild drought and control conditions and analyzed the growth reduction. To this end, a segregating population obtained by back crossing hemizygous GA20OX-1\textsuperscript{OE} plants to B104 was used. A growth reduction of 23% and 26% was observed in the non-transgenic and transgenic siblings, respectively, showing no significant difference in drought response. The mild drought assays were repeated using GA20OX-1\textsuperscript{OE} homozygous plants. Despite the fact that leaf growth of the GA20OX-1\textsuperscript{OE} plants under mild drought was enhanced compared to the control siblings grown under control conditions, the relative growth reduction caused by mild drought was comparable between the transgenic and non-transgenic siblings (growth reduction of 23% in the GA20OX-1\textsuperscript{OE} plants relative to 24.4% in the control plants, respectively). Again no significant interaction could be shown for the drought treatment. Kinematic analysis revealed that in the GA20OX-1\textsuperscript{OE} plants the mild drought effect caused a significant reduction in the size of the DZ (22%) similar as was observed for the non-transgenic controls (22.5%), indicating that high levels of GA were not sufficient to overcome the reduction of the size of the division zone caused by the mild drought (Table 2; Supplemental Table S1).

Table 2. Phenotypes of the GA20OX-1\textsuperscript{OE} transgenic and non-transgenic plants grown under mild drought.

<table>
<thead>
<tr>
<th>parameters</th>
<th>GA20OX-1\textsuperscript{OE} non-transgenic siblings</th>
<th>GA20OX-1\textsuperscript{OE} transgenic siblings</th>
</tr>
</thead>
<tbody>
<tr>
<td>LER (mm/h)</td>
<td>control\textsuperscript{a}</td>
<td>drought\textsuperscript{a}</td>
</tr>
<tr>
<td>FLL (mm)</td>
<td>560 ± 6.4</td>
<td>468.8 ± 13.2</td>
</tr>
<tr>
<td>Ldi (mm)</td>
<td>14.8 ± 0.5</td>
<td>11.5 ± 0.3</td>
</tr>
<tr>
<td>Ndi (cells)</td>
<td>694 ± 32</td>
<td>540 ± 16</td>
</tr>
</tbody>
</table>

\textsuperscript{a}: mean ± SE. Percentages of growth related parameters of drought relative to the well-watered plants for the segregating GA20OX-1\textsuperscript{OE} non-transgenic and transgenic siblings. P-values as obtained by Student-test between parentheses (n=3). LER=leaf elongation rate; FLL=final leaf length; Ldi=division zone size; Ndi=number of dividing cells.
These data indicated that elevated bioactive GA levels were not able to counteract the effect of the mild drought treatment which was caused by a basal shift of TZ1 and concomitant lowered levels of bioactive GA. One possible explanation why enhancing GA levels, and in this way relocating the TZ1, was not sufficient to render the plants more tolerant to drought, is that the elevated levels of GA in the GA20OX-1OE plants were insufficient to overcome the reduction of the leaf growth zone by mild drought. Alternatively, the observation that the basal shift in TZ1 caused by the mild drought treatment cannot merely be rescued by high endogenous GA levels, could indicate that another mechanism, besides GA biosynthesis was necessary during the mild drought response. We were unable to discriminate between these two possibilities as attempts to increase the GA levels further by expression GA20OX under the control of the strong BdEF1 promoter (Coussens et al., 2012) failed, likely because very high GA20OX overexpression impairs transformation or early plant development. None of the analyzed BdEF1α::GA20OX lines showed a higher expression of the GA20OX compared to the GA20OX-1OE line (Supplemental Figure S1a), and the highest overexpressing line from BdEF1α::GA20OX showed a phenotype comparable to that of GA20OX-1OE (Supplemental Figure S1b). Because mild drought might act through additional mechanisms besides GA biosynthesis to affect the position of the TZ1, we compared the genome-wide transcriptome with high resolution throughout the growth zone of mild drought treated and GA20OX-1OE plants.

*Leaf Growth Viewer (LGV) as a tool to query the high resolution transcriptome data on mild drought and GA20OX-1OE plants and their controls*

To further explore the dynamics of molecular changes within the growth zone, a transcriptome study was performed on samples taken with half cm intervals throughout the entire growth zone (five and eight from the base of the leaf in the mild drought and GA20OX-1OE experiment, respectively) (Figure 2). The mild drought experiment was complemented with a sample representative of the expansion zone (between cm 4 and 5) and one of the mature zone (between cm 8 and 9) (Figure S2). The high resolution and the multiple opportunities for comparative analysis between the different perturbations make that the transcriptome data contain a plethora of information. To date, many different research groups use the maize leaf as a model, albeit with a different biological question (Li et al., 2010; Jaskiewicz et al., 2011; Bonhomme et al., 2012; Nelissen et al., 2012; Facette et al., 2013; Ponnala et al., 2014; Tausta et al., 2014; van Wijk et al., 2014; Zhang et al., 2014). The higher resolution transcriptomics datasets provided in this manuscript, not only are of interest to the maize leaf community, but also to researchers studying growth processes, plant organ size and mild drought stress. Therefore, we provide an online search engine to make the data available and
searchable for the community. This online tool, called Leaf Growth Viewer (LGV) is accessible through the following link (https://psblgv01.psb.ugent.be) (Supplementary Figure S2). LGV allows for querying the dataset starting from gene identifiers to obtain the leaf growth zone specific expression profile, but also to query for genes that follow a specific expression profile within the maize leaf growth zone. The obtained expression profiles can be exported as image files (heat map of expression along the gradient under different perturbations) as well as data list files that can serve as input for other software, supporting applications such as clustering. All gene-identifiers are linked to PLAZA3.0 (Proost et al., 2014), so that each query automatically and in parallel with the expression profiles, results in a GO enrichment for the selected genes. Based upon these PLAZA data, both the GO-enrichments and the orthologs or homologs from Arabidopsis can be easily exported as tabular data after every query. LGV was used for the analysis of the transcriptome data in the remainder of the manuscript and additional features are explained throughout the results.

Transcriptional changes within the division and expansion zone discriminate each zone into a basal and distal part

The high resolution sampling throughout the growth zone allows to investigate the transcriptional changes that occur between consecutive samples along the leaf gradient. As the two control samples (well-watered from the mild drought experiment and non-transgenic from the GA20OX-1OE experiment) were both in the B104 background and some samples were in overlap, the samples were merged and statistically analyzed together. This resulted in eight continuous leaf samples of 0.5 cm each, starting from the base of the leaf (0-4 cm), followed by a sample from the expansion zone (4-5 cm) and the mature zone (8-9 cm) (Figure 2, control). 1284 genes were significantly upregulated along the leaf gradient (FC > 2; FDR < 0.05) of which 611 between the expansion zone and the mature zone and 1227 genes were significantly (FC < -2; FDR < 0.05) downregulated of which 661 between the expansion zone and the mature zone (Supplemental Table S2).

Although the distal expansion zone (between cm 4 and 5) is sampled consecutive with the growth zone (0-4 cm), a strong increase in transcriptional changes was observed, indicating that within the expansion zone, a discrimination should be made between early expansion zone and distal expansion zone. Similarly, in the division zone, that only comprises of approximately 1.5 cm, transcriptional changes can still be observed between the basal part and the more distal part. The genes that are higher expressed in the basal part of the division zone than in the subsequent half cm are enriched in nucleotide and amino acid biosynthesis and regulation of transcription (Figure 6; Supplemental Table S2). Ten transcription factors were specifically upregulated at the base of the division zone, among
which four Dof zinc finger transcription factors; GROWTH REGULATING FACTOR15; three TGACG
SEQUENCE_SPECIFIC BINDING PROTEIN (TGA) transcription factors, of which one is FASCIATED EAR4
(FEA4) and another one is LIGULELESS2; BEL1-like homeodomain transcription factor; and ETHYLENE
REGULATED FACTOR1 (Supplemental Table S2). Conversely, the genes that were lower expressed in
the basal half cm than in the next half cm were involved in GA mediated signaling (Gibberellin 3-beta-
dioxygenase 2-2 and GATA transcription factor 22) and translation (15 ribosomal proteins) (Figure 6).
These data indicated that the distal division zone is characterized by higher GA signaling and high
translation activity, for which the amino acid pools possibly were formed in the basal division zone.

Throughout the division zone, genes were upregulated with a function in tissue development,
epidermis, stomata and cell fate, growth and the maintenance of meristematic identity. Towards the
distal part of the division zone, the processes of replication, chromatin assembly cytokinesis, cell
cycle were enriched among the downregulated genes (Figure 6; Supplemental Table S2).

The downregulation of genes involved in DNA metabolism, cell cycle, cytokinesis persisted till after
the transition zone, while the process of meristem organization was specifically downregulated at
the transition and GA biosynthesis (Gibberellin 3-beta-dioxygenase 2-2) dropped directly after the
transition zone. The sample containing the transition zone is also characterized by the upregulation
of distinct transport pathways (oligopeptide, peptides, amide and water) and the GO category of
‘response to abiotic stimulus, light intensity, (far) red, UV-A, fatty acids (FA), blue light and radiation’.
After the transition zone, genes involved in lignin (mainly S-type lignin) biosynthesis and secondary
metabolism were upregulated, together with the biogenesis of cinnamic esters, indicating that
lignification starts soon after the transition zone. Later in the expansion zone, many genes involved
in phenylpropanoid metabolism and cell wall organization are differentially expressed along the leaf
gradient. Towards the distal part of the expansion zone genes involved in leaf senescence as well as
in negative regulation of cytokinin and GA catabolism (GA2-oxidase) are upregulated (Figure 6;
Supplemental Table S2).

Towards the distal expansion zone and the mature zone, genes involved in response to ions
(magnesium, calcium, potassium) and to mannitol and sorbitol were significantly downregulated,
while genes involved in thiamine biosynthesis were upregulated. At the transition between the
expansion and mature zone, growth, protein polymerisation, microtubule organization and response
to brassinosteroid (BR) hormones were downregulated (Figure 6; Supplemental Table S2).

Remarkably, the category of photosynthesis was significantly upregulated at all positions along the
leaf gradient, starting within the division zone. Although all GO categories are part of photosynthesis,
a distinction can be made between different sub-processes as cells progress through the different
zones. In all different comparisons along the leaf gradient, response to light stimulus was upregulated, while at the base of the leaf, precursors of photosynthetic pigments appear to be already synthesized, and ‘transcription from plastid promoters’ was upregulated at the basal half centimeter compared to the next half cm. Around the transition zone, an enrichment in the genes involved in light harvesting through photosystem I and subsequently photosystem II was observed. The GO categories ‘chloroplast ribulose bisphosphate carboxylase complex biogenesis’ and the ‘electron transport chain’ are upregulated after the transition, followed by ‘chloroplast relocation and organization’ in the basal expansion zone. In the distal part of the expansion zone, the GO categories ‘pentose-phosphate shunt’ and ‘carboxylic acid biosynthetic process’ are upregulated. In cm four to five, thus in the distal expansion zone, genes involved in the ‘circadian rhythm’ were significantly upregulated, while the genes involved in the actual production of sugars and starch are upregulated towards the mature zone (Figure 6; Supplemental Table S2).

Taken together, the high resolution sampling along the leaf gradient showed that many molecular processes occur gradually or in a consecutive order as cells move through cell division and cell expansion towards maturity. Even within one zone molecular changes were observed as well as around the transition zone between cell division and cell expansion, reinforcing the molecular reprogramming that occurs at the transition zone.
Figure 6. Schematic overview shows the main categories of transcriptional changes between the sequential samples in a growing maize leaf in well-watered condition. The GO categories of transcripts that were upregulated and therefore associating with the maturity of the tissue are indicated in green, and the GO categories of transcripts that were downregulated are indicated in yellow. The red vertical line indicates the transition zone (TZ1) between cell division and cell expansion. The slash indicates the 8-9 centimeter leaf sample is not continuous with the other samples. GO enrichment is characterized by PLAZA (Proost et al., 2014).

FA: fatty acid; BR: brassinosteroid; GA: gibberellin.
Mild drought specifically maintains the ability to grow by keeping the mitotic cell cycle machinery in stand-by for a longer period of time

Besides comparing consecutive samples, LGV also allows to query how the perturbations of the two transition zones (GA20OX-1OE plants and mild drought) affect the transcriptome along the leaf gradient. In order to implement the data in LGV, all samples were statistically analyzed together. A principle component analysis (PCA) of all the samples shows that the majority of the transcriptional changes can be explained by six principle components (Figure 7A). The genes that positively contribute to the first principle component (PC1) that separates the samples according to their position along the leaf, are enriched for the GO categories ‘photosynthesis’, ‘response to abiotic stimulus’, ‘oxidation-reduction process’, ‘very long-chain fatty acids metabolic process’ and ‘cell wall biogenesis’. The genes that negatively affect PC1 are enriched in DNA replication, cell cycle and nucleosome assembly and disassembly. PC2 also divides the samples according to their position along the leaf, but is highest for the samples in the basal expansion zone relative to the samples in the division zone and the mature zone. The genes that positively contribute to PC2 were enriched for lipid metabolic process, response to osmotic stress and auxin, while the genes that negatively contribute are mainly involved in leaf senescence, response to jasmonic acid and response to oxidative stress. Both PC1 and PC2 cause a little shift in the control and perturbed samples in both experiments; a difference that is opposite in both perturbations and that becomes more pronounced in the growth zone as the distance from the leaf base increases (Figure 7B). These shifts correspond to the microscopically determined position of the transition zone in mild drought, well-watered GA20OX-1OE plants and the non-transgenic siblings.

The genes that contribute to PC4 that discriminates between the growth zone, expansion zone and mature zone samples were enriched for hexose biosynthetic process, proline transport, lipid and phenylpropanoid metabolic process (Figure 7C). It is mainly PC6 that discriminates the drought samples from all well-watered samples. The genes that contribute to PC6 in the same direction as the mild drought classification are enriched in a plethora of responses to abiotic stimulus (including heat, reactive oxygen species), while the oppositely regulated genes were mainly involved in carbohydrate catabolic process and hexose transport (Figure 7D).

LGV can also be used to narrow down the number of genes identified as it allows to perform chained queries (or a query within a query) which was useful to search for genes that were differentially expressed under drought and additionally also in the GA20OX-1OE line. In this way, genes were searched for that have opposite expression profiles in the transition zone in GA20OX-1OE and mild drought stress plants, respectively. A direct comparison of the samples taken at a fixed distance from
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the base of the leaf showed that in sample 1-1.5cm many genes are differentially expressed in the mild drought samples and GA20OX-1OE samples as compared to WT samples (1111 genes were upregulated by drought and downregulated by GA and 711 genes were downregulated by drought and upregulated by GA). However, as the position of the transition zone changes in opposite direction in the mild drought samples and the GA20OX-1OE samples, above comparison is likely to reflect mainly developmentally regulated genes. To solve this problem and allowing for a better comparison of the samples, we performed a shift in both experiments, based on the cellular analyses that were used to determine the position of the transition zone (Table 1). In the mild drought samples, we compared the section that contains the transition zone (between 0.5 and 1.0 cm) to that of the well-watered that contained the transition zone (between 1.0 and 1.5 cm). Similarly, we compared the wild type section containing the transition zone (between 1.0 and 1.5 cm), with the GA20OX-1OE sample that includes the transition zone (between 1.5 and 2.0 cm). In this way, transcripts were identified that were specific for each perturbation of the transition zone.

Remarkably few genes were specifically (and not differentially expressed under mild drought) higher or lower expressed in GA20OX-1OE plants as compared to wild type (16 and 1 genes at the division zone, 4 and 7 genes at the transition zone and 5 and 10 genes at the expansion zone, respectively). The genes that were specifically higher expressed in the GA20OX-1OE plants than in the wild-type plants included Mannose-6-phosphate isomerase which involved in ascorbic acid biosynthesis; glucuronic acid decarboxylase 1, malate dehydrogenase in the division zone; and histone 2A, a methyltransferase, a beta-glucosidase and alpha-1,4-glucan-protein synthase involved in cellulose biosynthesis in the expansion zone. In the expansion zone, recA, a Glutamine synthetase, an L-2-hydroxyglutarate dehydrogenase and a RNG/U-box superfamily protein were significantly enriched among the downregulated genes in the GA20OX-1OE plants compared to wild-type(Supplemental Table S3).

In contrast to GA20OX-1OE, much more genes were zone-specifically up- or down-regulated the mild drought treatment: 140 and 76 at the division zone, 206 and 182 at the transition zone and 103 and 84 at the expansion zone, respectively). The genes that were upregulated by mild drought in the division zone were enriched for ‘response to light’, ‘regulation of gene expression’, ‘response to light intensity’, ‘response to reactive oxygen species’, ‘leaf morphogenesis’ and ‘stomatal complex formation’. The genes involved the ‘regulation of gene expression’ are several Dof (DNA-binding with one finger) transcription factors; two TGA; ethylene response factor1 (ERF1); NAC67; SPEECHLESS; KNOTTED1; and a B3 domain containing transcription factor. Remarkably, at the transition and expansion zone, the GO categories of DNA replication and cell cycle were significantly enriched among the genes that were upregulated by drought. This is opposite to what was observed at the
division zone, where mild drought downregulated the cell cycle machinery (Avramova et al., 2015b). We hypothesize that the reduced expression of cell cycle genes in the DZ is responsible for the lower LER but in contrast the higher expression of cell cycle related genes in the TZ might safeguard that the leaf has a higher LED. Besides CDKB1;1 and CDKB2;1 that are known to function in the G2/M transition of the cell cycle, many of these genes involved in cell cycle and DNA replication were previously shown to be targets of E2F/DP (Vandepoele et al., 2005; Verkest et al., 2014). At the transition zone, a significant enrichment was obtained as 31 of the 180 specifically mild drought upregulated genes show homology to Arabidopsis genes that were computationally and experimentally shown to be E2F/DP targets (Vandepoele et al., 2005; Verkest et al., 2014), and 6 E2F targets were specifically upregulated by mild drought right after the transition. These genes encode the histone chaperone ASF1B, many proteins of the MINICHROMOSOME MAINTENANCE COMPLEX (MCM), DEL1, RETINOBLASTOMA RELATED protein, CDC45, genes encoding Chromatin Assembly Factor-1 (CAF-1) p150 subunits and a protein required for sister chromatid cohesion and DNA repair. In addition, components of the condensing complex, shugoshin and TWO IN ONE (TIO) that plays a role in cytokinesis, were significantly upregulated under mild drought and directly after the transition. At the expansion zone, several histones (H4, H2A, H2B) were significantly upregulated under mild drought, together with genes involved in DNA replication and cell cycle (Supplemental Table S3).

Amongst the drought-mediated down-regulated genes at the division zone are GA3-oxidase; proline dehydrogenase involved in proline catabolism; and two transcription factors that were already associated with abiotic stress response. Both at the division and transition zone, genes involved in photosystem II, RUBISCO and light harvesting complex were downregulated upon mild drought (Supplemental Table S3). At the transition and expansion zone several aquaporins (PIP2-3, PIP2-4 and PIP2-5) were significantly downregulated.
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Figure 7: Principal component analysis (PCA) of the transcriptome data.

The numbers indicate the leaf samples: 1-8 in GA20OX-1\textsuperscript{OE} and its non-transgenic siblings are continuing samples (0.5 centimeter scale) from the basal leaf (0 centimeter) to 4 centimeters away from the leaf basis; 1-5 in well-watered and mild drought conditions indicate continuous samples (0.5 centimeter scale) from the leaf basis (0 centimeter) to 2.5 centimeter away from the leaf basis, while 5= 4-5 centimeter leaf sample and 9= 9-10 centimeter leaf sample from the leaf basis.

A: bar chart showing the explained variance for each component of PCA.

B-D: Principal components (PCs) representing the classifications of the transcriptome data.

WT=well-watered B104
D=mild drought treated B104
WT=GA20OX-1\textsuperscript{OE} non-transgenics
G=GA20OX-1\textsuperscript{OE} transgenics
Discussion

Detailed sampling combined with molecular profiling reveals the complex regulation of growth in the maize leaf

The key processes to determine organ size are cell division and cell expansion and these processes are spatially organized in the maize leaf. At the basis of a growing maize leaf, all cells are dividing and at a given position away from the leaf base, cell division ceases and cells continue to grow by cell elongation. A cellular analysis was developed to quantify the contribution of cell division or cell expansion to a given leaf growth phenotype. Depending on the mitotic figures and the cell length profiles, zones can be delineated in which cells are dividing or expanding, until they reach their mature size, resulting in a division zone (DZ), expansion zone (EZ) and mature zone (MZ) (Nelissen et al., 2012; Nelissen et al., 2013). However, this rather simplistic view of growth is very likely much more complex. High resolution transcriptome analysis and quantification of hormones showed that within the DZ and EZ, there are substantial differences between the basal and distal parts of these zones. Indeed, the levels of auxin and TZ declined throughout the division zone, to reach their basal level around TZ1 (Nelissen et al., 2012) (this work), reinforcing the concept that cells in the basal and distal part of the division zone are molecularly distinct, while the presence of mitotic figures in the kinematic analysis (Nelissen et al., 2013) classified them all as dividing cells. These data suggest that there are gradients of molecular processes within the growth zone that govern cell cycle progression. It is likely that such gradients are at least partially mediated by diffusible factors, such as auxin, originating from the basis of the leaf and gradually diminishing in concentration when cells getting displaced further away from the leaf base. The high resolution transcriptome data reinforced this concept and showed that also the cells in the expansion zone are molecularly distinct, depending on their position within that zone.

The concept that the basal division zone is different from the more distal division zone is exemplified by a number of transcription factors that were specifically expressed in the basal half centimeter of the growing maize leaf. Several of these transcription factors have an unknown function (Dof transcription factors, TGA) while others were previously described to have a role in leaf growth or development. The BEL1-domain containing protein that is an orthologue of the Arabidopsis BELRINGER; and FEA4, that is orthologous to Arabidopsis PERANTHIA, were both shown to regulate AGAMOUS (Bao et al., 2004; Pautler et al., 2015). FEA4 is expressed in the peripheral zone of the shoot apical meristem where it thought to promote differentiation in the meristem periphery.
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through auxin-based responses (Pautler et al., 2015). Remarkably, the expression of FEA4 during leaf growth coincides with the position where the highest levels of auxin were observed. In addition, FEA4 was shown to regulate many different transcription factors involved in leaf differentiation and polarity (Pautler et al., 2015). Other genes that are specifically highly expressed in the basal half centimeter are LG2, that is involved in leaf patterning along the proximo-distal axis (Walsh et al., 1998) and GROWTH REGULATING FACTOR15 (GRF15). Previously, we showed that GRF15 was indeed higher expressed in the division zone, relative to the expansion zone and the protein was identified as a significantly enriched part of the AN3 mediated chromatin remodeling SWI/SNF complex in the division zone (Nelissen et al., 2015).

The fine sampling strategy also highlights the transition zone between the adjacent cellular processes. Both at the transcript level and the hormone level, a lot of changes occur exactly at the transition between cell division and cell expansion suggesting that this process is an important integration point of molecular cues. The observation that mild drought affects the position of this TZ1 underscores the importance of this developmental transition. Previously the role of the local accumulation of GA at the TZ1 was shown to be functionally important as ectopic expression of GA20-Oxidase resulted in a more distal shift of this transition and hence in more cells in the division zone (Nelissen et al., 2012). Here we show that the more proximal shift of TZ1 under mild drought was associated with lowered levels of bioactive GA. This reduction in GA levels was also observed when analyzing the biosynthetic intermediates. Furthermore, the transcript levels of genes encoding enzymes operating after the GA13 oxidation step in the GA biosynthetic pathway were found downregulated by mild drought stress.

Some processes, such as photosynthesis, were found to be transcriptionally regulated in a gradient throughout the entire growth zone, however the high resolution sampling allows for discriminating sub-processes that show spatial specificity. Although the different events are not strictly delineated at one given position along the leaf, some photosynthesis-related processes are only differentially expressed till or from certain positions. At the base of the leaf, in the division zone, genes enriched in the transcription from plastid promoters and porphyrin biosynthesis, as well as genes involved in the biogenesis of the chloroplast ribulose bisphosphate carboxylase complex are upregulated. Genes involved in plastid gene expression and the regulation of photosynthesis were already shown to be expressed at the base of the growing maize leaf and typically anti-correrate to final leaf size and timing of growth related parameters (Baute et al., 2015, 2016). After TZ1, in the early expansion zone, genes involved in light harvesting in photosystem I and II are upregulated, while later in the expansion zone, the upregulated genes are enriched for functions in electron transport chain, sucrose transport, and chloroplast relocation and organization. Finally, and more distally, genes
involved in carbohydrate and starch metabolism are significantly upregulated. The transcriptome data show, that even from the base of the leaf, that is shielded from light by the surrounding older leaves, cells are preparing for their final role in photosynthesis. Together, our data show that the basal part of the growing leaf is instrumental to determine the final size and shape of the leaf as well as its role as powerhouse of the plant.

**Perturbations of the organization of the growth zone allows for identifying novel mechanisms**

The high-resolution sampling provides an integrative view on gradual and specific molecular changes that take place throughout the distinct positions along the growing leaf, but still the emerging picture remains static. Positively and negatively disturbing the organization of the growth zone, by selecting conditions that shift the TZ1 more basally and more distally, allowed to identify novel mechanisms. Remarkably, the cytokinin, iP, transiently increases in level in a zone corresponding to TZ2 in all three examined conditions, each distinctively affecting the position of the Tzs in the growth zone. The levels of iP are not changed in the three conditions, only the transient increase in cytokinin is shifted more basally or more distally, under mild drought and high GA, respectively. As cytokinins were shown to promote shoot development, to inhibit leaf senescence and to delay differentiation (Kieber and Schaller, 2014), it is unlikely that the increase in cytokinin is causing the transition between cell expansion and maturation. On the other hand, it is known that cytokinin influences the ultrastructure and the amount of chloroplasts as well as regulating chlorophyll biosynthesis (Cortleven and Schmülling, 2015). As the increase in cytokinin occurs at a position in the growing leaf, that is close to the point of leaf emergence from the sheet and that thus might already perceive incident light, we hypothesize that towards the end of the expansion zone, cytokinins are upregulated to steer the development and activity of chloroplasts. This increase in cytokinin levels is concomitant with an increase in expression level of a gene encoding an F-box protein that is the orthologue of KISS ME DEADLY-LIKE2 (KMD2). The expression level of the KMD2 orthologue is significantly upregulated at multiple successive samples in the expansion zone and between the expansion and the mature zone. Elevated expression of KMD2 targets key transcription factors in the cytokinin response, namely the B-type Arabidopsis Response Regulators (ARRs) for degradation (Kim et al., 2013). Together with the local GA accumulation at TZ1, the increase in cytokinin levels at TZ2, shows that the key transitions in the growth processes in the maize leaf are regulated by phytohormones.

SA was found to gradually accumulate within the division zone, a phenomenon that became more apparent when the levels of SA were elevated as a consequence of the mild drought treatment. Previously it was reported that SA can influence both plant growth and drought tolerance in a dose-
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dependent manner. When high concentrations of SA is applied to wheat seedlings, drought tolerance and plant growth is suppressed (Kang et al., 2012), while plant growth and cell division is enhanced by low levels of SA in wheat (Hamada, 2001). Pretreatment of plants with low SA levels can reduce water loss and increase drought tolerance (Kang et al., 2012; Kang et al., 2013). In general, SA is considered to be involved in the early stages of drought response and is involved in the biosynthesis of ABA and proline in barley leaves (Miura and Tada, 2014).

Growth reduction by mild drought stress cannot be suppressed by elevated GA levels

Since GA biosynthesis is hampered in mild drought treated plants, we expected that overexpression of a rate-limiting GA biosynthesis gene could alleviate the growth reduction caused by drought stress. However, merely increasing the levels of GA cannot render plants more tolerant to mild drought, suggesting there are additional factors, independent of GA, that are induced by drought. Remarkably, five of the ten transcription factors that were highly expressed at the base of the leaf, were significantly upregulated under mild drought (two Dof transcription factors, FEA4, a TGA6 transcription factor and ERF1). One of the upregulated Dof (Dof22) transcription factors was previously shown to be upregulated in maize seedlings under salt treatment (Chen and Cao, 2015), implying that this gene is an early response gene to multiple abiotic stresses during maize development. The ERF1 protein is orthologous to RELATED TO APETALA2 (RAP2.2), for which it was shown that elevated levels sustain ABA-mediated activation of stress response genes and thus plays a role in tolerance for multiple stresses (Papdi et al., 2015). Also the expression of KNOTTED1 (KN1) is specifically upregulated by mild drought at the DZ. Interestingly, KN1 was shown to negatively modulate the accumulation of GA through the control of GA2oxidase1, an enzyme that inactivates GA. In concert, overexpression of KN1 results on smaller plants (Bolduc and Hake, 2009), raising the possibility that the upregulated KN1 expression might be involved in the drought mediated growth reduction.

In contrast, genes involved in proline accumulation, photosynthesis and aquaporins were specifically downregulated by drought stress. The downregulation of PROLINE DEHYDROGENASE (ProDH) during stress is widely accepted to promote proline accumulation under stress (Verslues and Sharma, 2010). Our results are consistent with previous observations in Arabidopsis, where ProDH was downregulated at the transition from cell division to cell expansion under mild drought (Clauw et al., 2015). Genes involved in photosystem II (PS II) and light harvesting complex were downregulated at DZ and TZ1. Previously, many studies show that drought stress results in damage to PS II and affects PS II photochemistry in mature leaves (Lu and Zhang, 1999; Sperdouli and Moustakas, 2012).
Remarkably, the negative effect of mild drought on photosynthesis is already established in cells that were covered by the sheath of older leaves and most likely were never exposed to light and thus photosynthetically inactive. Transcriptome data also reveal that several genes encoding PLASMA MEMBRANE INTRINSIC PROTEINS (PIPs), belonging to aquaporins, that facilitate the water diffusion across cell membranes, were significantly downregulated at the TZ and EZ by drought stress. Under water stress, the expression of several PIPs in leaves was downregulated, and ABA represses aquaporin activity (Alexandersson et al., 2005; Shatil-Cohen et al., 2011). The downregulation of aquaporins might be a way for plant to minimize water flow through cell membranes and to maintain leaf turgor, that is required for both cell division and cell expansion (Chaumont and Tyerman, 2014).

Although fewer genes were differentially expressed in the GA20OX-1OE plants compared to the mild drought treated plants, some specific processes were identified by overexpression of the rate-limiting GA biosynthetic gene. One gene (GRMZM2G456471; Mannose-6 phosphate isomerase; MPI) involved in ascorbic acid biosynthesis was specially upregulated at DZ in the GA20OX-1OE plants. L-ascorbic acid is an important cofactor for several enzymes, among which 2-oxoacid dependent dioxygenases involved in GA biosynthesis (Smirnoff and Wheeler, 2000). In addition, ascorbic acid has been shown to be enriched in dividing tissues rather than the cell division-inactive tissues, and is necessary for the transition from G1 to S in the cell cycle (Citterio et al., 1994; Kerk and Feldman, 1995; Arrigoni and De Tullio, 2002). Lycorine, an alkaloid inhibiting ascorbic acid biosynthesis, can prevent cell division, while addition of ascorbic acid could restore cell division in Lupinus albus seedlings (Arrigoni et al., 1997). The decrease of endogenous ascorbic acid resulted in a reduced growth rate of tobacco BY-2 cells (Tabata et al., 2001) and smaller leaves and slower shoot growth in Arabidopsis (Veljovic-Jovanovic et al., 2001). Moreover, it has been shown that methyl jasmonate treatment increases the de novo synthesis of ascorbic acid in tobacco Bright Yellow-2 (BY-2) suspension cells by stimulating the transcription of at least two genes encoding enzymes for ascorbic acid biosynthesis (Wolucka et al., 2005). As higher jasmonic acid content was observed in GA20OX-1OE plants, it might trigger the increase of MPI expression.

At the EZ, alpha-1,4-glucan-protein synthase involved in cellulose biosynthesis was specifically upregulated in the expansion zone of growing leaves of GA20OX-1OE plants. Interestingly, the increased cellulose was observed in the stem of mature GA20OX-1OE plants (Voorend et al., 2016), suggesting an overall stimulation on cellulose biosynthesis by GA GA20OX-1OE.

Although increased GA levels could not compensate for the reduced cell division and cell expansion upon mild drought and consequently leaf growth in GA20OX-1OE plants is proportionally similarly affected by mild drought as wild-type leaves, the growth advantage of leaves from GA20OX-1OE
plants was maintained. Indeed, the leaf length of GA20OX-1OE plants under mild drought still exceeds that of the well-watered non-transgenic siblings. These data indicate that the effects of growth promoting genes can still persist under mild abiotic stress conditions, while the genes are not necessarily involved in stress tolerance or survival.

Leaf Elongation Duration (LED) as a compensatory growth mechanism for drought induced growth reduction in the maize leaf

In Arabidopsis, plants exposed to mild osmotic stress, a stress that mimics drought stress with low water potential, clearly reduced their growth. Osmotic stress initially causes a very quick cell cycle arrest in proliferating cells, but these cells are kept in a ‘pausing’ status, allowing them to recover when the environmental conditions improved (Skirycz et al., 2011a). However, when the stress persists, the cells are irreversibly pushed into differentiation (Skirycz et al., 2011a; Dubois et al., 2013).

In maize, our kinematic analysis showed that the cell division rate and the cell cycle duration were not significantly affected by mild drought, and also at the transcript level, no differential expression of cell cycle related genes was observed at the DZ. However, effects on cell division rate and downregulation of cell cycle genes were observed in another study that examined the effect of mild and severe drought in the maize leaf (Avramova et al., 2015b), indicating that the significance of differential gene expression for cell cycle genes might depend on the severity of the applied drought stress. The combination of our kinematic and expression data shows that, oppositely to what was observed in Arabidopsis, the ‘pausing’ mechanism was not observed in our mild drought conditions. Conversely, transcripts that are known as downstream targets of E2F/DP transcription factors, well-known regulators of the G1-S transition that upon ectopic expression result in an increased cell division duration (De Veylder et al., 2002), were significantly upregulated at TZ1 and the basal expansion zone under mild drought during steady state growth. These data suggest that the mild drought induced expression of E2F/DP targets around TZ1 might provide the possibility to maintain the capacity to resume growth upon water availability.

During osmotic stress in Arabidopsis leaves, the initial reduction in cell division was in part compensated by meristemoid divisions, generating extra pavement cells while forming stomata (Geisler et al., 2000; Bergmann and Sack, 2007; Skirycz et al., 2011a). Unlike Arabidopsis, where the meristemoids divisions contribute substantially to final leaf size (Gonzalez et al., 2012), the polarized stomatal divisions in maize barely affect leaf size (Larkin et al., 1997). Instead, our data showed that
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the prolonged duration of leaf growth (or leaf elongation duration; LED), at least partly, compensates for the mild drought induced growth reduction. The prolonged duration of growth was also suggested in another study that assessed the effects of mild and severe drought on maize leaves (Avramova et al., 2015b), suggesting that the prolonged LED is a more general mechanism to compensate the growth reduction under drought. More studies will be needed to examine the biological relevance of the prolonged LED as a compensation for the drought induced growth reduction and to study if the upregulation of the E2F/DP targets around TZ1 allows to resume growth upon re-watering.

Leaf Growth Viewer allows querying transcriptomic changes over the leaf growth gradient under mild drought and elevated GA levels

In this study, we developed a user-friendly and searchable tool, called “LGV”, to visualize our transcriptome data. Genes that are differentially expressed at a given position in the leaf and under a certain condition can be easily exported in a table-format, so they can be transferred to spreadsheets or clustering tools. GO enrichments of the selected queries are performed as a built-in option in LGV and can be easily exported, as well as the Arabidopsis orthologs. Alternatively, a set of unknown genes can be simultaneously queried for their expression profile in the maize leaf under standard, mild drought and GA20-Oxidase overexpressing plants. The accessibility of the data should allow researchers to come up with novel strategies to engineer for growth enhancement or drought tolerance, most likely with very temporal and spatial specific promoters.

LGV provides a scaffold that can be further developed to integrate multiple datasets and analytical tools. Recently, the AIM database in Arabidopsis (Wang et al., 2014b) exemplified the need and the power of integrating big datasets. Genes that are transcriptionally coordinated are often functionally related, indicating the importance to use different transcriptome studies to identify co-expressed genes (De Bodt et al., 2010; Mutwil et al., 2014). It was shown that a comparative analysis of co-expression networks over different species offers additional value to remove false positives and to increase the power of predictions (Mutwil et al., 2014). In addition, proteins can interact with many different interactors and thereby often affect distinct processes (Tucker et al., 2001). In species where there are still a limited number of experimentally validated protein-protein interactions, one solution is to infer interactions from model species, such as Arabidopsis, as was done in CORNET2.0 (De Bodt et al., 2012). However, more and more experimental data come available in the maize leaf on proteome (Facette et al., 2013), metabolome (Wang et al., 2014a) and protein-protein interactions (Bommert et al., 2013; Nelissen et al., 2015), together with the wealth of transcriptome
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data (Li et al., 2010; Mattiello et al., 2014; Tausta et al., 2014; Wang et al., 2014a) so it would be a great opportunity to boost the maize leaf as a model system by integrating these different levels of information. Such a multi-level integration, ultimately even complemented with phenotype data will provide a challenge towards the future and LGV might serve as a starting point.
Materials and methods

Plant Material and Growth Conditions

The constitutive overexpression of GA20-Oxidase transgenic line (GA20OX-1\textsuperscript{OE}) was described before (Nelissen et al., 2012). For strong overexpression of GA20OX-1, the GA20OX-1 gene was cloned behind the Brachypodium distachyon EF1α promoter (Coussens et al., 2012). BdEF1α::GA20OX construct was introduced into the B104 inbred background using Agrobacterium tumefaciens mediated transformation of immature embryos (Coussens et al., 2012). For all experiments, segregating transgenic lines were used, therefore the non-transgenic siblings were used as controls. Genotyping was done by an immunochromatographic assay detecting the PAT protein (AgroStrip, Romer) and leaf painting.

All experiments were executed in growth chambers with controlled relative humidity (55%), temperature (24°C), and light intensity (170 mmol m\textsuperscript{-2} s\textsuperscript{-1} photosynthetically active radiation at plant level) provided by a combination of high-pressure sodium vapour (RNP-T/LR/400W/S/230/E40; Radium) and metal halide lamps with quartz burners (HRI-BT/400W/D230/E40; Radium) in a 16-h/8-h (day/night) cycle. For mild drought treatments, water contents were allowed to drop after sowing to a soil water content of 70% of the well-watered condition, where they were maintained. The water potential of control condition was -23 kPa, and the water content of mild drought soil was -210 KPa.

Growth analysis

To calculate LER, we measured the length of the fourth leaf (n=5) from leaf emergence to maturity, using the soil level as a reference point. The fourth leaf at two days after its appearance from the whorl of leaf three was harvested for kinematic analysis (Nelissen et al., 2013). Kinematic analysis was performed based on Nelissen et al., 2013 with at least three plants.

Hormone Profiling

For hormone profiling, samples of 10 plants were combined and lyophilized. Extraction, purification and hormone metabolic profiling was performed as described (Nelissen et al., 2012).

Maize RNA extraction and qRT-PCR
The expression levels of GA20OX in transgenic line were detected by quantitative PCR. Total RNA was isolated with the guanidinium thiocyanate-phenol-chloroform extraction method using TRI-reagent (Sigma-Aldrich). First-strand cDNA was synthesized from 1 µg total RNA using the iScript™ Advanced cDNA Synthesis Kit for qRT-PCR (Bio-Rad Laboratories). The specific primers for quantifying GA20OX expression levels are CATCAACGTTCGACGTAGTTGATCTACG and GCGGCTCGTGTATTCATGAGCG. qRT-PCR was performed on a LightCycler 480 (Roche) on 384-well plates with LightCycler 480 SYBR Green 1 Master mix (Roche Diagnostics) according to the manufacturer’s instructions. PCR reactions were done in triplicate. For relative quantification, a threshold cycle was set at the same level for each reaction within the exponential amplification phase. For normalization, the transcript levels of the housekeeping gene, 18S rRNA were used as internal control, the primers for 18S rRNA are ACCTTACCAGCCTTGACATATG and GACTTGACCAAACATCTCACG.

Microarray analysis

The forth leaf of maize B104 plants grown under well-watered and mild drought conditions, respectively, were harvested two days after emergence from the sheath. The basal 2.5 cm leaf samples together with the 4-5 cm and 8-9 cm leaf samples were used for transcriptome analysis. The segregating GA20OX-1OE was grown under normal growth conditions, and the basal 4 cm of leaf four were dissected two days after emergence from the sheath for transcriptome analysis. Five plants were taken for one biological replicate, and three biological replicates were harvested for each genotype and each condition. Total RNA was isolated with the guanidinium thiocyanate-phenol-chloroform extraction method using TRI-reagent (Sigma-Aldrich). Total RNA was hybridized in two loop designs for drought and GA20OX-1OE, respectively, with dye swap on maize oligonucleotide two-color arrays printed by Maize Oligonucleotide Array Project Array (maizearray.org). The loop for drought experiment was designed to directly compare the samples under well-watered and mild drought treatment, and the adjacent samples in well-watered and mild drought conditions, respectively. The loop for GA20OX-1OE experiment was designed to directly compare the GA20OX-1OE samples with the non-transgenic leaf samples, and the adjacent samples in GA20OX-1OE transgenic and non-transgenic plants, respectively. Two biological replicates of each loop were performed. Hybridizations were done according to the protocols provided by the maizearray service. The microarray contains 42034 real probes corresponding to 42034 unique ProbeUID. The mapping of the probes to the genes was done using the Maize Microarray Annotation database (http://MaizeArrayAnnot.bi.up.ac.za/). Only probes were retained that identified a single gene after BLAST (Coetzer et al., 2011).

Background subtraction was performed using the normal-exponential model using a saddle-point
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Approximation (Ritchie et al., 2007; Silver et al., 2009). Within-array normalisation to correct for dye-bias was done with the Loess normalization method with default values for the span width (Yang et al., 2001; Yang et al., 2002; Smyth and Speed, 2003). Between-array normalisation was done using the Aquantile method developed by (Yang and Thorne, 2003). The Preprocessing steps were done at the probe level. Subsequently, the log2 expression values were averaged over probes targeting the same gene. After averaging, some filtering was performed: Only probes with a log2 expression intensity higher than 10% of the 95th quantile of the log2 expression values of the negative control were retained. One outlying sample (WT1) was removed based on a MDSplot. A linear model was fitted to the log2 intensity values of each of these retained genes. All combinations of the two factor levels genotype and layer were converted to single factor levels to facilitate making the a-priori group comparisons that motivated our study. Contrasts of interest were the identification of differentially expressed genes between subsequent layers in WT, and within the same layer, between WT and the treated condition and the mutant genotype. Moderated t-statistics were calculated with the empirical Bayes method (Smyth 2004). P-values were adjusted for each contrast separately using the FDR method from (Benjamini and Hochberg, 1995). A gene was called differentially expressed when the adjusted p-value was smaller than 0.05 and the log fold change was at least 1. All analyses have been done with the limma package for R (Ritchie et al., 2015; Team, 2015).

Principle component analysis

The principle component analysis (PCA) plot on transformed count data was done in R using ‘pca’ function.

Leaf Growth View

The Leaf Growth Viewer (LGV) (https://psblgv01.psb.ugent.be/) is a web-based query tool for analyzing maize transcriptome data. It consists of a back-end written in Python using the Django framework (https://www.djangoproject.com/) which connects to a MySQL database (https://www.mysql.com/), as well as a dynamic HTML front-end using java script. SQL queries are performed by the built-in ORM functionality of the Django framework. The inputs for LGV are microarray data of leaf samples from B104 under drought and control conditions, and GA20OX-1OE transgenic and non-transgenic siblings (illustrated in Figure 2). The LGV database stores gene expression values, precalculated fold change (FC) values between the selected samples and their
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respective false discovery rate (FDR) values. Metadata is stored in index-value pairs linked to samples and experiments.

LGV allows users three options for adding a filter to a query, listed on the left hand side of the screen: filter by gene name, expression value, FC/FDR. The specific settings of each filter can be changed in the corresponding group. The filter on gene name allows the user to enter a list of gene names as text. Alternatively, a list of genes from a previous query can be selected and copied. Duplicate genes are automatically removed from the list and there is a shortcut to check if the genes are defined in LGV. The expression value filter displays dropdowns for the selection of a dataset (or an experiment) and a sample. The selected sample is highlighted on an illustrated maize leaf. The user must also define a value range that applies to the selected sample. A histogram of the expression values of the selected dataset is displayed to give the user an idea of the profile of the expression values. The FC/FDR filter displays dropdowns for the selection of dataset, FC group and/or FC sample. There are separate value ranges for FC and FDR and either one can be disabled. Similar to the expression value filter, there are two histograms for both FC and FDR values and an illustrated maize leaf indicating both samples used in the selected FC sample. Multiple filters can be added to a single query, and each filter does not need to be applied to the same dataset. The resulting set of genes after performing the query will only contain those that pass all filters. Following the performance of the query, a heatmap of the resulting set of genes in the dataset used for filtering is presented. Visualizations are generated by the HighCharts (http://www.highcharts.com/) component. This dataset is a default choice and can be altered. The heatmap colors range from blue to red and the values corresponding with red and blue can also be altered. The result of the GO enrichment of the set of genes is also displayed, as well as along with the option to recalculate them with a different p-value or to change the visualization (list or chart). The export function allows the user to download the heatmap, the expression values (with or without FC data) or GO enrichments in csv or xlsx format. Additionally, the user can also export the list of the Arabidopsis ortholog or homologs. LGV allows to start a new query based on the existing set of genes found in the current query. This allows users to perform versatile selections based on their interests.
Supplementary data

**Supplementary Figure S1:** The relative expression level and leaf phenotype in two *GA20OX-1* overexpression lines. (A): the relative expression level of six independent transformation events of EF1α::GA20OX and GA20OX-1^{OE}. (B): The final leaf length of EF1α::GA20OX transgenic line (126-15) and GA20OX-1^{OE}. 
Supplementary Figure S2. Representation of Leaf Growth Viewer (LGV) implementation. A. The interface of LGV. LGV allows users to maintain the working histories, and can be easily accessed by selections on the right upper corner. The main query options of LGA are placed on the left. B. The interface displays hormone profilings of B104 under well-watered and mild drought conditions, and hormone levels of GA20OX-1OE plants compared to their non-transgenic siblings. C-D. LGV allows to view expression changes over the leaf development based on the filter by gene name (C) and by FC/FDR (D). (C) A list of genes can be entered to LGV, and their expression value can be further viewed and exported. (D) Genes are selected based on the differential expression values in different samples. The expression levels of the resulting set of genes can be viewed by a heat map function of LGV, and these genes can be functional clustered. The yellow arrow shows the work flow of LGV based on specific selections.
Supplemental Table S1. Effects of mild drought relative to the well-watered plants for (A) the inbreds B104 and B73 and (B) the segregating GA20OX-1\textsuperscript{OE} non-transgenic and transgenic siblings.

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<td>110 ± 4</td>
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<td>882 ± 142</td>
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Percentages of growth related parameters of drought relative to the well-watered plants for the inbreds B104 and B73 and the segregating GA20-OX1 non-transgenic and transgenic siblings. \(p\)-values as obtained by Student-test between parentheses (n=3). LER: leaf elongation rate; FLL: final leaf length; \(I_{\text{ma}}\): mature cell length; \(L_{\text{e}}\): expansion zone size; \(N_{\text{ex}}\): number of cells in expansion zone; P: cell proliferation rate; \(L_{\text{d}}\): division zone size; \(N_{\text{di}}\): number of dividing cells; D: cell division rate; Tc: cell cycle duration. a. Mean±SE

Supplementary Table S2. The differentially expressed transcripts along the leaf gradient in well-watered condition.

Supplementary Table S3. The specifically differentially expressed transcripts in GA20OX-1\textsuperscript{OE} and drought conditions.
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References


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Chapter 3

Altered expression of the maize cytochrome P450 CYP78A1/KLUH increases biomass and seed yield by an extended duration of cell division
KLUH enhances maize growth
ALTERED EXPRESSION OF THE MAIZE CYTOCHROME P450 CYP78A1/KLUH INCREASES BIOMASS AND SEED YIELD BY AN EXTENDED DURATION OF CELL DIVISION

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AUTHOR CONTRIBUTION

X.S., H.C., D.I. and H.N. designed the study. X.S., K.F., S.D., C.V., K.D., J.D.B. performed experiments. J.C. carried out the field trial and analyzed the field data in the U.S. under the supervision of M.M.. A.D.V., H.N. directed and X.S., K.F., T.V.H., C.V., K.D., J.D.B., A.D.V., H.N. together with some volunteers performed field observations, monitoring and measurements for the field trial in Belgium. X.S and H.N. analyzed the field data in Belgium. X.S., T.V.H., F.C. performed the RNA sequencing analysis. V.S. provided statistical support. O.N. and K.L. performed auxin measurements. G.C. and M.V.L. did the maize transformations. X.S., H.N. and D.I. wrote the chapter.

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Abstract

Maize is the highest yielding cereal crop, that is grown worldwide for grain yield or silage. We modulated the highly specific expression pattern of the growth enhancing ZmKLUH gene, encoding a cytochrome P450 (CYP78A1), by using a growth zone specific GA2-oxidase promoter. This resulted in increased organ growth, which in turn gave rise to enhanced seedling vigor and a higher stover biomass and seed yield. The engineered trait was robust as it improved yields in an inbred background as well as in a panel of hybrids, at several locations and over multiple seasons in the field. To unravel how ZmKLUH promotes organ size, the maize leaf was further examined. Transcriptome studies and hormone measurements show that ZmKLUH functions through an increase in auxin, of which the accumulation pattern in the leaf growth zone is highly similar to the ZmKLUH expression pattern. Detailed analysis of growth over time demonstrated that ZmKLUH stimulates the duration of leaf elongation by maintaining dividing cells for a longer period in a proliferative, undifferentiated state. This ZmKLUH mediated process is independent of the enhanced leaf growth that is observed by the overproduction of bioactive gibberellins that affects growth rate or leaf elongation rate, as combining ectopic ZmKLUH and GA20-Oxidase overexpression results in additive phenotypes. Furthermore, we demonstrate that the prolonged duration of growth serves as a compensation mechanism to maintain the growth potential when plants experience a growth rate reduction caused by abiotic stresses, such as mild drought or cold nights.
Introduction

Leaves are important plant organs as they determine plant architecture and convert sunlight into energy by photosynthesis. The carbohydrates that are produced by the photosynthetic process in leaves sustain both the life of the plant itself, but also of animals and humans. Although quite some genes are identified to play a role in leaf growth, it is largely unknown how they interact with each other and if there are additional mechanism yet to be discovered.

Leaf growth is coordinated by the interaction between genotype and environment, and is mainly driven by two processes, cell division and cell expansion. The interplay between cell division and cell expansion or how different aspects of these processes contribute to observed growth phenotypes, can be routinely studied by using the maize leaf. During the first days after leaf appearance from the pseudostem formed by older leaves, the leaf grows at a steady state rate, during which dividing, expanding and mature cells are present simultaneously in a gradient from the base to the tip of the leaf. As the maize growth zone encompasses several centimeters, this linear organization allows for sampling sections enriched for dividing or expanding cells, that could suffice even for analytical approaches that require larger input quantities. In addition, a cellular analysis called kinematic analysis, based upon growth measurements over time, DAPI staining of mitotically dividing cells and cell length measurements, allows to pinpoint whether the number, the duration or the rate of dividing or expanding cells was altered to cause the changes in growth or final organ size (Nelissen et al., 2013; Avramova et al., 2015a). In this way, several growth regulators have been identified to control maize leaf growth (Nelissen et al., 2012; Kir et al., 2015; Nelissen et al., 2015), of which gibberellins (GAs) were shown to play an important role in determining the transition from cell division to cell expansion. The increase in final leaf growth in the lines overexpressing the rate-limiting GA biosynthetic enzyme GA20-Oxidase was caused by an enhanced leaf elongation rate (LER), due to a higher number of dividing cells (Nelissen et al., 2012).

The coordination of growth processes within multi-cellular organisms is highly complex and can be controlled through many different signaling cascades that act locally or over a longer distance, between tissue layers, organs or sectors. Depending on whether the origin and perception of the signal occur in one cell, or multiple cells, growth regulation can be considered cell-autonomous or non-cell-autonomous. The majority of the growth processes known today are regulated in a cell-autonomous manner (Powell and Lenhard, 2012). During leaf growth, the majority of the molecular pathways (Gonzalez et al., 2012) are cell-autonomous, such as the proteolytic control of cell cycle regulators (Eloy et al., 2012), the function of expansins during cell expansion and the transcriptional regulation of meristemoid cell divisions through PEAPOD (White, 2006; Gonzalez et al., 2015).
other hand, phytohormones represent the best documented non-cell autonomous growth control (Santner et al., 2009), but also transcriptional activators, such as ANGUSTIFOLIA3, were shown to control growth over a longer distance (Kawade et al., 2010). Over the last decade, a novel mobile signal with organ growth promoting activity resulting from cytochrome P450 activity was identified (Nogueira et al., 2007; Bak et al., 2011; Dotto et al., 2014).

CYP78A, one of the subfamilies of cytochrome P450s, has been described to play a role in controlling vegetative and reproductive organ growth across different plant species. In Arabidopsis (Arabidopsis thaliana), CYP78A5/AtKLUH stimulates seed, leaf and flower growth by promoting cell proliferation, and acts to some extent redundantly with CYP78A7 (Anastasiou et al., 2007; Wang et al., 2008; Adamski et al., 2009; Eriksson et al., 2010). CYP78A6/ENHANCER OF da1-1 3 (EOD3), CYP78A8, CYP78A9 and CYP78A10 stimulate cell proliferation during seed and integument development, while CYP78A6 affects cell expansion (Fang et al., 2012; Sotelo-Silveira et al., 2013). In the moss Physcomitrella patens, the functional AtKLUH orthologs CYP78A27 and CYP78A28 affect protonema growth and gametophore development (Katsumata et al., 2011). In rice (Oryza sativa), CYP78A11/PLASTOCHRON1 (PLA1) positively controls plant size, and CYP78A13/GIANT EMBRYO (GE) was shown to enhance both vegetative growth and grain yield (Miyoshi et al., 2004; Yang et al., 2013). Moreover, in tomato (Solanum lycopersicum), up-regulation of SIKLUH predominantly resulted in large fruits caused by an increased cell number of pericarp and septum tissues (Chakrabarti et al., 2013). Finally, both soybean (Glycine max) GmCYP78A10b and wheat (Triticum aestivum L.) TaCYP78A3 positively regulate seed size (Ma et al., 2015; Wang et al., 2015). In maize, three genes were identified as CYP78A subfamily members, ZmGE1, ZmGE2 and ZmCYP78A1 (Larkin 1994), of which GE2 plays a role in kernel composition, as overexpression of GE2 significantly decreased the embryo to endosperm ratio (Zhang et al., 2012).

In this study, we describe the ZmKLUH/CYP78A1 (KLUH) as a time keeper of cell division during leaf growth in a GA independent manner. Constitutive overexpression of KLUH severely affects plant architecture by generating very large leaves but fails to reproduce. More moderate ectopic expression of KLUH results in fertile maize plants that show an increased maize growth, leaf and plant size and ear length in lab and field conditions. Using the maize leaf, we were able to show the cellular mechanism by which KLUH affects cell division as moderate overexpression of KLUH results in an increased duration of maximal growth rate during the steady state growth, while the opposite is observed in kluh mutants. Genetic and molecular evidence is presented to show that this increased duration of cell division is an independent mechanism to the increased number of dividing cells and leaf elongation rate (LER) that was previously shown to determine leaf size (Nelissen et al., 2012). Using two types of environmental stress, cold nights and mild drought, we were able to show that
the increased duration of growth serves as a compensatory mechanism under abiotic stress conditions that lower LER. Transcriptome studies, combined with hormone measurements showed the involvement of auxin in this compensatory mechanism.

Results

*Strong constitutive overexpression of KLUH stimulates leaves to keep on growing*

To study the role of *ZmKLUH* (*KLUH*), encoding a cytochrome P450 of the subclass of CYP78A1, we constitutively overexpressed *KLUH* under control of the constitutive UBI-L promoter. Two transgenic plants, referred to as UBIL::KLUH-P1 and UBIL::KLUH-P2 and both showing high KLUH expression (Supplementary Figure S1A) were examined in detail. Both transgenic plants grew slowly and produced reproductive organs after an extended growing period, but they failed to produce pollen or silks, making it impossible to obtain subsequent generations. Therefore, phenotypic analyses were performed on the primary transformants and compared to B104 plants and a control plant that simultaneously went through the same steps of tissue culture in the transformation platform (referred to as control transformant).

The UBIL-KLUH plants produced less leaves than B104 (14 leaves in UBIL::KLUH-P1, 17 in UBIL::KLUH-P2 as compared to 20 in B104), but the leaves were very long and broad, a phenotype that was correlated to the overexpression level of *KLUH* (Supplementary Figure S1A-C). The increased leaf length and width resulted in a more pronounced increase in leaf blade area, as is exemplified for leaf 9 for which the area was increased with 109% and 170% for UBIL::KLUH-P1 and UBIL::KLUH-P2, respectively, compared to the average leaf area for B104 (Figure 1 A, B). This remarkable difference in leaf size severely affected the plants morphology and stature (Figure 1C).

To determine the cellular basis of the KLUH mediated growth enhancement, we measured the average mature cell length of leaf 4, for which extensive information on final cell size in B104 was available (Nelissen et al., 2012; Voorend et al., 2014). The average epidermal cell length of leaf four of both UBIL::KLUH-P1 and UBIL::KLUH-P2 plants was significantly (*p* <0.001) reduced (78.5 ± 0.9 µm and 92.1 ± 1.3 µm, respectively) compared to that of wild type B104 plants (123.6 ± 1.5 µm) and the control transformant (136.8 ± 2.0 µm) (Figure 1D). As leaves with increased length consisted of smaller mature cells, we hypothesized that strong constitutive expression of *KLUH* stimulated cell division, producing more, but less expanded cells.
Figure 1. Phenotypes of the UBIL::KLUH plants. (A) Fully grown leaf nine of eight B104 plants compared to UBIL::KLUH-P1 and UBIL::KLUH-P2. Bar indicates 50 cm. (B) Leaf area of leaf nine of the two UBIL::KLUH plants compared to the average of eleven B104 plants. The error bar indicates the standard error for B104 plants. (C) Growing UBIL::KLUH showed altered plant architecture (UBIL::KLUH-P1 is at 241 days and UBIL::KLUH-P2 is at 255 days after transformation). (D) Average epidermal cell length for mature leaf tissue of UBIL::KLUH plants compared to the control plants (B104 and the control transformant). The error bars indicate the standard error. Significant differences P<0.001 (independent two-tailed T-test) are indicated with asterisks (***)

*Moderate overexpression of KLUH in maize robustly increases growth, biomass and seed yield*

As constitutive overexpression of KLUH results in too much cell proliferation, leading to infertile plants with very large leaves, we aimed to more subtly enhance KLUH expression to still promote growth without interfering with reproductive development. In steady state growing wild type leaves, KLUH shows a maximal expression level at the base of the leaf (first 0.5 cm) with a dramatic decrease at the next position along the growth zone (1 cm), although the latter is still part of the zone in which cells divide (Supplementary Figure S2A). We reasoned that extending the expression profile of KLUH along the growth zone could stimulate growth in a more balanced manner as compared to
constitutive expression. To this end, we cloned the 2046bp promoter sequence, upstream of the ATG start codon of GA2-oxidase (GA2ox) encoding gene, that was shown to be specifically expressed at the transition from cell division to cell expansion (Supplementary Figure S2A) (Nelissen et al., 2012).

Three independent, single-locus events, showed ectopic overexpression of KLUH in the growth zone of leaf four (referred to as GA2ox::KLUH-P1, GA2ox::KLUH-P2, GA2ox::KLUH-P3) (Supplementary Figure S2B). GA2ox::KLUH-P3 had the highest expression level which was 35 times more than that of the endogenous KLUH expression in its non-transgenic sibling at the basal part of the leaf (Supplementary Figure S2B). Opposite to the constitutive overexpression lines, the GA2ox::KLUH transgenic maize plants had a normal morphology, were fertile and had a comparable number of leaves as the non-transgenic siblings (21 leaves). In all three independent transgenic lines, the more subtle overexpression of KLUH resulted in an increased seedling vigor (Figure 2A) which was due to an increase in leaf length, width and area (Figure 2C-E). On average, 55% increase was observed in leaf four blade area (Figure 2A, C), that emanated from a 20% increase in leaf length and a 32% augmented leaf width (Figure 2D, E). The growth enhancing phenotypes were maintained throughout plant development since the transgenic plants showed an increase in leaf length and leaf width that was more pronounced with increasing leaf number (as illustrated for GA2ox::KLUH-P2 in Figure 2F-G). Also final plant height was significantly increased with values varying from 6.8% to 13.4% (Figure 2B, 3A; Supplementary Figure S3A). The transgenic lines were somewhat delayed in flowering, which was more pronounced for silking (on average 72 days in GA2ox::KLUH and 66 days in non-transgenic plants) than pollen shedding (on average 74 days in GA2ox::KLUH and 71 days in non-transgenic plants), resulting in a shorter anthesis-silking interval (ASI) (on average 1.4 days in GA2ox::KLUH versus 4.7 days in non-transgenic siblings; p<0.01) (Figure 3A; Supplementary Figure S3A). Final plant fresh weight was increased in the three lines, but was only significant in GA2ox::KLUH-P1 in greenhouse conditions (Supplementary Figure S3A).

To examine whether the greenhouse observations of increased leaf length and final plant height persisted in field evaluations and to study the effect on seed yield related parameters, the three independent GA2ox::KLUH lines (all in the inbred B104 genetic background) were grown in Ames, Iowa (USA) in 2014. The adult leaf below the main cob (ear leaf) was significantly longer (between 8.7% and 26.3% depending on the line) and wider (between 12.2% and 26.1% depending on the line) (Figure 3B; Supplementary Figure S3A). The effect on plant height was more pronounced in the field (significant increase ranging from 19% to 22% compared to the segregating non-transgenic siblings). Similar to the greenhouse, more time was needed for pollen shedding (on average 4.2 days) and silking (on average 6.8 days), and the ASI was shorter (on average 1.5 days) in GA2ox::KLUH in the
field (Figure 3B; Supplementary Figure S3A). Remarkably, the overall growth enhancement was also observed for the ear as the ear length was 9 to 23% longer, leading to 5.3% to 22% more florets (Figure 3B; Supplementary Figure S3A). The observations were confirmed for the GA2ox::KLHUH-P2 line in the growing season of 2015 (Supplementary Figure S3C and S4).

To further evaluate the robustness of the growth promoting effect of KLUH, we examined the KLUH-mediated phenotype in hybrid backgrounds by crossing homozygous GA2ox::KLHUH-P2 transgenic (T) and non-transgenic (NT) siblings to the inbred lines, CML91, H99 and W153R, that all are genetically distant of B104 and from each other (Liu et al., 2003) in greenhouse. In the presence of the KLUH transgene, the hybrids showed a 12.5% to 12.8% increase in final leaf length, a 7% to 14% in leaf width and a 24% to 49% expanded leaf blade area as compared to the hybrids without the KLUH transgene in greenhouse condition (Supplementary Table S1).

As the hybrid with CML91 resulted in the most pronounced absolute effects on leaf length (Supplementary Table S1), homozygous transgenic (T) GA2ox::KLHUH-P1; homozygous GA2ox::KLHUH-P2 and the respective null segregates (NT) each crossed to CML91 were assessed in the field in Wetteren, Belgium. In Belgium, the significantly augmented leaf length and width (measured for leaf 4 and ear leaf), leaf four blade area, plant height and stem width (Figure 3C-F; Supplementary Figure S3B-C) contributed to more plant fresh weight in the two transgenic hybrids compared to their non-transgenic hybrids (8%-35%; exemplified by GA2ox::KLHUH-P2×CML91 in Figure 3C). Moderate but consistent increases in the number of kernels per row (7%-14%) and the volume of the individual kernels (6%-16%) resulted in a significant increase cob length (17%-26%) and cob dry weight (18%-27%) in the two independent transgenic hybrids (Figure 3D; Supplementary Figure S3B).

GA2ox::KLHUH-P2_TxCML91 and GA2ox::KLHUH-P2_NTxCML91 hybrids were also grown in the field in Ames, Iowa, USA in 2015. In the U.S., the GA2ox::KLHUH-P2 transgenic hybrids were characterized by significant enhancement of plant height, leaf length and width (Figure 3E, Supplementary Figure S3C). Similar to what was seen in the inbred lines in the US and the hybrids in Belgium, more time was needed for KLUH transgenic hybrids for pollen shedding (on average 4 days) and silk emergence (on average 3 days), but the ASI was dramatically reduced (83%) relative to the non-transgenic hybrids (Figure 3F). Also in the US field trials, the transgenic hybrid plants showed a significantly increased cob length (9.3%) with a higher seed yield (total kernel dry weight per cob 13%), resulting from increased kernel number (5.8%) and kernel dry weight (7%) (Figure 3F; Supplementary Figure S3C). The presence of the GA2ox::KLHUH construct had a positive effect on stover biomass, seed yield and the synchronization of flowering in the B104 inbred as well as the B104xCML91 hybrid background in field conditions at two distinct locations over multiple growing seasons.
Figure 2. Phenotype of the GA2ox::KLUH plants. (A) Seedlings (12 days after sowing) and (B) mature plants (96 days after sowing) of GA2ox::KLUH-P3 segregating population, white arrows indicate the newly appeared leaf four. (C-E) Fully grown leaf four phenotypes in three independent GA2ox::KLUH segregating lines. The error bars indicate the standard error. Two-tailed Student t-tests revealed significant higher leaf area, leaf length and leaf width in the transgenic lines compared to the non-transgenic line. (F) Leaf length of GA2ox::KLUH-P2 compared to its non-transgenic siblings and B104 (n=3). A linear spline mixed model with a knot at leaf 2 showed a significant interaction between leaf number and genotype (p=0.015) and a significant change in slope at leaf 2 (p<0.0001). Wald tests indicated significant larger leaf lengths in the transgenic line compared to the non-transgenic line and B104 at indicated leaves (adjusted for multiple testing with the MaxT method). (G) Leaf width of GA2ox::KLUH-P2 compared to its non-transgenic siblings and B104 (n=3). A linear spline mixed model on the log2 transformed leaf width data with a knot at leaf 2 showed a significant main effect of genotype (p<0.0001), a significant slope for leaf (p=0.0023) and a significant change in slope at leaf 2 (p<0.0001). Wald tests indicated significant larger leaf widths in the transgenic line compared to the non-transgenic line (p<0.0001) and B104 (p<0.0001, adjusted for multiple testing with the Dunnett method). In the absence of an interaction term, this difference was the same at each leaf. * indicated significant difference (P<0.05) between GA2ox::KLUH-P2 and its non-transgenic siblings; † indicated significant difference (P<0.05) between GA2ox::KLUH-P2 and B104. All statistics are calculated based on two-tailed student T-test.
Figure 3. Phenotypic evaluations of the GA2ox::KLUH-P2 transgenic lines in the greenhouse, field and different genetic backgrounds. Phenotypes of the GA2ox::KLUH-P2 plants in B104 background, relative to the segregating non-transgenic siblings (A-B) in the greenhouse (A) and the US field trial 2014 (B). Vegetative and reproductive phenotypes of GA2ox::KLUH-P2 (T) X CML91 and the non-transgenic GA2ox::KLUH-P2 (NT) X CML91 hybrids grown in the field at Belgium (C-D) and U.S. (E-F) in 2015. The scale of the spider web is from -15% to 125%, data are presented as plant traits measurements from plants containing the GA2ox::KLUH construct relative to the non-transgenic controls.

**KLUH stimulates growth by extending the duration of cell division**

To determine the cellular mechanism by which KLUH stimulates growth, the fourth leaf was monitored over time by determining the leaf elongation rate (LER), the size of the division zone through DAPI staining and cell length profiles. Together, these measurements, typically done at steady state growth two days after appearance of leaf four, form the primary observations for a kinematic analysis that allows for quantifying the contributions of cell division and expansion (Nelissen et al., 2013). Strikingly, the kinematic analysis (Supplementary Table S2) on GA2ox::KLUH-P1 and GA2ox::KLUH-P2 revealed no statistical significant differences in cell production, the size of dividing cells, the number of dividing cells and the size of the division zone. In both transgenic lines, a small but significant increase in mature cell length (10% in GA2ox::KLUH-P1, P=0.02 and 8.6% in GA2ox::KLUH-P2, P=0.03) was found, however this difference was too small to explain the 19% to 20.5% increase in final leaf length. Indeed, no significant difference was observed between transgenic and non-transgenic siblings for the maximal leaf elongation rate at steady state growth, suggesting that the difference in final leaf length are due to a prolonged duration of growth. To
quantify the latter, we determined the leaf elongation duration (LED) (Voorend et al., 2014) that is defined as the time interval in which the leaf grows from 100mm until fully grown. The LED was significantly increased in the GA2ox::KLUH transgenic plants as compared to the non-transgenic siblings (13.7% for GA2ox::KLUH-P1; 12.3% for GA2ox::KLUH-P2) (Supplementary Table S2). The prolonged duration of growth was reproducible between the three GA2ox::KLUH lines, reflected by the LER profiles (Figure 4A, Supplemental Figure S5), where the period of steady state growth is prolonged by about one day. Together, these data suggest that KLUH promotes cell division by extending the time at which the division zone stays active, rather than affecting the absolute size of the division zone. To test this hypothesis, we followed the growth of leaf four and the size of the division zone over time, from when leaf 4 was still growing in the pseudo stem formed by the older leaves until leaf four stopped growing (Figure 4B). At the day before leaf four appeared, the growth rate and the division zone reached approximately two-thirds of their maximum, but both parameters increased gradually. The maximal size of the division zone was maintained between two days and four days after appearance of leaf four, while the LER reached steady state growth from day one until day five after appearance of leaf four in the non-transgenic siblings (Figure 4B). From day five onwards the division zone size decreased but the leaf continued to grow one more day at maximal rate.

In the GA2ox::KLUH-P3 transgenic plants, the size of the division zone was not always significantly larger than in the non-transgenic siblings after leaf initiation (Figure 4B), in accordance with the observations of the kinematic analysis (Supplementary Table S2). However, when leaf development proceeded, the LER and size of the division zone remained higher in the GA2ox::KLUH plants as compared to the non-transgenic siblings (Figure 4B). The interaction between genotype and LER over time was highly significant (two-way mixed model, P=0.0175), as well as the interaction between genotype and division zone size over time (two-way ANOVA, P=0.021). The observation that the difference in leaf growth between the transgenic and non-transgenic siblings became more pronounced during the later phases of leaf four growth, was supported by the progression of leaf length over time. The differences in final leaf length became significant (P= 0.003) at the time point where LER started to decrease (day 6 in Figure 4B and 4C).

Opposite phenotypes were observed in a homozygous kluh mutation caused by insertion of the Mutator transposon (UniformMu; McCarty et al., 2005). The kluh homozygous mutant plants displayed 9.2% shorter final leaf length (P=0.02) than WT, an overall decrease in the size of the division zone and a premature decline of the maximal growth rate as compared to WT (Figure 4D).
KLUH enhances maize growth

Figure 4. Leaf four growth phenotype of GA2ox::KLUH-P3 and kluh over time. T: transgenics; NT: non-transgenics. (A) Leaf elongation rate (LER) of leaf 4 of GA2ox::KLUH-P3 from day 1 till day 11. A two-way mixed model revealed a significant interaction between genotype and days (p<0.0001). Wald tests indicated significant faster LER in the transgenic line compared to the non-transgenic line at indicated days (adjusted for multiple testing with the MaxT method). (B) Division zone size and LER measurements over time in segregating GA2ox::KLUH-P3 from day -1 till day 8. A two-way ANOVA model fit to the division zone size data revealed a significant interaction between genotype and days (p=0.021). Wald tests indicated significant larger division zone sizes in the transgenic line compared to the non-transgenic line at indicated days (adjusted for multiple testing with the Sidak method). (C) Leaf length measurements over time in segregating GA2ox::KLUH-P3. A two-way ANOVA model revealed a significant interaction between genotype and days (p<0.0001). Wald tests indicated significant larger leaf lengths in the transgenic line compared to the non-transgenic line at indicated days (adjusted for multiple testing with the Sidak method). (D) Division zone size and LER measurements over time in the kluh mutant and WT. A two-way ANOVA model fit to the division zone size data revealed a significant interaction between genotype and days (p<0.0001). Wald tests indicated significant smaller division zone sizes in the kluh mutant compared to WT at indicated days (adjusted for multiple testing with the Sidak method). *p<0.05.

KLUH controls the developmental timing of cell division as a compensatory mechanism that is independent of the GA-dependent increase in the number of dividing cells.
Phenotypic analysis showed that KLUH plays a role to stimulate the developmental time window for cell division and thus in maintaining steady state growth over time. To determine how the duration of steady state growth is related to the maximal steady state growth, the GA2ox::KLUH plants were challenged by conditions in which the maximal steady state growth was positively or negatively affected.

Previously, the high levels of bioactive gibberellic acid (GA) increased the LER caused by an enlarged size of the DZ (Nelissen et al., 2012; Voorend et al., 2014), while here we show that KLUH enlarges the time window of cell proliferation. In order to investigate how these two mechanisms relate to each other, crosses were made between the segregating GA20ox-1 line (UBIL::GA20OX) and the segregating GA2ox::KLUH-P2. The characteristics of both parental lines, being the GA-mediated high maximal LER and the KLUH-mediated prolonged growth duration, were observed in the plants expressing both transgenes (Figure 5A), resulting in a final leaf size and plant height increase that are additive in the growth chamber and field grown plants, respectively (Figure 5B-C). Also other phenotypic traits, such as leaf four area, were additive (Supplementary Table S3).

Alternatively, it was shown that mild drought conditions (Chapter 2) and cold nights (Rymen et al., 2007; Material and Methods) resulted in a reduction in LER, which is comparable for the two stresses. In order to study how the KLUH-mediated growth mechanism functions when the maximal growth rate is lowered by stress conditions, the growth of GA2ox::KLUH (segregating) lines was monitored under mild drought and cold nights. In the non-transgenic siblings, mild drought resulted in a significant reduction of LER (ranging from -25.0% to -27.9% reduction) which is partly compensated by a 13.6% to 18.2% prolonged LED (Supplementary Table S4A). In the KLUH transgenic plants, the reduction in LER (ranging from -30.4% to -34.6% reduction) was slightly more severe compared to the respective non-transgenic siblings, while the compensation from LED was more pronounced (ranging from 23.4% to 28.8%) in the presence of the KLUH transgene (Figure 5D; Supplementary Table S4A). In cold nights, a similar decrease in LER (-25.6% for GA2ox::KLUH-P3, -25.4% for non-transgenic siblings) was observed in both transgenic and non-transgenic plants. In contrast to mild drought stress, the cold-induced reduced LER was not compensated by an extended LED in the wild-type. However, although not as pronounced as the increased LED extension in GA2ox::KLUH induced by drought, a 9.8% extended LED was observed in cold-treated GA2ox::KLUH-P3 (Figure 5E; Supplementary Table S4B). These data indicate that the KLUH-mediated growth mechanism is part of the compensatory growth mechanisms that operates in B104 plants when the growth rate is reduced by adverse environmental conditions.
KLUH enhances maize growth

Figure 5. (A-C) Leaf phenotypes of UBIL::GA20OX × GA2ox::KLUH: leaf elongation rate (A) and final length (B) of leaf 4 of UBIL::GA20OX × GA2ox::KLUH in greenhouse condition; plant height (C) of UBIL::GA20OX × GA2ox::KLUH in the field condition. (D) Leaf elongation rate of GA2ox::KLUH under mild drought stress. A three-way mixed model fitted to the LER data revealed a significant interaction between genotype, condition and days (p=0.0012). Simple tests of effect for each genotype at each day between the drought and control condition indicated significant differences in both directions (adjusted for multiple testing with the MaxT method). (E) Leaf elongation rate of GA2ox::KLUH under cold nights stress. A three-way mixed model fitted to the LER data revealed a significant interaction between genotype, condition and days (p<0.0001). For the treatments see Materials and Methods. T: transgenics; NT: non-transgenics.
The KLUH mediated growth stimulation involves in auxin metabolism

To examine the molecular changes within the division zone over time and the effect of modulated ZmKLUH expression, the transcriptome of the most basal half centimeter in GA2ox::KLUH-P3, entirely consisting of dividing cells, was profiled at two different time points during leaf development. The first time point was during the steady state growth (2 days after the appearance of leaf 4, Figure 4B). The second time point was when the differences in both LER and the size of DZ were maximal between the transgenic and non-transgenic siblings (6 days after the appearance of leaf 4, Figure 4B).

The specific transcriptomic changes (FC>2 and FDR<0.05) between time points (2444 transcripts in non-transgenic siblings, and 1845 transcripts in GA2ox::KLUH transgenics) were much more pronounced than between the genotypes (115 transcripts at day 2, and 533 transcripts at day 6). The highest upregulated transcript was ZmKLUH, 51.8 and 47.5 times higher than the endogenous KLUH transcript level at day 2 and day 6, respectively.

The genes that were downregulated over time in non-transgenic siblings were enriched for the GO category “cell cycle arrest”, “auxin metabolic process” and “cytokinin biosynthetic process” (Supplementary Table S5A). Conversely, the GO categories of “photosynthesis” and “secondary cell wall biogenesis” were upregulated in the later time point, suggesting that the division zone started to differentiate over time. The GO category of “auxin mediated signaling pathway” was also upregulated over time, represented by significantly upregulated expression of nine SMALL AUXIN-UP RNA (SAUR) genes as the size of the division zone decreased over time (Supplementary Table S5B). In addition, genes involved in auxin efflux were significantly upregulated over time (Supplementary Table S6). These data suggest that already some differentiation is taking place in the division zone as the leaf progressed its growth and that the division zone became consumed, a process that might be regulated by auxin. In the GA2ox::KLUH-P3 transgenic plants, the majority of the GO categories that changed over time (including “photosynthesis”, “auxin signaling”, “cell cycle arrest”) were similar to those in the non-transgenic siblings (Supplementary Table S5B).

During steady state growth, the genes that were differentially downregulated in GA2ox::KLUH-P3 relative to the non-transgenic plants belonged to the GO category “aromatic amino acid transport” and “floral organ development”, while the upregulated genes were involved in “lactate transport” and “oxidation-reduction process” (Supplementary Table S5C). At day 6, the upregulated genes in the transgenics compared to the non-transgenic control plants were enriched for the GO categories “negative regulation of DNA binding transcription factor activity” and “regulation of hormone levels” while the downregulated genes were enriched for “terpenoid catabolic process” and “auxin polar
transport” (Supplementary Table S5D). Several genes encoding auxin efflux transporters were downregulated in GA2ox::KLUH-P3 as compared to the non-transgenic siblings, including PIN1b, PIN10a and BIF2, a positive regulator of cellular auxin efflux (Supplementary Table S6). The transcriptome data suggest that the auxin homeostasis is altered in the GA2ox::KLUH-P3 transgenic plants compared to wild type.

To further analyze the effect of ectopic KLUH expression on auxin metabolism, the levels of IAA and auxin precursors and conjugates were determined in the growth zone of GA2ox::KLUH-P3 transgenic plants in comparison with non-transgenic siblings. Samples were taken with 0.5 cm intervals along the most basal 3 cm of the growth zone. The auxin content was highest at the leaf basis and decreased gradually along the growth zone, similar to what previously was observed (Nelissen et al., 2012; Chapter 2) (Supplemental Figure S2A). In GA2ox::KLUH-P3, auxin levels are consistently higher than in the non-transgenic siblings (Figure 6). Also a significant increase in the levels of auxin biosynthesis precursors such as tryptophan (TRP) and indole-3-pyruvic acid (IPyA) as well as the auxin conjugates (IAA-Glu; IAA-Asp) and the auxin inactivation product (oxlIAA) were observed in the GA2ox::KLUH-P3 transgenic plants (Figure 6). An independent experiment using samples from 0.5 cm to 1 cm and 2.5 cm to 3 cm from the leaf basis confirmed the higher levels of auxin related metabolites in the KLUH transgenic plants (Supplementary Figure S6).
Figure 6. Bioactive IAA level including IAA precursors and its inactivation products in GA2ox::KLUH-P3. Leaf 4 of transgenic GA2ox::KLUH-P3 plants and non-transgenic siblings were harvested at two days after its appearance. The x-axis indicates different zones in the leaf. Two-way mixed models were fitted to the data. Only for IPyA a significant interaction between genotype and zone was detected (p=0.0073). Wald tests indicated significant higher levels of IPyA in the transgenic siblings compared to the non-transgenic siblings (adjusted for multiple testing with the MaxT method) at 2-2.5cm (p=0.04) and 2.5-3cm (p=0.0003). For IAM, IAA, oxIAA, IAA-Asp and IAA-Glu significant main effects were detected for both genotype and zone. Corresponding p-values for genotype are 0.0018, <0.0001, 0.022, 0.0001, 0.0006, for IAM, IAA, oxIAA, IAA-Asp and IAA-Glu respectively. For TRA only a significant genotype effect was detected with a significant higher level in the non-transgenic line compared to the transgenic line (p=0.0007). For ANT and TRP only a significant effect of the zone was detected.

ANT, anthranilate; TRP, tryptophan; TRA, tryptamine; IAM, indole-3-acetamide; IPyA, indole-3-pyruvic acid; IAA, indole-3-acetic acid; oxIAA, 2-oxoindole-3-acetic acid; IAA-Asp, IAA-aspartate; IAA-Glu, IAA-glutamate.
Discussion

KLHUH represents a novel mechanism in leaf growth

Leaf growth is driven by cell division and cell expansion and the spatial or temporal regulation of these processes seem to be species-specific. Leaf growth in Arabidopsis occurs sequentially in time: the leaf initially consists of fully dividing cells and as the leaf grows, cell division ceases from the tip to the base of the leaf, resulting in a cell cycle arrest front moving across the leaf (Kazama et al., 2010; Andriankaja et al., 2012). In contrast, in maize, growth is primarily considered spatially with a linear organization in which sequentially the division zone (DZ) and expansion zone (EZ) occur from the base to the tip of the maize leaf during steady state growth. The proportions of DZ and EZ stay stable during the steady state growth, and play an important role in determining the final leaf length, as shown by the effect of changes in DZ size on final leaf length in the GA insensitive mutants and GA20OX overexpression lines (Nelissen et al., 2012, Chapter 2). Besides GA, mutations in BRASSINOSTEROID INSENSITIVE1 (BRI1) and GROWTH-REGULATING FACTOR1 (GRF1), and stress growth conditions have been shown to affect maize leaf growth by altering the DZ size (Avramova et al., 2015b; Kir et al., 2015; Nelissen et al., 2015).

In addition to the growth regulators that control the spatial distributions of the cellular processes, we identified a role for CYP78A1/KLUH in the temporal regulation of maize leaf growth. Both strong and mild expression KLUH resulted in longer leaves with longer growth period comparing to controls. Detailed cellular analysis on mild KLUH overexpression plants revealed that KLUH controls the duration of cell division and therefore affects the duration of leaf elongation (LED). Conversely, in kluh mutants, the size of the DZ decreases faster than in wild type. The analysis of leaf size and division zone size over time revealed that there is also a temporal regulation of the growth processes in the maize leaf, reminiscent to that observed in the Arabidopsis leaf. On the other hand, at a given time point during Arabidopsis leaf growth, the cell cycle arrest front indicates that cell division (at the base) and cell expansion (toward the tip of the leaf) occur in a similar spatial organization that is typical for monocot leaves. These data indicate that the spatial and temporal regulation of leaf growth is more conserved in monocots and dicots than originally thought.

Gibberellin and auxin are involved in two mechanisms that regulate maize leaf growth
Interestingly, the spatial and temporal regulation of maize leaf growth appears to be controlled by two hormones. GA promotes leaf growth by increasing the maximal growth rate (LER), whereas auxin is downregulated over time and increased auxin levels in the GA2ox::KLUH transgenic lines associate with a prolonged LED. Auxin has been implicated in virtually every aspect of plant growth and development, including lateral root initiation, root hair elongation, leaf patterning and flower bud formation (Okada et al., 1991; Sieburth, 1999; Aloni et al., 2006; Velasquez et al., 2016). In maize, auxin inhibits root length and promotes lateral root and crown root formation (McSteen, 2010), and mediates leaf primordia initiation (Tsiantis et al., 1999; Johnston et al., 2015). However, the function of auxin in the growth zone of the maize leaf is not yet elucidated. In accordance with previous observations (Nelissen et al., 2012), we show here that the endogenous auxin is highly abundant at basal DZ and decreases to a basic level at the distal boundary of the DZ, which is parallel to the expression profile of KLUH. Moreover, hormone and transcriptome analysis shows that auxin content and response are consistently associated with KLUH expression level, suggesting that auxin is involved in the KLUH mediated regulation of growth duration. Interestingly, overexpressing the auxin induced gene AUXIN REGULATED GENE INVOLVED IN ORGAN GROWTH (ARGOS) resulting in larger organs is partly due to the prolonged cell division period (Hu et al., 2003). Additionally, chorismate synthase that catalyzes the auxin precursor chorismate as well as auxin transporter ZmPIN1b are downregulated in GA2ox::KLUH compared to the non-transgenic sibling after steady state growth stage, and are negatively correlated with LED (Baute et al., 2015). Together, the data show that auxin is highly involved in determining the duration of maize leaf growth. Additionally, chorismate synthase that catalyzes the auxin precursor chorismate, as well as auxin transporter ZmPIN1b are downregulated in GA2ox::KLUH compared to the non-transgenic sibling after steady state growth stage, and are negatively correlated with LED (Baute et al., 2015). Together, the data show that auxin is highly involved in determining the duration of maize leaf growth.

The phenotypes of the double transgenics containing UBI::GA20OX and GA2ox::KLUH were additive compared to the single transgenes suggesting that both LER and LED are two independent processes regulating leaf growth. A similar conclusion was reached when the natural variation of LER and LED were studied in the B73×H99 RIL population and a MAGIC (multiparent advanced generation intercross) population, as both processes were highly correlated to final leaf length but not correlated to each other (Baute et al., 2015; Baute et al., 2016). The interplay between auxin and GA is rather complicated as there are reports of the effect of DELLA mediated GA action on auxin signaling, such as SMALL AUXIN UPREGULATED RNA36 (SAUR36) and SAUR9 in the proliferating Arabidopsis leaf (Claeys et al., 2014). GA biosynthesis is also required for normal auxin responses during Arabidopsis hypocotyl elongation, although the transcriptome data indicate that auxin also
acts in part through pathways independent of GA (Chapman et al., 2012). These studies show that both auxin and GAs affect growth at cellular level, and that the interaction between them might be context dependent, such as depending on the species, organ and developmental stage.

**KLUH involved in a compensation mechanism in maize under stress conditions**

Previously, reduction of the cell division rate and cell production are observed when maize plants are subjected to water deficit conditions (Avramova et al., 2015c), while only cell cycle duration is affected in cold nights (Rymen et al., 2007). In Arabidopsis ethylene acts on cell cycle progression and causes very quickly a reversible pause on cell cycle in young proliferating leaves under mild osmotic stress. When the stress persists, the pausing of the cell cycle will become irreversible and cells will enter the differentiation process. The shutdown of the cell cycle could be compensated by additional cell divisions from meristemoid development when the stress is released (Skirycz et al., 2011). However in maize, the contribution from stomatal formation to leaf size is limited (Vatén and Bergmann, 2012). In addition, the maize leaf reduces the growth rate rather than a pause of growth under drought stress, and grows for a longer period (Avramova et al., 2015, Chapter 2). A similar phenotype with reduced LER but prolonged LED was observed for leaves of plants grown under mild drought and cold stress conditions in this study. Detailed cellular analysis showed that mild drought reduces the size of DZ at steady state growth stage, and consequently leads to a smaller leaf (Avramova et al., 2015, Chapter 2). Therefore, instead of completely shutting down cell division, maize leaves under stress conditions reduce the number of dividing cells (and thus the LER) but remain their dividing capacity for a longer period (prolonged LED). It might be that the plants lower the growth rate and invest in a longer period of growth during stress conditions to maintain a longer ability to resume growth when the conditions become more favorable. Alternatively, the plant adjusts its growth program to form its organs to a predefined size, when stress conditions reduce LER, by prolonging another mechanism to stimulate leaf growth such as LED. Future experiments are needed to further unravel the biological function of this compensation mechanism.

**The leaf growth and yield increase potential depends on the expression level of KLUH**

We generated two KLUH overexpression lines (UBIL::KLUH and GA2ox::KLUH) in this study, both of which increased the length and width of the leaves. The increase in leaf area is more pronounced in UBIL::KLUH than in GA2ox::KLUH, indicating that KLUH acts dose dependent. The dose-dependency of the KLUH gene is also observed by the effects on mature cell length and reproductive growth. The
mature cell length is increased in the GA2ox::KLUH mild overexpression (10% in GA2ox::KLUH-P1 and 9% in GA2ox::KLUH-P2), whereas the mature cells are significantly smaller than that of B104 in the two strong KLUH overexpression lines (-39% in UBIL::KLUH-P1 and -28% in UBIL::KLUH-P2). As KLUH stimulates the cell division period, the small cells in UBIL::KLUH might be due to overproliferation leading to many, smaller cells that fail to go through the complete cell expansion phase. The prolonged leaf growth in KLUH expression lines also is reflected on the complete developmental time window of the maize plants. A slight delay in reproductive timing was observed in GA2ox::KLUH, but this mild overexpression of KLUH stimulates cob development, leading to increased cob size with higher grain yield. In contrast, the female and male flowers did not develop properly in the strong KLUH expression lines. The impairment of the reproductive organ in UBIL::KLUH might be due to the long vegetative growth period, resulting in an even further delay in reproductive growth. Alternatively, the cell division in the reproductive organs might last so long that the necessary differentiation was not completed, thereby causing the infertility in UBIL::KLUH. In the moss Physcomitrella patens, strong overexpression of CYP78A27 and CYP78A28 resulted in reduced fertility caused by delayed reproductive growth (Katsumata et al., 2011).

Besides the members in CYP78A family, several other growth regulating genes also cause retarded plant growth by over-stimulating cell division (Blomme et al., 2014). When the cell cycle gene CYCD3;1 is overexpressed in Arabidopsis, the mature leaf epidermis of the transgenic plants consists of a large number of very small cells due to overproliferation in the absence of cellular differentiation (Dewitte and Murray, 2003). Additionally, plants overexpressing the cell cycle regulating transcription factor E2Fa have larger cotyledons with increased cell number and decreased cell size, while overexpression both E2Fa and DPα severely affected the plant stature due to cellular overproliferation (De Veylder et al., 2002). In these cases, the growth stimulation highly depends on the expression level of growth regulators. Therefore, using tissue-specific or inducible promoters driving gene expression can overcome unfavorable effects instead of using constitutive promoters. The use of specific promoters to drive gene expression has been recently discussed in crops (Dutt et al., 2014; Nuccio et al., 2015), and here the specific expression of KLUH provides another successful example of the importance of the promoter choice in the construction of transgenes.

**KLUH universally promotes growth in different conditions and genetic backgrounds**

Mild overexpression of KLUH did not only positively affect leaf and plant size and finally stover yield, but also seed yield. The overall increased kernel number and kernel weight result in a final significantly increased grain production. It was reported that in Arabidopsis elevated
**CYP78A5/AtKLHU** levels produced bigger seeds by promoting integument cell proliferation (Adamski et al., 2009). The tomato CYP78A5/KLUH homolog increases fruit size by producing more cells in pericarp and septum tissues (Chakrabarti et al., 2013). Here, we demonstrated that the knowledge-based expression of the KLUH gene has resulted in a simultaneous increase in both stover and seed yield, showing that the KLUH mediated growth mechanism is conserved in different maize organs.

The effect of a transgene, shown in a lab strain, is often lost when being introduced in elite varieties or hybrids. Previously, many studies that assessed the expressivity of transgenes in hybrids focused on traits that are not interfering with the core plant metabolism and development, such as herbicide tolerance (Agapito-Tenfen et al., 2014; Zhang et al., 2014). However, since plant growth is a complex trait, the development of transgenic crops to improve growth and yield, might be more difficult than the first generation of GM crops. As there might be an interaction between the inserted transgene and the genetic background, that can affect the expected phenotype, the transgene expressivity should be studied in more genetic backgrounds prior to commercialization. So far, only few growth-related genes were introduced into different genetic backgrounds in maize. Maize ARGOS1 (ZAR1) and GROWTH-REGULATING FACTORS10 (GRF10), which stimulate growth in inbred genetic background, persisted their growth regulating capacity in one tested hybrid background (Guo et al., 2014; Wu et al., 2014). Ectopic overexpressing the rice trehalose-6-phosphate phosphatase (TPP) in maize displays drought tolerance in many hybrid genetic backgrounds, resulting in a general better yield production (Nuccio et al., 2015). Also GA2ox::KLHU consistently positively affected plant growth and yield in different genetic backgrounds. In addition, the effects in both inbred and hybrid background were observed in both the greenhouse and two independent field trials that differ in growing season or geographical location, strengthening the expressivity of the KLUH mediated effects on biomass and seed yield.
Materials and methods

Gene cloning and maize transformation

The maize cytochrome P450 CYP78A1 is the closest homolog of Arabidopsis CYP78A5/AtKLUH (Miyoshi et al., 2004). Therefore, we assigned maize CYP78A1 as ZmKLUH following the Arabidopsis nomenclature. Extention overlap PCR was used for ZmKLUH (KLUH) cloning, primers were designed with NCBI Primer-Blast. PCR primers for amplification KLUH are ATGGCGATGGCCTCCGGCCTTGCTCATG and TTATATGCATGTACAGTATGTTTTTTTCAG; CTGAAAAAAACATACTGTACATGCATATAA and TCAGGCGGTGCGGGGGATGG. For overexpression of KLUH, the KLUH gene was cloned behind the UBIL promoter (Christensen and Quail, 1996) and GA2-oxidase (GA2ox) promoter into the vector pBbm42GW7 respectively (Karimi et al., 2013) (http://gateway.psb.ugent.be/). GA2-oxidase (GRMZM2G031724; GA2ox) is a gene that encodes an enzyme involved in GA degradation (Nelissen et al., 2012) and 2046bp in 5’UTG from the start coding of GA2ox was cloned as promoter. Primers for cloning the GA2ox promoter are GAGGATTGCAGCTCCTGGATC, GGCTGTCTGGCCGTGCGGATG. Immature embryos of the maize inbred line B104 were transformed by Agrobacterium tumefaciens cocultivation as described previously (Coussens et al., 2012). For UBIL::KLUH, two independent events were obtained from transformation, and both were used for further analysis. For GA2ox::KLUH, transgenic lines backcrossed to B104, in which the T-DNA was present in a single locus were used for further analysis.

Isolation of maize genomic DNA and PCR analysis

Genotyping was done by visual screening for the transgenic lines by an immunochromatographic assay detecting the PAT protein (AgroStrip, Romer) and a PCR-based method detecting the BAR selectable marker and the transgene. Total genomic DNA was isolated from mature leaf tissue with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Primers against selection marker and transgen specific primers were used for transgenic plants genotyping. All T0 plants contain the BAR selection marker, encoding for the phosphinothricin acetyltransferase (PAT) protein. The presence of the PAT protein in T0 plants was tested by an immunochromatographic assay (AgroStrip, Romer). The primary transformants (T0 shoots) were tested for the presence of the construct in their gDNA by means of PCR. The primers were designed flanking promoter and terminator region. For UBIL::KLUH, forward primer was TACGCTATTTATTTGCTTGG and reverse primer was ATCGACGTCGATGCTC; for GA2ox::KLUH, forward primer was AGTTATTTGATGAGCCGAGTC and reverse primer was GAACGCCATGAAGCTCTGCTTGG. The PCR condition for amplifying the UBIL::KLUH
construct fragment was as followed: initial denaturation at 98°C for 3 min, followed by 35 cycles of 98°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min 20 s, and a final extension step of 5 min at 72°C. The detection PCR conditions for amplifying the fragment in GA2ox::KLUH construct was as followed: initial denaturation at 98°C for 3 min, followed by 35 cycles of 98°C for 10 s, annealing at 57°C for 30 s, extension at 72°C for 30 s, and a final extension step of 5 min at 72°C.

Growth conditions in growth chamber and greenhouse

Plants for leaf growth monitoring were grown under growth chamber condition with controlled relative humidity (55%), temperature (24°C/18°C), and light intensity (170 mmol m-2 s-1 photosynthetic active radiation at plant level) provided by a combination of high-pressure sodium vapour (RNP-T/LR/400W/S/230/E40; Radium) and metal halide lamps with quartz burners (HRI-BT/400W/D230/E40; Radium) in a 16h/8h (day/night) cycle. Plants under mild drought treatment shared the same growth condition as control but with 60% water deficiency. The water content of control condition was 1.23 g water g-1 dry soil, and the water content of mild drought soil was 0.738 g water g-1 dry soil, corresponding to -23kPa and -1025 kPa, respectively. For the cold treatment, plants were grown in the same growth condition during photoperiod but 4°C in the dark for 8 hours. A gradual decrease and increase of radiation intensity was implemented over 0.5 h to mimic dusk and dawn. Plants were grown under controlled greenhouse conditions (26°C/22°C, 55% relative humidity, light intensity of 180 mmol m-2 s-1 photosynthetic active radiation, in a 16h/8h day/night cycle) for adult plant traits.

Plant materials and phenotype evaluation in growth chamber

Plants were measured daily to determine the leaf elongation rate (n=5). The leaf blade was cut and scanned to determine the leaf area by ImageJ (Schneider et al., 2012). Kinematic analysis was performed based on Nelissen et al., 2013. For determination of the division zone size over time, leaf 4 was harvested daily before emergence from the sheath of leaf 3 until fully grown. The time point was determined by the day when leaf 4 was initially visualized from the whorl of leaf 3. The size of the division zone was determined by the distance between the base and the most distally observed mitotic figure in DAPI-stained leaves along the proximal-distal axis with a fluorescence microscope (AxioImager, Zeiss). For every analysis, at least three technical repeats were taken.
Field experiment trial design and plant trait analysis

Field experiments were conducted in 2014 and 2015 at Ames, Iowa, U.S. and 2015 at Wetteren, Belgium. In U.S. 2014, three GA2ox::KLUH segregating progenies were grown in 3 rows, 20 plants per row, the plant traits were measured in all plants. In 2015, GA2ox::KLUH-P2 segregating population and its segregating transgenic hybrid GA2ox::KLUH-P2×CML91 were grown in 5 and 10 rows, respectively: each row contained 20 plants, plant traits and grain yield were performed on 12 representative plants per row. In Belgium, two transgenic hybrids GA2ox::KLUH-P1×CML91 and GA2ox::KLUH-P2×CML91 and their non-transgenic control were grown in randomized block design with three replicates. Each replicate contained 4 rows and 40 plants per row. All the plants were measured for vegetative traits analysis, except ear leaf width and stem width were measured on 20 and 5 represented plants per replicates, respectively. Cob component data were determined by harvesting five representative ears per replicate. All the seeds per cob were weighted and divided by their number, the volume of the seeds was measured by immersion 50 seeds per cob in water.

Maize RNA extraction and qRT-PCR

Total RNA was isolated with the guanidinium thiocyanate-phenol-chloroform extraction method using TRI-reagent (Sigma-Aldrich). First-strand cDNA was synthesized from 1 µg total RNA using the iScript™ Advanced cDNA Synthesis Kit for qRT-PCR (Bio-Rad Laboratories). KLUH specific primers are CTCTGCAACGGCATGGTGGTC and GGCGGCATCGTGGGTTATGG. qRT-PCR was performed on a LightCycler 480 (Roche) on 384-well plates with LightCycler 480 SYBR Green I Master mix (Roche Diagnostics) according to the manufacturer’s instructions. PCR reactions were done in triplicate. For relative quantification, a threshold cycle was set at the same level for each reaction within the exponential amplification phase. For normalization, the transcript levels of the housekeeping gene 18S rRNA was used as internal control.

RNA sequencing analysis

Library preparation was done using the TruSeq RNA Sample Preparation Kit v2 (Illumina). In brief, poly(A)-containing mRNA molecules were reverse transcribed, double-stranded cDNA was generated and adapters ligated. After quality control using a 2100 Bioanalyzer (Agilent), clusters were generated through amplification using the TruSeq PE Cluster Kit v3-cBot-HS kit (Illumina) followed by sequencing on an Illumina HiSeq2000 with the TruSeq SBS Kit v3-HS (Illumina). Sequencing was performed in paired-end mode with a read length of 100 nt. The quality of the raw data was verified.
with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, version 0.9.1). Next, quality filtering was performed using the trimmomatic: reads were globally filtered so that for at least 75% of the reads, the quality exceeds Q10 and 3’ trimming was performed to remove bases with a quality below Q10, ensuring a minimum length of 20 bp remaining. Repairing was performed using a custom perl script. Reads were subsequently mapped to v3 of the maize B73 reference genome (http://ftp.maizesequence.org/B73_RefGen_v3/) using GSNAP 2.0.0 (Wu and Nacu, 2010). The concordantly paired reads that uniquely map to the genome were used for quantification on the gene level with htseq-count from the HTSeq.py python package (Anders et al., 2014).

These genes were subjected to differential analysis with the R software package edgeR (Robinson et al., 2010; Team, 2014) (R version 3.2.3). Only genes with an expression value higher than 1 cpm (corresponding to 5 read counts) in at least 3 samples were retained for the analysis (20284 genes were kept out of 39323). TMM normalization (Robinson and Oshlack, 2010) was applied using the calcNormFactors function. Variability in the dataset was assessed with a MDSplot. All three biological replicates clustered nicely together and there was a clear separation between the four factor level combinations. Trended negative binomial dispersion parameters were estimated with the default Cox-Reid method based on a model with main effects of treatment, time and replicate and an interaction term between time and treatment using the estimateGLMTrendedDisp function, followed by the estimation of the empirical bayes dispersion for each transcript. A negative binomial regression model was then used to model the overdispersed counts for each gene separately with fixed values for the dispersion parameter as outlined in (McCarthy et al., 2012) and as implemented in the function glmFit using the above described model. A likelihood ratio test (LRT) was performed to compare this model with a model without replicate to assess possible replicate (batch) effects. After false discovery rate adjustments of the p values with the method described by (Benjamini and Hochberg, 1995), only 8 genes were found to have a significant batch effect as was expected by the MDS plot. The estimate of the dispersions and the fitting of the model was repeated with now only the main effects of time and treatment and their interaction. The significance of the interaction term was assessed with a LRT test comparing the full model with the main effects model. To test user-defined hypotheses, the model was reparameterized. The factors were combined to 1 factor with 4 levels, and a no intercept single factor model was fitted to the data. With this design, dispersions were re-estimated and the model was refit. The four contrasts of interest were in the difference between the time points for each genotype, and the difference between the genotypes at each time point. Significance was assessed with a LRT test and as before, FDR adjustments of p values were applied. All edgeR functions were applied with default values.
IAA metabolic profiling

Leaf four of GA2ox::KLUH-P3 transgenic plants and non-transgenic siblings were harvested at the second day after the appearance from the whorl. Five plants were taken for one biological replicate, and three biological replicates were harvested. Extraction, purification and IAA metabolic profiling was performed as described (Novak et al., 2012).
Supplementary data

Supplementary Figure S1. Leaf phenotype in two *KLUH* overexpression lines UBIL::KLUH-P1 and UBIL::KLUH-P2. (A) Relative KLUH expression levels for B104 compared to UBIL::KLUH-P1 and UBIL::KLUH-P2 in mature samples of leaf eight. (B-C) Final leaf length and maximal leaf width of the two UBIL::KLUH plants compared to the average of eleven B104 plants.
Supplementary Figure S2. KLUH expression level in wild-type and KLUH overexpression lines. (A) KLUH and GA2-oxidase expression along maize leaf four in B104 (wild type) relative to 18S. The dotted line indicates the mature zone that continues after centimeter 5. (B) Relative expression of KLUH at the basal 0.5 cm leaf of three GA2ox::KLUH transgenic lines, compared to their corresponding leaf samples in non-transgenic siblings. DZ: division zone; EZ: expansion zone; MZ: mature zone.
Supplementary Figure S3. Bar chart showing the phenotypes of GA2ox::KLUH in the greenhouse and field. A-B: plant and ear related phenotypes of three GA2ox::KLUH B104 segregating populations grown in greenhouse (A) and field (U.S.) (B) conditions in year 2014. C: plant and yield traits of GA2ox::KLUHxCML91 grown in field (Belgium) in year 2015. D: plant and yield traits of GA2ox::KLUH-P2 B104 segregating line and GA2ox::KLUH-P2xCML91 grown in field (U.S.) in year 2015. Data are presented as measurements from plants containing the GA2ox::KLUH construct relative to the non-transgenic controls. Asterisks indicate statistically significant differences in the transgenic plants versus the non-transgenic plants according to a student t-test (*, **, and *** correspond to P-values of 0.05>p>0.01, 0.01>p>0.001, and p< 0.001, respectively).
Supplementary Figure S4. Phenotypic evaluations of the GA2ox::KLUH-P2 transgenic lines in the U.S. field trial 2015. The scale of the spider web is from -80% to 120%. Data are presented as measurements from plants containing the GA2ox::KLUH construct relative to the non-transgenic controls.
Supplementary Figure S5. Leaf elongation rate of leaf 4 in (A) GA2ox::KLUH-P1 and (B) GA2OX::KLUH-P2 relative to the non-transgenic siblings. A two-way mixed model fitted to the LER data of GA2ox::KLUH-P1 and the non-transgenic sibling revealed a significant interaction between genotype and days (p=0.0057). Wald tests indicated significant faster LER in the transgenic line compared to its non-transgenic sibling at indicated days (adjusted for multiple testing with the MaxT method). *p<0.05. A two-way mixed model fitted to the LER data of GA2ox::KLUH-P2 and the non-transgenic sibling revealed only a significant main effect of genotype (p=0.0073) and days (p<0.0001). LER was significantly faster in the transgenic line compared to its non-transgenic sibling.
Supplementary Figure S6. Endogenous auxin content in GA2ox::KLUH-P3 transgenic plants. Two segments of the growth zone of the fourth leaf (0.5-1 cm and 2.5-3 cm) of transgenic plants and non-transgenic siblings were harvested at the second day after leaf appearance. Error bars indicate SD (n = 5). Asterisks indicate statistically significant difference in the transgenic plants versus the non-transgenic plants in paired t-test (*, ** correspond to P-values of 0.05>p>0.01, 0.01>p>0.001, respectively).

ANT, anthranilate; TRP, tryptophan; TRA, tryptamine; IAM, indole-3-acetamide; IPyA, indole-3-pyruvic acid; IAA, indole-3-acetic acid; oxIAA, 2-oxoindole-3-acetic acid; IAA-Asp, IAA-aspartate; IAA-Glu, IAA-glutamate.
Supplementary Table S1. The effect of GA2ox::KLUH transgenics and non-transgenics on leaf growth in different hybrids.

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<th>Leaf area (mm^2)</th>
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<th>p-value</th>
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<td><strong>GA2ox::KLUH-P2_T x H99</strong></td>
<td>12106.97±428.19</td>
<td></td>
<td></td>
<td>821.29±11.50</td>
<td></td>
<td></td>
<td>28.49±0.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GA2ox::KLUH-P2_NT x CML91</strong></td>
<td>13567.88±940.26</td>
<td>24.1</td>
<td>0.013</td>
<td>875.38±33.94</td>
<td>12.8</td>
<td>0.014</td>
<td>28.49±0.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GA2ox::KLUH-P2_T x CML91</strong></td>
<td>16832.34±387.16</td>
<td></td>
<td></td>
<td>987±14.75</td>
<td></td>
<td></td>
<td>28.89±0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GA2ox::KLUH-P2_NT x W153R</strong></td>
<td>10435.11±178.72</td>
<td>48.7</td>
<td>8.5E-07</td>
<td>721±11.77</td>
<td>12.5</td>
<td>1.7E-05</td>
<td>25.04±0.61</td>
<td>13.4</td>
<td>0.017</td>
</tr>
<tr>
<td><strong>GA2ox::KLUH-P2_T x W153R</strong></td>
<td>15521.12±209.61</td>
<td></td>
<td></td>
<td>811±9.83</td>
<td></td>
<td></td>
<td>28.41±0.81</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Average±SD; n=4. Statistics were calculated based on student T-test.
Supplementary Table S2. Kinematic analysis of GA2ox::KLHU-P1 and GA2ox::KLHU-P2 transgenic and non-trangenic siblings.

<table>
<thead>
<tr>
<th>Growth parameters a</th>
<th>GA2ox::KLHU-P1 NT</th>
<th>GA2ox::KLHU-P1 T</th>
<th>P_value b</th>
<th>GA2ox::KLHU-P2 NT</th>
<th>GA2ox::KLHU-P2 T</th>
<th>P_value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final leaf length (mm)</td>
<td>585.38</td>
<td>705.14</td>
<td>0.00</td>
<td>20.46</td>
<td>572.27</td>
<td>680.92</td>
</tr>
<tr>
<td>LERmax (mm.h⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LED (h)</td>
<td>232.3</td>
<td>264.17</td>
<td>0.00</td>
<td>13.71</td>
<td>235.26</td>
<td>264.17</td>
</tr>
<tr>
<td>Division zone size (cm)</td>
<td>1.26</td>
<td>1.29</td>
<td>0.82</td>
<td>2.26</td>
<td>1.23</td>
<td>1.31</td>
</tr>
<tr>
<td>Dividing cell size (µm)</td>
<td>17.41</td>
<td>17.07</td>
<td>0.52</td>
<td>-1.93</td>
<td>17.76</td>
<td>18.09</td>
</tr>
<tr>
<td># dividing cell</td>
<td>737.77</td>
<td>757.75</td>
<td>0.79</td>
<td>2.71</td>
<td>709.41</td>
<td>739.04</td>
</tr>
<tr>
<td>Cell production (Cell.h⁻¹)</td>
<td>21.58</td>
<td>21.46</td>
<td>0.83</td>
<td>-0.58</td>
<td>20.09</td>
<td>21.08</td>
</tr>
<tr>
<td>Cell division (Cells cell⁻¹h⁻¹)</td>
<td>0.03</td>
<td>0.03</td>
<td>0.70</td>
<td>-4.04</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Cell cycle duration (h)</td>
<td>23.76</td>
<td>24.45</td>
<td>0.80</td>
<td>2.87</td>
<td>24.51</td>
<td>24.30</td>
</tr>
<tr>
<td>Mature cell size (µm)</td>
<td>109.67</td>
<td>120.61</td>
<td>0.02</td>
<td>9.98</td>
<td>105.69</td>
<td>114.82</td>
</tr>
<tr>
<td>Elongation zone size (cm)</td>
<td>2.53</td>
<td>2.72</td>
<td>0.64</td>
<td>7.37</td>
<td>2.59</td>
<td>2.96</td>
</tr>
<tr>
<td>Elongating cell size (µm)</td>
<td>62.76</td>
<td>64.82</td>
<td>0.78</td>
<td>3.28</td>
<td>59.39</td>
<td>68.62</td>
</tr>
<tr>
<td># elongation cell</td>
<td>406.77</td>
<td>417.89</td>
<td>0.81</td>
<td>2.73</td>
<td>436.15</td>
<td>432.18</td>
</tr>
</tbody>
</table>

a Mean value for each parameters were determined for 3 plants, final leaf length and leaf elongation rate were determined using 5 plants. All parameters were determined at 48 h after leaf emergence from the sheath.

b Statistical significance based on student T-test. Numbers in bold indicate parameters that are statistically different.

Supplementary Table S3. Leaf phenotype of transgenic plants expressing both GA2ox::KLUH and UBIL::GA20OX, relative to the single overexpression plants and the null segregants.

<table>
<thead>
<tr>
<th></th>
<th>leaf 4 length (mm)</th>
<th>leaf 4 width (mm)</th>
<th>leaf 4 area (mm^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBIL::GA20OX_T</td>
<td>770,5±36</td>
<td>17,3±2</td>
<td>8965,0±283</td>
</tr>
<tr>
<td>UBIL::GA20OX_NT</td>
<td>565,2±7</td>
<td>20,4±1</td>
<td>8634,0±152</td>
</tr>
<tr>
<td>GA2ox::KLUH_T</td>
<td>707,7±15</td>
<td>25,4±3</td>
<td>10866,7±436</td>
</tr>
<tr>
<td>GA2ox::KLUH_NT</td>
<td>548,3±9</td>
<td>22,2±1</td>
<td>8287,5±82</td>
</tr>
<tr>
<td>UBIL::GA20OX_T×GA2ox::KLUH_T</td>
<td>912,3±8</td>
<td>24,2±1</td>
<td>13216,7±222</td>
</tr>
<tr>
<td>UBIL::GA20OX_NT×GA2ox::KLUH_NT</td>
<td>608,5±6</td>
<td>19±2</td>
<td>8876,7±167</td>
</tr>
</tbody>
</table>

The cross of GA2ox::KLUH and UBIL::GA20OX was made by crossing segregating GA2ox::KLUH with segregating UBIL::GA20OX.

T: transgenics; NT: non-transgenics.

Average± SD; n>3. Statistical significance based on student T-test.
Supplementary Table S4. Leaf growth parameters of GA2ox::KLUH segregating lines under mild drought and GA2ox::KLUH-P3 segregating line under cold nights conditions.

| A | Transgenic line | | Non-transgenic line | |
|---|---|---|---|---|---|---|---|---|---|---|
| | control | mild drought | Δ% | P value | control | mild drought | Δ% | P value | |
| FLL (mm) | | | | | | | | | |
| GA2ox::KLUH-P1 | 703.4±8.71 | 589.9±25.36 | -16.1 | 0.0004 | 540.3±10.98 | 491.8±24.1 | -10.2 | 0.1 | |
| LERmax (mm/h) | 3.4±0.07 | 2.2±0.11 | -34.6 | 3.3E-09 | 3.1±0.09 | 2.3±0.24 | -25.0 | 0.04 | |
| LED (h) | 237.5±5.57 | 293.2±15.86 | 23.4 | 0.004 | 193.4±5.0 | 219.2±13.9 | 14.3 | 0.16 | |
| FLL (mm) | 643.8±8.68 | 573.8±11.46 | -9.1 | 0.003 | 557.5±15.85 | 478.6±13.55 | -14.3 | 0.002 | |
| GA2ox::KLUH-P2 | LERmax (mm/h) | 3.4±0.07 | 2.4±0.16 | -30.4 | 0.002 | 3.1±0.12 | 2.2±0.13 | -27.9 | 0.0002 | |
| LED (h) | 209.4±3.6 | 268.5±18.09 | 28.2 | 0.029 | 191.6±4.48 | 217.6±16.16 | 13.6 | 0.004 | |
| FLL (mm) | 677.7±7.75 | 560.5±14.9 | -13.7 | 0.0004 | 536.3±8.34 | 487.4±12.85 | -8.9 | 0.01 | |
| GA2ox::KLUH-P3 | LERmax (mm/h) | 3.3±0.08 | 2.2±0.13 | -34.4 | 2.47E-05 | 3.0±0.07 | 2.2±0.11 | -25.0 | 0.0001 | |
| LED (h) | 230.6±7.95 | 297.0±8.26 | 28.8 | 0.0001 | 187.9±2.46 | 222.2±6.34 | 18.2 | 0.001 | |
| B | control | cold nights | Δ% | P value | control | cold nights | Δ% | P value | |
| FLL (mm) | | | | | | | | | |
| GA2ox::KLUH-P3 | 791.8±11.9 | 671.8±7.5 | -15 | 0 | 634.9±10.8 | 513.0±10.8 | -19 | 0 | |
| LERmax (mm/h) | 3.38±0.04 | 2.5±0.04 | -25.6 | 0 | 3.04±0.03 | 2.24±0.05 | -25.4 | 0 | |
| LED (h) | 271.0±4.2 | 299.5±4.5 | 9.8 | 0.0003 | 227.9±3.5 | 233.5±4.5 | 2.4 | 0.37 | |


FLL: final leaf length; LERmax: maximal leaf elongation rate; LED: leaf elongation duration.
Supplementary Table S5. Enrichment analysis of gene ontology (GO) terms for differentially expressed transcriptions in GA2ox::KLUH-P3 segregating plants during different developmental stages.

<table>
<thead>
<tr>
<th>A</th>
<th>genes upregulated in W6 compared with W2</th>
<th>genes downregulated in W6 compared with W2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>membrane lipid catabolic process</td>
<td>negative regulation of cyclin-dependent protein serine/threonine kinase activity</td>
</tr>
<tr>
<td></td>
<td>photosynthesis</td>
<td>positive regulation of phytoalexin biosynthetic process</td>
</tr>
<tr>
<td></td>
<td>sulfate transport</td>
<td>cell cycle arrest</td>
</tr>
<tr>
<td></td>
<td>salicylic acid metabolic process</td>
<td>cytokinin biosynthetic process</td>
</tr>
<tr>
<td></td>
<td>cellulose metabolic process</td>
<td>regulation of systemic acquired resistance</td>
</tr>
<tr>
<td></td>
<td>secondary cell wall biogenesis</td>
<td>auxin metabolic process</td>
</tr>
<tr>
<td></td>
<td>ATP hydrolysis coupled proton transport</td>
<td>maintenance of meristem identity</td>
</tr>
<tr>
<td></td>
<td>response to light</td>
<td>hormone biosynthetic process</td>
</tr>
<tr>
<td></td>
<td>photosynthetic electron transport chain</td>
<td>stem cell maintenance</td>
</tr>
<tr>
<td></td>
<td>anion transport</td>
<td>nucleobase metabolic process</td>
</tr>
<tr>
<td></td>
<td>auxin mediated signaling pathway</td>
<td>glutamine family amino acid biosynthetic process</td>
</tr>
<tr>
<td></td>
<td>response to karrakin</td>
<td>phylloyme development</td>
</tr>
<tr>
<td></td>
<td>organic acid transport</td>
<td>shoot system development</td>
</tr>
<tr>
<td></td>
<td>carboxylic acid transport</td>
<td>regulation of cellular biosynthetic process</td>
</tr>
<tr>
<td></td>
<td>lipid localization</td>
<td>regulation of nitrogen compound metabolic process</td>
</tr>
<tr>
<td></td>
<td>response to cold</td>
<td>response to endogenous stimulus</td>
</tr>
<tr>
<td></td>
<td>ion transport</td>
<td>regulation of macromolecule metabolic process</td>
</tr>
<tr>
<td></td>
<td>response to oxidative stress</td>
<td>cellular response to stress</td>
</tr>
<tr>
<td></td>
<td>response to organic substance</td>
<td>heterocycle biosynthetic process</td>
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<td></td>
<td>lipid metabolic process</td>
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</table>

<table>
<thead>
<tr>
<th>B</th>
<th>genes upregulated in K6 compared with K2</th>
<th>genes downregulated in K6 compared with K2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>photosynthesis</td>
<td>regulation of phytoalexin biosynthetic process</td>
</tr>
<tr>
<td></td>
<td>sulfate transport</td>
<td>cotyledon vascular tissue pattern formation</td>
</tr>
<tr>
<td></td>
<td>response to light</td>
<td>detection of fungus</td>
</tr>
<tr>
<td></td>
<td>auxin mediated signaling pathway</td>
<td>benzoate metabolic process</td>
</tr>
<tr>
<td></td>
<td>response to karrakin</td>
<td>cellular response to high light intensity</td>
</tr>
<tr>
<td></td>
<td>organic acid transport</td>
<td>cell cycle arrest</td>
</tr>
<tr>
<td></td>
<td>carboxylic acid transport</td>
<td>regulation of systemic acquired resistance</td>
</tr>
<tr>
<td></td>
<td>response to cold</td>
<td>primary root development</td>
</tr>
<tr>
<td></td>
<td>cellular glucan metabolic process</td>
<td>abscisic acid metabolic process</td>
</tr>
<tr>
<td></td>
<td>glucan metabolic process</td>
<td>apocarotenoid metabolic process</td>
</tr>
<tr>
<td></td>
<td>response to abiotic stimulus</td>
<td>auxin metabolic process</td>
</tr>
<tr>
<td></td>
<td>lipid metabolic process</td>
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</tr>
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<td>transmembrane transport</td>
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<td></td>
<td>carbohydrate metabolic process</td>
<td>cellular response to gibberellin stimulus</td>
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<td></td>
<td>single-organism transport</td>
<td>disaccharide biosynthetic process</td>
</tr>
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<td></td>
<td></td>
<td>negative regulation of developmental process</td>
</tr>
<tr>
<td></td>
<td></td>
<td>response to auxin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>shoot system development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>regulation of cellular biosynthetic process</td>
</tr>
<tr>
<td></td>
<td></td>
<td>regulation of nitrogen compound metabolic process</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C</th>
<th>genes upregulated in K2 compared with W2</th>
<th>genes downregulated in K2 compared with W2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>lactate transport</td>
<td>aromatic amino acid transport</td>
</tr>
<tr>
<td></td>
<td>oxidation-reduction process</td>
<td>floral organ development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>perianth development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pyrimidine nucleobase salvage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>positive regulation of epidermis development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>positive regulation of stomatal complex development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>regulation of growth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pattern specification process</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D</th>
<th>genes upregulated in K6 compared with W6</th>
<th>genes downregulated in K6 compared with W6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>negative regulation of DNA binding</td>
<td>terpenoid catabolic process</td>
</tr>
<tr>
<td></td>
<td>transcription factor activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>regulation of hormone levels</td>
<td>auxin polar transport</td>
</tr>
<tr>
<td></td>
<td>regulation of transcription, DNA-dependent</td>
<td>response to karrakin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lipid transport</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fatty acid biosynthetic process</td>
</tr>
</tbody>
</table>
K2 and K6 are basal (0.5cm) leaf samples harvested at two days and six days after the emergence of leaf four, respectively, in GA2ox::KLUH-P3; W2 and W6 are basal (0.5cm) leaf samples harvested at two days and six days after the emergence of leaf four in GA2ox::KLUH-P3 non-transgenic siblings. (A-B) GO enrichment of the 2444 and 1845 differentially expressed transcripts in non-transgenic (A) and GA2ox::KLUH-P3 (B) leaf samples over time, respectively; (C-D) GO enrichment of the 115 and 533 differentially expressed transcripts in GA2ox::KLUH-P3 compared to the non-transgenic siblings at day 2 (C) and day 6 (D), respectively.
GO enrichment is characterized by PLAZA (Proost et al., 2014) with a P-value<0.01 as cut-off, and are listed from the highest enriched to less enriched categories.

Supplementary Table S6. Expression level of auxin efflux transporters in GA2ox::KLUH-P3 segregating plants during different developmental stages.

<table>
<thead>
<tr>
<th>geneID</th>
<th>K2 vs W2</th>
<th>K6 vs W6</th>
<th>K6 vs K2</th>
<th>W6 vs W2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAX3 GRMZM2G149481</td>
<td>-1.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZmPIN1a GRMZM2G098643</td>
<td>-1.48</td>
<td>-1.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZmPIN1b GRMZM2G074267</td>
<td>-1.17</td>
<td>1.20</td>
<td>1.87</td>
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<tr>
<td>PIN1c GRMZM2G149184</td>
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<td>4.77</td>
<td>4.93</td>
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<tr>
<td>PIN10a GRMZM2G126260</td>
<td>-1.52</td>
<td>1.55</td>
<td>2.93</td>
<td></td>
</tr>
<tr>
<td>PIN1d GRMZM2G171702</td>
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<td>-1.15</td>
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</tr>
<tr>
<td>ZmPINY GRMZM2G050089</td>
<td></td>
<td>1.12</td>
<td>1.43</td>
<td></td>
</tr>
<tr>
<td>BIF2/positive regulator of cellular auxin efflux GRMZM2G103559</td>
<td>-1.12</td>
<td>1.29</td>
<td>2.34</td>
<td></td>
</tr>
<tr>
<td>BIF2/positive regulator of cellular auxin efflux GRMZM2G177812</td>
<td></td>
<td></td>
<td>1.06</td>
<td></td>
</tr>
</tbody>
</table>

K2 and K6 are basal (0.5cm) leaf samples harvested at two days and six days after emergence of leaf four, respectively, in GA2ox::KLUH-P3; W2 and W6 are basal (0.5cm) leaf samples harvested at two days and six days after their emergence, respectively, in GA2ox::KLUH-P3 non-transgenic siblings.

The expression level was calculated by log fold change (logFC) and indicated the change for the front samples compared with the next. purple: logFC>1; blue: logFC<1.
References


KLUH enhances maize growth


Chapter 4

Metabolic characterization of cytochrome P450

CYP78A1/ZmKLUH
Metabolic characterization of cytochrome P450
CYP78A1/ZmKLUH

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AUTHOR CONTRIBUTION

The plants were grown and sampled by X.S. and J.D.B under the supervision of H.N. and D.I.. K.M. and J.P. performed the LC-MS measurements and analysis under guidance of B.V., A.G. and W.B.. X.S. wrote the chapter with contributions from K.M., H.N. and D.I.
Abstract

Maize CYP78A1/ZmKLUH belongs to the CYP78A subfamily of cytochrome P450s. The members of the CYP78A family stimulate cell division during vegetative and reproductive growth in many species. Previously, we showed that both the maize ZmKLUH and its Arabidopsis homolog CYP78A5/AtKLUH stimulates cell proliferation in maize and Arabidopsis leaves, respectively. We postulate that the substrate and reaction product of the maize and Arabidopsis KLUH are conserved between dicots and monocots. Here we further characterized by LC-MS the metabolic changes of maize lines which overexpress ZmKLUH in comparison to wild-type maize and a maize line in which CYP78A1/ZmKLUH is mutated. We found the content of chlorogenic acid (CGA) was higher in the ZmKLUH overexpressing line and reduced in Zmkluh mutant plants. Next, the effect of CGA was analyzed by feeding CGA to Arabidopsis wild-type, Atkluh mutants and AtKLUH overexpression lines. Cell division in Arabidopsis rosette leaves was highly stimulated by CGA treatment, however not to the same extent as observed in Arabidopsis overexpressing AtKLUH. We postulate that both maize and Arabidopsis KLUH have a role in catalyzing biosynthesis of CGA or related metabolites.
Introduction

As one of the largest gene families in plants, Cytochrome P450s (CYPs) are hemethiolate enzymes involved in numerous biosynthesis and xenobiotic pathways found in all organisms (Bak et al., 2011). CYPs are classified as monooxygenase and catalyze extremely diverse reactions, but usually incorporate a hydroxyl group into substrates with the use of NAD(P)H (Bernhardt, 2006). For many CYPs, genetic analysis has uncovered the biological process they are involved in, but the substrate often remains unknown and cannot be deduced from the sequence (Bak et al., 2011). One of these P450 families with unknown molecular function is the CYP78A subfamily, but the genetic and cellular analysis reveal that the CYP78A family members share a conserved function in controlling vegetative and reproductive organ growth in land plants. In Arabidopsis (Arabidopsis thaliana), rice (Oryza sativa) and maize (Zea mays), CYP78A5/AtKLUH, CYP78A11/PLASTOCHRON1 (PLA1), and CYP78A1/ZmKLUH affect leaf growth and plant morphology respectively (Miyoshi et al., 2004; Wang et al., 2008); and several Arabidopsis CYP78As, including CYP78A5, CYP78A6/ENHANCER OF da1-1 3 (EOD3), CYP78A8 and CYP78A9, rice OsCYP78A13/GIANT EMBRYO (GE), wheat (Triticum aestivum L.) CYP78A3, have been well described to positively control seed size through cell proliferation (Ito and Meyerowitz, 2000; Adamski et al., 2009; Fang et al., 2012; Sotelo-Silveira et al., 2013; Yang et al., 2013; Ma et al., 2015). Additionally, rice CYP78A13/GE can rescue the smaller inflorescence and silique phenotype in the Arabidopsis loss of function mutant cyp78a5/Atkluh, suggesting that the CYP78As may have conserved function in both monocots and dicots (Xu et al., 2015).

Another interesting feature of the CYP78A subfamily members is that they mediate organ growth in a non-cell autonomous manner. CYP78A5/AtKLUH is thought to generate a mobile growth-promoting signal traveling over large distances, since AtKLUH activity extends not only between floral organs within one flower, but also within inflorescences (Anastasiou et al., 2007; Eriksson et al., 2010). Additionally, CYP78A13/GE affects the cell division and the maintenance of the shoot apical meristem (SAM), but GE mRNA is absent from either embryonic SAM or seedling SAM (Yang et al., 2013). Moreover, CYP78A6/EOD3 and CYP78A9 were not detected in the maternal integuments of developing seeds, although small seeds were observed in eod3, cyp78a9 and a eod3 cyp78a9 double mutant (Fang et al., 2012). The differences between the expression patterns of CYP78As and their functions suggested that CYP78As stimulate organ growth in a non-cell autonomous manner, probably by generating a conserved plant signaling molecule.

However, the substrates, end products and the mode of action of CYP78As remain elusive. It was reported that the recombinant maize CYP78A1/ZmKLUH protein in yeast catalyzed the 12-hydroxylation of lauric acid (C12) (Imaishi et al., 2000), and the Arabidopsis CYP78A5/AtKLUH,
CYP78A7 and CYP78A10 were confirmed to catalyze the hydroxylation of short chain fatty acids, with lauric acid being the preferred substrate in vitro (Kai et al., 2009). However, the cyp78a5/Atkluh phenotype was not rescued by the application of 12-hydroxyl-lauric acid, suggesting that there might be other substrates in plants (Kai et al., 2009; Sotelo-Silveira et al., 2013). Other evidence suggests that the CYP78A subfamily is involved in the phenylpropanoid metabolism pathway. Two components of the phenolic biosynthesis pathway, shikimic acid and quinic acid, were reduced in the rice mutant with high expression level of CYP78A13 (Xu et al., 2015). Arabidopsis CYP78A9 was predicted to be a flavonoid 3’-monooxygenase, involved in the flavonol biosynthesis pathway, and alterations of flavonoids contents were observed in cyp78a9 and CYP78A9-overexpressing plants (Sotelo-Silveira et al., 2013). However, abolishing flavonoid biosynthesis did not alter the CYP78A9 overexpression phenotype, suggesting that the perturbations in flavonoid levels are not responsible for the CYP78A9 function (Sotelo-Silveira et al., 2013).

In chapter 3, we described that CYP78A1/ZmKLUH controls organ growth as a time keeper for cell division duration. Specifically, a mild overexpression of ZmKLUH under the maize GA2-oxidase (GA2ox) promoter (GA2ox::KLUH) results in large leaves caused by a prolonged duration of cell division. In concert with this phenotype, bigger rosette leaves containing more cells were obtained when CYP78A5/AtKLUH was overexpressed under its endogenous promoter (AtKLUH::AtKLUH) in Arabidopsis (Anastasiou et al., 2007). However, when AtKLUH was continuously overexpressed under the control of the 35S promoter (35S::AtKLUH), plants with smaller leaves were obtained containing many more cells (Claeys et al., unpublished data). Probably the KLUH mediated cell proliferation prohibits proper cell growth. Opposite to the phenotype of AtKLUH overexpression plants, AtKLUH mutants have smaller leaf size resulting from the reduction of cell numbers (Claeys et al., unpublished data). Therefore, AtKLUH and ZmKLUH seem to regulate cell division during leaf development in similar ways, both in Arabidopsis and maize. Consequently, it is expected that both in Arabidopsis and maize KLUH uses the same substrate and converts it to a similar, if not identical, mobile growth regulator. Here we describe experiments aiming at identifying the nature of the KLUH substrate and end product(s).
Results

CYP78A-like CYPs are implicated in phenylpropanoid metabolism or fatty acid metabolism

Plant cytochrome P450s (CYPs) are initially classified into two clades: the A-type and the non-A-type. The A type CYPs, also known as the CYP71 clan, are mainly involved in biosynthesis of secondary metabolites, whereas the non-A-type P450 clade consists out of individual clades that sometimes show more local similarity to non-plant CYPs than to other plant CYPs (Paquette et al., 2000; Bak et al., 2011). The CYP78A family belongs to the A-type P450 clade, and contains an intron that is conserved in many CYP71 clan families. In order to investigate the possible substrate of CYP78As, we therefore summarized the known substrates of Arabidopsis A-type CYPs that are closely related to CYP78As in a phylogenetic tree.

Six gene families are closely related to CYP78As within the CYP71 clan, including CYP701A, CYP77, CYP89A, CYP73A, CYP703A and CYP79 (Figure 1). Except for the CYP89A and CYP78A subfamilies, substrates of some members of the other five families have been identified. CYP701A3 is ent-kaurene oxidase that catalyze important steps in gibberellin biosynthesis (Helliwell et al., 1999). CYP77A4 and CYP77A6 of the CYP77A family, and CYP703A2 of the CYP703A family were identified as fatty acid hydroxylases (Morant et al., 2007; Li-Beisson et al., 2009; Sauveplane et al., 2009). CYP79F1 and CYP79F2 of CYP79 family catalyze short- and long chain methionine-derived glucosinolates (Reintanz et al., 2001; Chen et al., 2003), while another two members of CYP79 family CYP79A2 and CYP79B2 were shown to catalyze the N-hydroxylation of the phenylalanine and tryptophan (Mikkelsen et al., 2000; Wittstock and Halkier, 2000). CYP73A5, which shares the highest phylogenetic identity with the CYP78As, is identified as cinnamic acid 4-hydroxylase catalyzing the hydroxylation of cinnamic acid to 4-coumaric acid (Mizutani et al., 1997) (Figure 1). As two out of six CYP78A-like CYPs (including the CYP78A closest homolog) catalyze reactions of the phenylpropanoid metabolism, it was thus tempting to speculate that the substrate of CYP78A enzymes was derived from phenylalanine as well.

The expression level of genes encoding enzymes of the phenylpropanoid pathway was affected by ZmKLUH overexpression

Ectopic expression of CYP78A1/ZmKLUH in maize under the control of the maize GA2-oxidase promoter (GA2ox::KLUH) exhibits growth promotion in vegetative and reproductive stages and displays a slight delay in development (chapter 3). To narrow down the potential KLUH substrate, a transcriptome analysis was performed on the division zone of the growing maize leaf. The most basal
half centimeter of the growing leaf in GA2ox::KLUH plants and control plants, consisting entirely of dividing cells, was profiled at two different time points. The first time point was during the steady state growth (2 days after the appearance of leaf 4). The second time point was when the differences in both LER and the size of DZ were maximal between the transgenic and non-transgenic siblings (6 days after the appearance of leaf 4) (as described in chapter 3). Twenty-three genes were upregulated in GA2ox::KLUH plants in both time points, including ZmKLUH as the highest upregulated transcript, while nine genes were consistently downregulated in both time points. Next to ZmKLUH itself, fourteen out of the thirty-two differentially expressed genes encode enzymes, indicating that ZmKLUH overexpression causes a significant metabolomic change (Table 1). The ten enzyme encoding genes that were upregulated in GA2ox::KLUH have diverse functions, including a serine-type peptidase; the CYP89A6 with unknown function; calmodulin-dependent protein kinase; DIMBOA glucosidase, ACC oxidase; a 3-beta hydroxysteroid dehydrogenase; two enzymes related to glutathione system (a glutathione S-transferase; glutaredoxin-C8) and two enzymes that are involved in phenylpropanoid metabolism (cyanidin 3-O-rutinoside 5-O-glucosyltransferase-like protein; cinnamate beta-D-glucosyltransferase) (Table 1). Four enzyme-encoding genes were downregulated in the GA2ox::KLUH samples. Two of them share 96% protein identity and are part of the GDSL-like lipase superfamily (Chepyshko et al., 2012), while the other two downregulated genes encode O-methyltransferase and share 40% protein identity (Table 1).

Previously, a preliminary metabolic screen in the empty siliques (es1-D) mutant (Marsch-Martinez et al., 2002), the Arabidopsis transposon activation-tagged mutant that overexpresses CYP78A9 and consequently results in larger organ size in Arabidopsis revealed a pronounced influence of CYP78A9 on flavonol contents across different organs (Sotelo-Silveira et al., 2013). Furthermore, lower shikimic acid and quinic acid content were determined in cyp78a13 mutants than in wild-type in rice (Xu et al., 2015). These data, together with the phylogeny of CYP78As, motivated us to explore whether ZmKLUH could influence the phenolic pathway in maize. The expression levels of the transcripts involved in phenolic pathway were determined in GA2ox::KLUH using transcriptome data (Figure 2). At day 2, GA2ox::KLUH and its non-transgenic siblings both contain the maximal DZ size and the difference between the two DZ sizes was small. The difference of the DZ size between GA2ox::KLUH and non-transgensics expanded with leaf growth, and reached the maximum at day 6. The enlarged difference of DZ size was associated with increased transcriptional changes of enzymes involved in phenylpropanoid pathway at day 6 comparing to day 2 (Figure 2). As the few enzyme encoding genes with differential expression at day 2 persisted their expression trend at day 6, we therefore focused on the transcriptome changes at day 6.
The initial steps of the phenolic metabolic pathway that leads to the phenylalanine biosynthesis were barely affected, but the enzymes that directed phenolic metabolism from phenylalanine were significantly affected by ZmKLUH overexpression (Figure 2), as shown by the downregulated genes encoding phenylalanine ammonia-lyase (PAL) and 4-coumarate:CoA ligase (4CL) at day 6. In the following steps of the phenylpropanoid pathway, p-coumaroyl CoA is the common substrate of two enzymes, which lead the metabolic routes to, on one hand, flavonoids and on the other hand of phenylpropanoids. Chalcone synthase (CHS) catalyzes the formation of the flavonoid skeleton, while hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferases (HCTs) lead to the biosynthesis of two major lignin building units (coniferyl and sinapyl alcohol) (Figure 2). Seven genes are predicted to encode HCTs in maize (Morohashi et al., 2012) and were expressed in our database. Three of them were significantly downregulated in GA2ox::KLUH (Figure 2). In contrast, although CHS was barely affected by ZmKLUH overexpression, the gene encodes chalcone isomerase (CHI, enzyme that catalyzes the next step after CHS) was significantly upregulated (Figure 2). As there are opposite transcriptional changes of genes encoding between enzymes directing lignin monomer biosynthesis and the flavonoid pathway in GA2ox::KLUH (Figure 2), it seems that ZmKLUH overexpression affected both flavonoids and phenylpropanoid compounds biosynthesis. The genes encoding enzymes that direct lignin monomer biosynthesis (CAD, cinnamyl alcohol dehydrogenase) and sinapic acid synthesis (ALDH, Aldehyde dehydrogenase), also showed differential expression suggesting that ZmKLUH overexpression had a profound influence on the phenolic pathway in maize.
Metabolic characterization of KLUH

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<td>N-hydroxylase</td>
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<tr>
<td></td>
<td>mono-penta-hexahomomethionine</td>
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Figure 1. Characteristics of reactions catalyzed by part of the A-type CYPs in Arabidopsis.

Different cytochrome P450s families are labeled with different colors.

The neighbor-joining bootstrap tree of the A-type CYPs is adapted from Bak et al., 2011.
Table 1. Significantly differentially expressed genes in GA2ox::KLUH compared to wild-type.

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<th>logFC_ K6 versus W6</th>
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K2 versus W2: comparisons between leaf four of GA2ox::KLUH and its non-transgenic siblings at two days after the fourth leaf emergence from the pseudo stem; K6 versus W6: comparisons between leaf four of GA2ox::KLUH with the non-transgenic siblings at the six days after leaf four emergence.

Annotation is based on the PLAZA description (Proost et al., 2014).
Figure 2. ZmKLHU significantly altered the expression level of enzymes in phenolic metabolism at the division zone. Transcriptional comparison on four basal leaf samples (0.5cm) was performed between GA2ox::KLHU transgenic plants and their non-transgenic siblings at the second day (A) and the sixth day (B) after leaf four emergence. The pathway was drawn based on (Niggeweg et al., 2004; Vanholme et al., 2012; Payyavula et al., 2015) and online tools MaizeCyc (http://maizecyc.maizegdb.org/) and KEGG (http://www.genome.jp/kegg/). The solid arrows represent direct pathways, dashed arrows represent indirect pathways. The transcripts that encode enzymes were annotated based on literature (Morohashi et al., 2012; Tohge et al., 2013), MaizeCyc and PLAZA (Proost et al., 2014). All genes that express in maize leaves are indicated by a square. Expression of these genes was determined via RNA sequencing, and was indicated based on their Log fold changes (logFC): genes indicated in red are upregulated in GA2ox::KLHU-P3, genes in blue are downregulated in GA2ox::KLHU-P3, and genes in white did not show differential expression. The enzyme transcripts not detected in our samples using RNA sequencing are not indicated.

AD, arogenate dehydrogenase; ADT, arogenate dehydratase; ALDH, Aldehyde dehydrogenase; AS, anthranilate synthase; AT, amino transferase; ATs, acyltransferases; 4CL, 4-coumarate:CoA ligase; 3CH, p-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; CHI, chalcone isomerase; CHS, chalcone synthase; CM, chorismate mutase; COMT, caffeic acid O-methyltransferase; CS, choromate synthase; DAHPS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; DFR, dihydroflavonol 4-reductase; DHS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; DHQD/SD, 3-dehydroquinate dehydratase/shikimate dehydrogenase; DQS, 3-dehydroquininate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; F2H, flavanone 2-hydroxylase; F3H, naringenin 3-dioxygenase; FSH, ferulate 5-hydroxylase; FLS, flavonol synthase; GTS, glycosyltransferases; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; ICS, isochorismate synthase; IGPS, indole-3-glycerol phosphate synthase; LAR/ANR, leucoanthocyanidin reductase/anthocyanidin reductase; PAI, phosphoribosylanthranilate isomerase; PAL, phenylalanine ammonia-lyase; PAT, phosphoribosylanthranilate transferase; PDT, prephenate dehydrogenase; SGT, sinapate 1-glucosyltransferase; SK, shikimate kinase; SMT, sinapoylglycine:malate sinapoyltransferase; SST, sinapoylglycosesinapoylglycose sinapoylglycosetransferase; TSA, Trp synthase a-subunit; TSB, Trp synthase b-subunit; UFGT, flavonoid-3-O-glucosyltransferase; UGTS, UDP-glucosyltransferase.

Chlorogenic acid accumulates in GA2ox::KLHU plants

To search for putative ZmKLHU substrates and end products, we performed a metabolome analysis using the maize leaf because of the high sampling resolution within the growth zone that can be obtained. Since ZmKLHU expression is particularly high at the basal division zone of the growing maize leaf, the product of ZmKLHU is expected to accumulate at the division zone as well. The first centimeter of the fourth leaf, which consists of dividing cells, was harvested during steady growth, and the general metabolome was profiled doing two comparisons: GA2ox::KLHU transgenic plants
Metabolic characterization of KLUH compared to its non-transgenic siblings (GA2ox::KLUH_T versus GA2ox::KLUH_NT) and Zmkluh mutant plants compared to WT (Zmkluh versus WT).

Metabolite profiling using liquid chromatography-mass spectrometry (LC-MS) yielded 10892 and 11315 m/z features in GA2ox::KLUH_T versus GA2ox::KLUH_NT and Zmkluh versus WT, respectively (Figure 3). Based on the characterization of ZmKLUH as a cytochrome P450 monooxygenase, the putative substrate and product should have a mass difference corresponding to that of an oxygenation or oxidation reaction. Recently, an algorithm has been developed that searches m/z feature pairs of which the mass difference and elution order correspond to those of enzymatic reactions. Such m/z feature pairs are called Candidate Substrate Product Pairs (CSPP) (Morreel et al., 2014). The CSPP algorithm was used to assemble as a list of CSPP corresponding with candidate substrate and product m/z features of oxygenation/oxidation reaction. About 500-600 CSPPs were generated for each comparison group (GA2ox::KLUH and Zmkluh) and each conversion type (oxygenation and oxidation), of which 436 CSPPs existed in both comparisons (Figure 3). These CSPPs were further filtered by the substrate and product ratio, as the concentration of the substrate is expected to be higher than the concentration of the product in kluh mutants (substrate/product in Zmkluh > substrate/product in WT). Oppositely, the concentration of the ZmKLUH reaction product is expected to be more abundant than the concentration of the substrate in GA2ox::KLUH plants as compared to control plants (substrate/product in GA2ox::KLUH_T < substrate/product in GA2ox::KLUH_NT). Based on the expected substrate/product ratio change in both comparison group, the MS/MS spectral similarity of the substrate and product m/z features and statistical analysis (P<0.05, student t-test), 14 of these 436 CSPPs were retained as possible substrate-product candidates (Figure 3). Upon matching with an in-house database and/or de novo elucidation (Morreel et al., 2014), the MS^n fragmentation spectra associated with two CSPPs could be elucidated: chlorogenic acid and DIMBOA-Glc (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside) (Jonczyk et al., 2008) derived from 5-O-p-coumaroylquinic acid and DIBOA-Glc (2,4-Dihydroxy-1,4-benzoxazin-3-one glucoside), respectively (Figure 3).
Figure 3. Candidate substrate and product of ZmKLUH Selection scheme from LC_MS results. (A): the selection flow of candidate substrate and product of ZmKLUH from the features generated from LC-MS. Selection is based on: Substrate and product ratio ($S/P; S/P_{ZmKLUH} > S/P_{WT}$ and $S/P_{GA2ox::ZmKLUH_T} < S/P_{GA2ox::ZmKLUH_NT}$); t-test ($P$ value < 0.05) in both comparisons, or discrete peaks (product absent in mutant). (B): the predicted reactions catalyzed by ZmKLUH.

CSPP: candidate substrate product pairs; DIMBOA glucoside: 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside.

Genes controlling the biosynthesis of DIMBOA has been characterized in maize, the hydroxylation step from DIBOA to DIMBOA is catalyzed by 2-oxoglutarate-dependent dioxygenase (ZmBx6) (Jonczyk et al., 2008; Makowska et al., 2015). DIMBOA and DIBOA are benzoxazinoids found in a multitude of species of the family Poaceae (Gramineae). Outside the Poales, benzoxazinoids are sporadically detected in two distant orders of the eudicots, the Ranunculales and the Lamiales (Schullehner et al., 2008). Moreover, both DIBOA and DIMBOA treatments on Arabidopsis seedlings caused impaired growth, even at very low concentration (Dick et al., 2012). On the other hand, CGA, suggested as a metabolically transient intermediate in the formation of lignin precursors, is barely detected in Arabidopsis (Schoch et al., 2001). But considering the fact that KLUH works in both eudicots and monocots and that the toxic effect of benzoxazinoids during the growth of Arabidopsis seedlings...
(Anastasiou et al., 2007; Dick et al., 2012; Chakrabarti et al., 2013), we first examined the CGA function as a potential product of ZmKLUH.

**Chlorogenic acid enhances growth by stimulating cell division**

Given the prediction that CGA might be the functional product of ZmKLUH that promotes growth, we further examined the effect of CGA in Arabidopsis rosette leaves. To this end, the effect of CGA ranging in concentrations from 5µM to 50µM was tested on two lines overexpressing Arabidopsis CYP78A5/AtKLUH (35S::AtKLUH and AtKLUH::AtKLUH), and the mutant Atkluh, with Columbia-0 as control (WT). Unfortunately, the Atkluh mutant had to be excluded from the interpretation of the experiment due to a poor germination rate and severe variations in growth. The experiment will be repeated with a fresh seedbatch of the kluh mutants. The rosette area from the other lines was measured. For WT and the two KLUH overexpression lines, no significant difference in the rosette area was observed following CGA treatments (5µM and 10µM) (Figure 4A). However, 50µM CGA had a clear growth promoting effect as shown by the significant larger rosette area observed in 35S::AtKLUH (38%, P=0.001) and AtKLUH::AtKLUH (38%, P=0.001) (Figure 4A).

To further confirm the growth enhancement of CGA, the growth experiment with the two AtKLUH overexpression lines and WT was repeated with 50µM CGA treatment. It was reported that mutations in AtKLUH reduced the length of the plastochron resulting in more leaves during the same growth period (Anastasiou et al., 2007; Wang et al., 2008). If CGA is the product of AtKLUH, the external application of CGA might also affect the plastochron and consequently result in an altered speed of development. Thus, in order to avoid the bias due to developmental differences, the size and number of all rosette leaves were measured at 22 DAS by making leaf series. In accordance with previous observations (Claeys et al., unpublished data), untreated AtKLUH::AtKLUH plants contained bigger leaves comparing to WT, whereas 35S::AtKLUH had smaller leaves than WT. However, the plastochron length was not affected by the upregulation of AtKLUH, as is seen in the leaf series (Figure 4B-C). External application of CGA also did not affect the plastochron but led to plants with larger rosette leaves (Figure 4B-C). The growth enhancement of CGA seemed to be reinforced by higher AtKLUH expression levels, since the cotyledons and leaf 1 to 4 were consistently enlarged in 35S::AtKLUH (from 20% to 81%, P<0.05), following exposure to CGA, while cotyledons and leaf 2 and 3 were significantly larger in AtKLUH::AtKLUH (from 24% to 39%, P<0.05) and WT (from 19% to 30%, P<0.05) plants, following growth on CGA containing media (Figure 4C).

In order to gain a better insight into the cellular and developmental basis of the growth promoting effect caused by CGA, the growth experiment was repeated once more using WT and 35S::AtKLUH,
Metabolic characterization of KLUH

following the 50µM CGA treatment. Again, in this experiment, CGA significantly stimulated leaf growth, as shown by increased leaf area (40%, $P=0.02$ in WT, 78%, $P=0.008$ in 35S::AtKLUH), but with different effects at the cellular level: CGA in WT increased both cell division and cell expansion, resulting in more cells (25%, $P=0.2$) with larger size (11%, $P=0.2$); while CGA in 35S::AtKLUH significantly increased cell division but slightly inhibited cell expansion, as shown by the presence of more cells (104%, $P=0.002$) with smaller size (-10%, $P=0.4$) (Figure 4D). The stomatal index, which represents the number of stomata as a fraction of the total number of cells, was not significantly affected in 35S::AtKLUH (6%, $P=0.4$) following CGA treatment, but the size of stomata complex was reduced (-22%, $P=0.06$). Therefore, the development of stomata in 35S::AtKLUH was in accordance with the effect of CGA on the pavement cells. In contrast, both stomata index (-16%, $P=0.3$) and stomata size (0.1%, $P=1$) was not significantly affected by CGA in WT (Figure 4D). To conclude, CGA treated Arabidopsis seedlings resulted in larger rosette leaves as a consequence of a stimulation of cell division.

Figure 4. Rosette leaf phenotype of Arabidopsis AtKLUH overexpression lines with chlorogenic acid treatments. 
A: rosette area was determined at 22 DAS. B-C: leaf series of WT, AtKLUH::AtKLUH and 35S::AtKLUH under 50µM CGA treatment at 22 DAS. Significant differences (student t-test; *, **, *** correspond to $P$-values of 0.05 > $P > 0.01$, 0.01 > $P > 0.001$, $P < 0.001$, respectively; n>10) are indicated with an asterisk. Error bars represent standard error (SE). D: leaf area, pavement cell number and cell area, were determined in the abaxial epidermis
of leaf 3 for 35S::AtKLUH and WT with 50µM CGA treatment, represented as relative change compared to 35S::AtKLUH and WT without CGA treatment, respectively. For each line, three representative plants are depicted. Significant differences (student t-test; *, ** correspond to P-values of 0.05 > p > 0.01, 0.01 > p > 0.001, respectively; n=3) are indicated with an asterisk. Error bars represent standard error (SE). WT, wild-type. DMSO, Dimethyl sulfoxide. cot, cotyledon.

**Chlorogenic acid feeding assays failed in maize**

In order to determine whether CGA could stimulate maize cell division, we performed a feeding assay to *in vitro* grown maize. Maize seeds (transgenic GA2ox::KLUH and non-transgenic siblings) were sowed in transparent boxes, containing the nutrient medium (with agar to fix the maize seed and sustain the further maize seedling growth), CGA and the corresponding solvent (DMSO). Higher concentration of CGA (100µM) was used in maize compared to the Arabidopsis treatment. The effect of CGA was determined by measuring the final leaf size of leaf 2 and leaf 3. No significant difference in final leaf length caused by CGA was found (P>0.05; Table 2), indicating that there is either no uptake of CGA in maize, or alternatively no response to CGA in maize.

**Table 2. Leaf phenotype on segregating GA2ox::KLUH-P3 transgenic plants following 100µM chlorogenic acid treatment.**

<table>
<thead>
<tr>
<th></th>
<th>leaf2 area (mm²2)</th>
<th>leaf2 length (mm)</th>
<th>leaf3 area (mm²2)</th>
<th>leaf3 length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA2ox::KLUH_NT-CGA</td>
<td>1296.8</td>
<td>149.7</td>
<td>2668.1</td>
<td>283.3</td>
</tr>
<tr>
<td>GA2ox::KLUH_NT+CGA</td>
<td>1119.1</td>
<td>148.3</td>
<td>2790.0</td>
<td>288.7</td>
</tr>
<tr>
<td>P_value</td>
<td>0.1</td>
<td>0.2</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>GA2ox::KLUH_T-CGA</td>
<td>2092.7</td>
<td>219.5</td>
<td>3970.5</td>
<td>378.3</td>
</tr>
<tr>
<td>GA2ox::KLUH_T+CGA</td>
<td>1897.5</td>
<td>195.0</td>
<td>3843.9</td>
<td>370.2</td>
</tr>
<tr>
<td>P_value</td>
<td>0.2</td>
<td>0.1</td>
<td>0.7</td>
<td>0.7</td>
</tr>
</tbody>
</table>

P_value is calculated based on student t-test (n=3).

NT, non-transgenics; T, transgenics; CGA, chlorogenic acid.
Discussion

*The altered transcription of genes encoding enzymes involved in flavonol biosynthesis might influence auxin transport in GA2ox::KLUH*

In the previous chapter, we showed that auxin accumulated in the growth zone of GA2ox::KLUH transgenic leaves, while several auxin efflux transporters were significantly downregulated at the transcriptional level. Here, we found that the transcription of genes encoding enzymes that are involved in phenylpropanoid metabolism were severely altered by ZmKLUH expression in GA2ox::KLUH transgenic plants. Especially lower and higher expression levels were observed for *hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT)* and *chalcone isomerase (CHI)* that direct the biosynthesis of lignin monomers and flavonols respectively. These changes in the expression level of auxin transporters and flavonol content are reminiscent of several reports, showing that flavonols can alter auxin transport during plant development. The flavonol-less *tt4* mutant of Arabidopsis shows a reduced gravitropic response and is affected in the transport of auxin (Buer and Muday, 2004). This observation might be related to the altered cycling of the auxin efflux facilitator PIN1 in the *tt4* mutant background (Peer et al., 2004). Other mutants with elevated levels of flavonols inhibit auxin transport. Silencing of *HCT* results in smaller plants with accumulated flavonols and decreased auxin transport. Suppression of flavonoid accumulation by chalcone synthase (CS) repression in HCT-deficient plants restored normal auxin transport (Besseau et al., 2007). The hyponastic cotyledons, aberrant shape of pavement cells, and deformed trichomes in *rol1-2* (for *repressor of lrx1*) mutants of Arabidopsis are suppressed by a flavonol-induced modification of auxin transport (Ringli et al., 2008; Kuhn et al., 2011). A suppressor screen on the *rol1-2* mutant allowed for the identification of several genes in flavonols biosynthesis and could rescue the *rol1-2* phenotype (Kuhn et al., 2011). Currently, it is still not clear how flavonols interfere with auxin transport, but flavonols were shown to compete with the auxin transport inhibitor 1-N-naphthylphthalamic acid for a high-affinity binding site in a protein complex belonging to the group of ATP-binding cassette transporters (Jacobs and Rubery, 1988; Noh et al., 2001; Murphy et al., 2002). Taken together, the significant downregulation of *HCT* and upregulation of *CHI* might result in altered flavonol content in the growth zone of GA2ox::KLUH leaves, and might consequently lead to local auxin accumulation.

*CGA mimics the effect of AtKLUH in stimulating cell division*
In this chapter, we described that CGA can facilitate growth by stimulating cell division, but whether it is a direct effect of CGA is still unclear. The best known feature of CGA is as an anti-oxidant. In animals, CGA can limit low-density lipid (LDL) oxidation and remove reactive oxygen species (Laranjinha et al., 1994; Sawa et al., 1999). In plants, transgenic tomatoes with elevated levels of CGA show a slower and more limited cell death when infected with bacteria and an enhanced tolerance for oxidative damage (Niggeweg et al., 2004). However, a growth stimulating effect of CGA was not reported.

*In vitro* CGA treatment increased Arabidopsis rosette leaf size by enhanced cell division, showing a similar function as AtKLUH. However, the CGA treatment does not completely resemble plants with elevated *AtKLUH* expression levels. Under normal condition, mild overexpression of *AtKLUH* promotes growth through increased cell division, whereas strong expression of *AtKLUH* results in smaller leaves with more cells but smaller size (Claeys et al., unpublished data). Therefore if CGA is the product of KLUH, a consistent and reinforced leaf area reduction could be expected when adding external CGA to 35S::AtKLUH. However, although cell size was further reduced in 35S::AtKLUH upon CGA treatment, the leaves became larger due to an increased cell division. A possible explanation for this result is that the cell size in CGA treated 35S::AtKLUH meets the size limitation and therefore the strongly increased cell division overcomes the defect on cell expansion and eventually increased leaf size. There is also a possibility that CGA is not the product of KLUH, therefore the CGA effect is different from KLUH. In addition, loss of AtKLUH function was reported to shorten the plastochron, leading to an increased number of leaves (Wang et al., 2008). This effect seems to be more pronounced in *Atkluh* mutant plants as compared to *AtKLUH* overexpression plants, as no significant plastochron effect was observed in 35S::KLUH. As neither WT nor 35S::KLUH following CGA treatment showed a plastochron effect compared to the non-treated plants, for future experiments, *Atkluh* might be better than the *AtKLUH* overexpression lines to determine whether CGA could cause plastochron effect.

*The possible enzymatic function of ZmKLUH if chlorogenic acid is the substrate*

In plants CGA is synthesized by at least two routes as shown in Figure 2. The close correlation between the induction of HQT activity and the synthesis of CGA in tomato suggests that HQT directs the most likely pathway for CGA (Strack and Gross, 1990; Niggeweg et al., 2004). But some studies have proposed the existence of additional pathways for CGA biosynthesis (Chang et al., 2009; Sonnante et al., 2010; Payyavula et al., 2013; Payyavula et al., 2015). For instance, silencing of HQT in
PAL overexpressing tobacco plants only resulted in a 50% reduction of CGA (Chang et al., 2009). No correlation between HQT expression and CGA concentration was observed among potatoes with naturally varying amounts of CGA, or in potatoes with increased CGA levels due to development, drought or other environmental cues (André et al., 2009; Payyavula et al., 2013). The biochemical characterization of hydroxycinnamoyl-CoA transferase (HCT) and p-coumarate 3-hydroxylase (C3H) in Arabidopsis suggests the CGA can be synthesized through this route (Schoch et al., 2001). However, both HCT and C3H are active in Arabidopsis, which does not accumulate CGA, making this route unlikely to be used by Arabidopsis to accumulate significant amounts of CGA. Moreover, silencing of HCT or C3H severely alters the lignin structure of the plants, with an increase of p-hydroxyphenyl (H) units (Franke et al., 2002; Besseau et al., 2007), suggesting this route is mainly involved in lignin biosynthesis instead of CGA accumulation. In maize, genes encoding HCTs and C3Hs have been characterized (Fornalé et al., 2015; Wang et al., 2015). Similar to the observation in Arabidopsis, downregulation ZmC3H1 in maize exhibits increased amounts of H units, indicating that there might be another enzyme leading CGA biosynthesis. In this study, we showed that CGA accumulates in GA2ox::ZmKLUH and decreases in the Zmkluh mutant, oppositely, the precursor (p-coumaroyl quinic acid) was reduced in ZmKLUH overexpression plants and accumulated in the mutant. Moreover, CGA promotes, at least in Arabidopsis, cell division in a similar way to ZmKLUH. Therefore, one possibility is that KLUH is an alternative enzyme to catalyze the C3H step in the HCT-C3H route of CGA biosynthesis (Figure 2).
Materials and Methods

Plants growth conditions

Maize plants were grown in soil under growth chamber conditions with controlled relative humidity (55%), temperature (24°C/18°C), and light intensity (170 mmol m-2 s-1 photosynthetically active radiation at plant level) provided by a combination of high-pressure sodium vapour (RNP-T/LR/400W/S/230/E40; Radium) and metal halide lamps with quartz burners (HRI-BT/400W/D230/E40; Radium) in a 16h/8h (day/night) cycle. For in vitro growth experiment, maize seeds were sowed in transparent plastic boxes containing Murashige and Skoog medium (Murashige and Skoog, 1962), supplemented with 1% sucrose. Seedlings were grown in a 16-h day (80 µmol/m2/s)/8-h night regime at 24°C.

For Arabidopsis, all experiments were performed in vitro by growing plants in half-strength Murashige and Skoog medium (Murashige and Skoog, 1962), supplemented with 1% sucrose. For shoot growth, 32 seeds were sowed at equal distances on 14-cm-diameter round plates that were placed horizontally. All genotypes were grown on the same plate to allow comparison. Seeds were stratified in the dark at 4°C for at least 48h. Seedlings were grown in a 16-h day (80 µmol/m2/s)/8-h night regime at 21°C.

Chemical treatment

For Arabidopsis, chlorogenic acid (sigma aldrich) was dissolved in DMSO, and therefore an equal amount of DMSO as need for the dilutions was added to control plates. At 22 DAS, pictures were taken of each plate, and projected rosette areas were measured using ImageJ v1.46 (NIH; http://rsb.info.nih.gov/ij/). The plants were harvested afterwards, and leaf series were made by cutting each individual leaf of the rosette and ranking them from old to young on a square agar plate. After 21 DAS (days after stratification), the third leaf was removed from the rosette and placed sequentially on agar plates, which were photographed. For cellular analysis, the harvested leaf 3 was cleared with 70% ethanol, mounted in lactic acid on microscopy slides. Abaxial epidermal cells (50-300) were drawn for five representative leaves with a DMLB microscope (Leica) fitted with a drawing tubus and a differential interference contrast (DIC) objective. Photographs of leaves and cell drawings were used to measure leaf area and cell size, respectively, using ImageJ v1.46, from which the cell numbers were calculated.
For maize, chlorogenic acid was added to growth medium, and equal amount of DMSO was added to control medium. Final leaf length and leaf blade area of leaf 2 and 3 were measured to determine the effect of chlorogenic acid.

Metabolome analysis

The basal division zone (1cm) of leaf four from GA2ox::KLUH-P3 transgenic and non-transgenic plants, Zmkluh and WT were harvested during steady state growth (at the second day after their appearance from the whorl). Metabolites were extracted from single plants, and calibrated by ten biological replicates. Plant material was extracted with methanol at room temperature for 10 minutes. The methanol extract was evaporated under vacuum, and the residue was redissolved in water/cyclohexane (1:1). 200μL of the resulting aqueous phase was analyzed by liquid chromatography electrospray ionisation Fourier transform ion cyclotron resonance mass spectrometry (LC-ESI-FT-ICR-MS), and coupled to an LTQ FT Ultra (Thermo Electron Corporation) via an electrospray ionization source. For LC-ESI-FT-ICRMS analysis, reversed-phase liquid chromatography was carried out using an Acquity UPLC HSS C18 column (150 x 2.1 mm, 1.7 μm; Waters) mounted on an Accela LC system consisting of an Accela pump (Thermo Electron Corporation) and an Accela autosampler (Thermo Electron Corporation). A gradient (time 0 min, 1% solvent B; 30 min, 50% solvent B; 33 min, 100% solvent B) was run using water:acetonitrile (99:1, v/v) (solvent A) and acetonitrile/water (99:1, v/v) (solvent B), both acidified with 0.1% (v/v) acetic acid. The loop size, injection volume, flow rate and column temperature were 25μL, 10μL, 300μL/minute and 55_C, respectively. Negative ionization was obtained using a capillary temperature of 300_C, sheath gas of 30 (arbitrary units), auxiliary gas of 10 (arbitrary units), and a spray voltage of 3.5 kV. Full FT-ICR-MS spectra between m/z 120-1,200 were recorded at a resolution of 100,000. To facilitate compound identification, full FT-ICR-MS scans were interchanged with dependent MS2 scan events, in which the most abundant ion of the previous FT-MS scan was fragmented, and two dependent MS3 scan events in which the two most abundant daughter ions of the MS2 scans were fragmented. The collision energy was set to 35%. Full FT-ICR-MS spectra were extracted from the LC-MS chromatograms using the RecalOffline software package (Thermo Electron Corporation) and integrated and aligned with the XCMS software package (Smith et al., 2006) in R (version 2.6.1) using the following parameter values: xcmsSet (fwhm=7, max=300, snthresh=2, mzdiff=0.01), group (bw=10, max=300), retcor (method=loess, family=symmetric).
References


Metabolic characterization of KLUH


Chapter 5

Investigation of Gibberellin-DELLA downstream targets by a glucocorticoid-mediated transcriptional inducible system in maize
DELLA inducible system
Investigation of Gibberellin-DELLA downstream targets
by a glucocorticoid-mediated transcriptional inducible system
in maize

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\textbf{AUTHOR CONTRIBUTION}

The research was designed by X.S., H.N., D.I.. The experiments were carried out by X.S. and H.T.. X.S. wrote this chapter with contributions from H.T., H.N. and D.I.
Abstract

The GA-DELLA module reacts upon multiple endogenous signals and environmental cues to regulate many growth processes, including cell division and cell expansion. As DELLAs lack a DNA-binding domain, they function through the interaction with transcription factors and transcriptional regulators to affect the expression of downstream target genes. To investigate which genes are the downstream targets of DELLA proteins in the maize growth zone, we explored the use of the glucocorticoid receptor-based inducible gene expression system and fused the dominant negative mutated DELLA (d8) with the glucocorticoid receptor (GR). From the six independent lines, two lines showed a growth reduction, reminiscent to that of the d8 mutants without induction. These data indicated that in some transformation events the construct could result in a leaky activity of the dominant negative d8 DELLA protein. A more pronounced growth reduction was observed upon induction of the leaky lines, while the non-leaky lines failed to show phenotypes upon induction. From the two glucocorticoid analogs that were tested, estradiol could more reproducibly induce d8 mediated growth inhibition compared to dexamethasone. Molecularly, the cell cycle gene CDKB1 was consistently downregulated 4 and 24 hours after estradiol induction, indicating that cell division was consistently inhibited by DELLA. Our data show that the glucocorticoid receptor-based induction system has the potential to work in maize seedlings when estradiol is used.
Introduction

Gibberellin (GA) is an essential endogenous regulator of plant growth. GA was first discovered in fungi in 1935 and was later also isolated from higher plants. GA influences a variety of developmental processes in plants, including germination, stem elongation, flowering time, leaf growth and fruit development (Richards et al., 2001; Hussain and Peng, 2003; Pimenta Lange and Lange, 2006). In cereals, mutants with perturbed GA action or production showed semi-dwarf phenotypes with a spectacularly increased harvest index, and formed a major component of the first green revolution (Peng et al., 1999; Hedden, 2003; Daviere and Achard, 2013).

Since the green revolution, the molecular pathways involved in GA signaling, from GA perception until its degradation, have been revealed (Sun, 2011; Hedden and Thomas, 2012; Daviere and Achard, 2013). In the absence of GA, GA responses are repressed by nuclear transcriptional regulators, called DELLA proteins. When bioactive GA is present, GA binds to its receptor (GA INSENSITIVE DWARF1, GID1), and induces a conformational change in GID1 allowing it to bind DELLA proteins. This complex enables an SCF E3 ubiquitin ligase to recognize the DELLA proteins and to trigger their degradation by the 26S proteasome (Daviere and Achard, 2013). The degradation of DELLAs allows for changing the activity of transcription factors that induce or inhibit expression of GA response genes and therefore regulates growth (Ikeda et al., 2001; Silverstone et al., 2001). Besides GA, other hormones, such as ethylene and auxin can induce the degradation of DELLA proteins, making the DELLA proteins important integration components for different hormonal signaling pathways (Alvey and Harberd, 2005).

As key intracellular repressors of GA responses, DELLAs have the potential to induce and repress very different transcripts, depending on the tissue, developmental stage, and environmental conditions (Cao et al., 2006; Zentella et al., 2007; Hou et al., 2008; Claeyts et al., 2012; Claeyts et al., 2014). As DELLAs have no DNA-binding domain, they function by interaction with a wide array of transcription factors and transcriptional regulators to inhibit their functions or to activate transcription through an association with DNA (Zentella et al., 2007; de Lucas et al., 2008; Feng et al., 2008; Hou et al., 2008; Josse et al., 2011; Zhang et al., 2011; Kazan and Manners, 2012). The genome of Arabidopsis encodes five DELLA proteins: REPRESSOR OF GA (RGA), GA INSENSITIVE (GAI) and three REPRESSORS OF GA LIKE (RGLs) (Gallego-Bartolome et al., 2010). Loss-of-function Arabidopsis quadruple DELLA mutants *gai rga rgl1 rgl2* are larger due to increased leaf cell number and cell size, suggesting that DELLAs affect both cell division and cell expansion (Achard et al., 2009). The Arabidopsis quintuple DELLA mutants *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* show early flowering than WT (Cheng et al., 2004). During vegetative growth, DELLAs have been shown to inhibit cell proliferation by inducing the expression of...
the cell-cycle inhibitors *SIAMESE* (*SIM*), *SIM-RELATED1* (*SMR1*), *SMR2* and *KIP-RELATED PROTEIN 2* (*KRP2*) (Achard et al., 2009). DELLAs also negatively regulate SPATULA (SPT), a basic helix-loop-helix (bHLH) transcription factor, to control cotyledon cell expansion (Josse et al., 2011). To identify DELLA downstream targets, an inducible system was created in Arabidopsis consisting of the fusion between the dominant negative DELLA protein (*gai*), which is resistant to GA-induced degradation, and the rat glucocorticoid receptor (GR). In the absence of steroid hormones, the fused proteins are inactive in the cytosol, and are translocated to the nucleus when GR interacts with external steroids, thereby exerting their functions (Picard, 1993; Aoyama and Chua, 1997). In this way, GAI proteins are expected to be functional when being activated in the presence of steroid hormones such as dexamethasone (DEX) or estradiol. This system works very well in Arabidopsis, where additional downstream targets of DELLA including growth regulators controlling cell proliferation (*CYP78A5/KLUH* (*AtKLUH*), *ANGUSTIFOLIA3* (*AN3*), *GROWTH-REGULATING FACTOR 5* (*GRF5*), *AINTEGUMENTA*), and several TEOSINTE BRANCHED1-CYCLOIDEA-PCF (TCP)-type TFs have been identified through time course transcriptome analysis after DEX induction (Claeys et al., 2012; Claeys et al., 2014). These transcriptional responses can be very fast as shown by a significant reduction in *AtKLUH* transcript levels which occurs already one hour after DELLA activation (Claeys et al., 2014 and unpublished data). The GR inducible system has been shown to work successfully in inducing gene expression in several species, including Arabidopsis, tobacco and rice (Aoyama and Chua, 1997; Ouwerkerk et al., 2001; Corrado and Karali, 2009).

DELLAs are highly conserved among different species, as the dwarf phenotype caused by GA-insensitive mutants in both Arabidopsis and maize exhibit similar mutations in the conserved DELLA domain (Peng et al., 1999; Daviere and Achard, 2013). In maize, two DELLA encoding genes are present, known as *DWARF8* (*D8*) and *DWARF9* (*D9*) (Peng et al., 1999; Lawit et al., 2010). D8 and D9 share high protein sequence similarity, but when mutated differences in plant stature (from very mild to very severe dwarfism) were observed (Cassani et al., 2009b; Lawit et al., 2010). The growth inhibition in the *d8* mutant was further studied in the maize leaves and revealed an important role for GA-DELLA signaling in determining the transition from cell division to cell expansion during leaf growth (Nelissen et al., 2012).

In this study, we applied the GR-based gene expression inducible system by fusing the dominant negative mutated DELLA (*d8*) with GR. Through using two glucocorticoid analogs, dexamethasone and estradiol, the induced d8 protein activity was explored by monitoring leaf growth. The expression of downstream target of d8 (*CDKB1;1*) was examined to test whether the estradiol mediated activation of d8 could also be observed molecularly.
Results

Growth experiments in a hydroponic system compared to soil

In order to facilitate maize plants to absorb the steroids and control the application of chemicals, we established a hydroponic system for maize growth. The maize seeds were germinated in a paper roll submerged in sterilized water, and transferred to 50mL falcon tubes containing Hoagland liquid medium for further growth (See material and method).

In order to investigate whether the hydroponic system could sustain maize seedling growth, wild-type (B104) maize were grown in parallel in soil and in liquid culture under the same environmental conditions and the impact of the cultivation methods on growth were assessed. Leaf elongation rate (LER) and leaf elongation duration (LED) (Voorend et al., 2014) based on the daily leaf length measurements are used to evaluate growth. The hydroponic system allows the fourth leaf to complete its growth without leaf wilting, but a 31% reduction (P=0.00) in final leaf four length in liquid medium compared to soil grown plants was observed. A significant reduction in maximal LER (LERmax) (36%, P=0.00) rather than leaf elongation duration (LED; 4.9%, P=0.3) caused this leaf reduction in liquid solution. Although the total growth is decreased, plants kept similar morphologies and growth profiles as compared to soil grown plants enabling us to use the hydroponic system for further tests (Figure 1).

![Figure 1. Leaf growth of the fourth leaf in maize seedlings grown in soil and liquid solution. LED: leaf elongation duration; LERmax: the maximal leaf elongation rate.](image-url)
A growth reduction, reminiscent to that of the maize d8 mutant was observed in some transgenic lines without induction

A construct was designed to express the maize dominant negative DELLA protein (d8) fused to the rat glucocorticoid receptor (GR) under the control of the constitutive promoter bdEF1α, which shows strong expression levels in different maize organs (Coussens et al., 2012). After transformation of maize B104, we expected to find plants with a normal appearance in contrast to the dwarf phenotype that is found in plants that contain a dominant mutation in the d8 protein. Six independent transgenic events containing single locus insertion of the T-DNA were obtained after transformation.

Expression analysis on the six transgenic lines using qRT-PCR showed that all six transgenic lines contain overexpression of d8-GR, compared to the non-transgenic siblings (Supplementary Figure 1). Although there was no significant difference (P>0.05) between each transgenic line, phenotypic differences were observed. In the absence of steroid hormones, transgenic lines with the highest d8-GR expression levels (line 1 and line 2) already showed phenotypes that have a striking resemblance to those of the naturally occurring d8 maize mutant (exemplified by plants from line 1 in Figure 2A). This phenotype is likely explained by either the leakiness of the construct or a cytosolic function of d8. The other four transgenic lines (line 3-6) with lower transgene expression levels resembled the non-transgenic plants and are represented by line 3 in Figure 2A. The transgenic lines showing a dwarf phenotype are therefore called leaky lines, and the rest with a wild-type phenotype are non-leaky lines.

The primary hemizygous transformants were backcrossed to B104, a growth experiment was performed on the segregating leaky line 1 and non-leaky line 3 containing the EF1α::d8-GR construct to quantify their growth in the hydroponic system compared to growth in soil conditions without induction. Compared to plants grown in soil, the liquid medium caused a visible reduction in the LERmax of transgenic (-19% in line 1, P=0.005; -28% in line 3, P=0.003) and non-transgenic plants (-26% in line 1, P=0.001; -25% in line 3, P=0.0004) (Figure 2B). In addition, a severe growth reduction was observed between the transgenic and non-transgenic progenies of the leaky line 1. In soil, the reduced LERmax (36%, P=0.00) led to a significant reduction on final leaf length (31%, P=0.00) in the transgenic leaky plants compared to the non-transgenics, and a similar decline of LERmax (30%, P=0.0003) and final leaf length (28%, P=0.006) were observed in liquid medium (Figure 2B). This reduction in LER was reminiscent of the previously reported d8 phenotype (Nelissen et al., 2012), indicating that d8 was functional in the leaky lines. On the contrary, the LER of plants from the non-
leaky line 3 was similar between plants with and without transgene when grown in the same condition, indicative of no d8 activity without induction (Figure 2B).

Figure 2. Phenotypes of the d8 maize mutant and the transgenic lines of EF1α::d8-GR without induction. A. morphology of the d8 mutant and two transgenic lines of EF1α::d8-GR without induction at 14 days after sowing. B. LER of two independent EF1α::d8-GR lines grown in soil and liquid medium. Error bars indicate standard error (n = 3 in all panels). NT=non-transgenic plants, T= transgenic plants.

**Ethanol, the solvent of the glucocorticoids, severely affects leaf growth**

Next, the glucocorticoid DEX, which has been successfully used for the induction of activity of GR fusion proteins in Arabidopsis, tobacco and rice (Aoyama and Chua, 1997; Ouwerkerk et al., 2001), was tested in our liquid medium setup on leaky line 1. DEX was dissolved in ethanol to make a 50mM stock solution, and the stock solution was further diluted into the liquid medium according to the applied DEX concentration. A gradient of three concentrations of DEX (2μM, 10μM and 50μM) was initially used to determine the best working concentration, and these were dissolved in ethanol of which 2μL, 10μL, 50μL, respectively, was added in 50 ml liquid medium. Liquid medium with the
same amounts of ethanol (mock medium) and without ethanol (control medium) were taken along with the DEX treatments. The segregating maize seeds of the leaky line 1 were germinated in paper rolls and similar amounts of the segregating seedlings were distributed over the three conditions (control, mock and induction). The growth effect was monitored by daily measuring the length of the third and fourth leaf.

Leaf 4 wilting was observed in plants grown in both mock and DEX containing solutions but not in control medium, indicating that the amounts of ethanol added to the medium are detrimental for leaf 4. Therefore, leaf 3 was used to quantify the growth effect for each treatment. For non-transgenic plants, a gradual decrease of final leaf length was associated with the increasing DEX concentration compared to plants grown in control medium (-10%, -15%, -26% for plants grown in 2μM, 10μM and 50μM DEX containing medium). The effect on final leaf length was mainly due to a reduction of maximal LER (-15%, -20%, -30%) rather than LED (3%, 2%, -2%) (Figure 3A, 3C; Supplementary Figure 2A). As the DEX medium contained increased ethanol to dissolve increased DEX, we assumed that the ethanol might have a toxic effect on maize growth. Indeed, the growth of leaf 3 was significantly affected by ethanol when mock-treated plants were compared to control plants. Comparing to control medium, extended decreases of LERmax (-9%, -18%, -25%) were observed with the increase of ethanol content (2μL, 10μL, 50μL ethanol per tube respectively) in non-transgenic plants (Figure 3A). The growth duration was not significantly affected by ethanol (P>0.05) (Supplementary Figure 2A). Excluding the ethanol effect by comparing the non-transgenic plants grown in DEX containing medium with mock medium, as expected, no significant growth inhibition was caused by DEX in non-transgenic plants (P>0.05) (Figure 3C).

For transgenic plants, similarly, an increasingly reduction on LERmax (-5%, -11.5%, -27%) was observed associated with the increasing DEX levels compared to plants in control medium. The ethanol also consistently repressed the growth rate (-3%, -11%, -17%) with increasing concentration (Figure 3B, 3D). Additionally, comparing to growth in control medium, significant reductions on the LED were observed in transgenic plants grown on 50μM DEX treatment (-10.5%) and corresponding mock medium (-11%) (Supplementary Figure 2B). To eliminate the ethanol effect on growth inhibition, we evaluated if the induced growth inhibition due to the induction by adding DEX exceeds the growth reduction by ethanol. No significant growth inhibition (P>0.05) on either LERmax or LED was observed for the transgenic plants that contain the EF1α::d8-GR construct (Figure 3D; Supplementary Figure 2B). Nevertheless, a slightly higher reduction (-10%) of LERmax was observed with by 50μM DEX compared to 2μM DEX (-2%) and 10μM DEX (-0.3%) treated plants, suggesting that steroids might induce d8 accumulation with higher concentrations. However, the amounts of
ethanol needed to dissolve such high levels of steroids have a severe impact on plant growth as the growth of leaf 3 is severely reduced and the growth of leaf 4 is compromised by wilting.

Figure 3. The growth rate of EF1α::d8-GR line 1 following treatment with different DEX concentrations. LER of leaf 3 from non-transgenic (A) and transgenic (B) plants of segregating line 1 grown in control, DEX and corresponding ethanol (mock) solutions. The maximal LER of leaf 3 from non-transgenic (C) and transgenic (D) plants of segregating line 1 grown in control, DEX and corresponding ethanol solutions. Significance was calculated based on student T-test (n>3; *, **, and *** correspond to P-values of 0.05>p>0.01, 0.01>p>0.001, and p<0.001, respectively). T refers to transgenic plants and NT refers to non-transgenic siblings.

Limited induced phenotypes were observed by DEX and estradiol application to two leaky lines in the hydroponic system

The experiment with DEX application showed that a steroid concentration higher than 50µM might be needed to activate the induction of d8-GR in maize. Furthermore, in the previous experiments DEX was applied after seed germination. We therefore repeated the DEX induction experiment with
both leaky lines (line 1 and line 2), and treatments with 50μM and 100μM DEX now applied already during seed germination, and the solvent ethanol contents were 50μL and 100μL. Therefore, the maize seeds of the two lines were germinated and continued to grow in medium with DEX and two control solutions with and without ethanol (mock and control, respectively). The LER of leaf 3 was determined to evaluate the effect of DEX. No significant germination difference was observed between each treatment. Because both maximal LER and LED can be affected by on one hand the growth repressing effect of the ethanol and on the other hand the activity of the d8 DELLA protein, a two-way ANOVA analysis incorporating these two parameters was used to decipher whether there are significant differences in overall LER (Table 1).

Similar to the previous observations, ethanol significantly affected growth in both transgenic and non-transgenic plants, characterized by a significant reduction in LER of leaf 3 in mock solutions compared with control solutions (ranging from -13.7% to -43.7%; Table 1). The effect of DEX was then analyzed by comparing the effect of DEX dissolved in ethanol with the effect of a mock treatment with the corresponding ethanol concentration. For non-transgenic plants, no significant growth difference was observed between mock and DEX containing media (p-value ranging from 0.46 to 0.6) (Table 1). In contrast, transgenic plants of line 1 showed a significant reduction in LER (-17%, P=0.001; Table 1) upon treatment with 50μM DEX. The DEX treated plants grew slower and during a shorter period than non-DEX treated plants (Figure 4A). However, this growth reduction did not persist when plants were treated with a higher DEX concentration, as no significant effect (P=0.54) was shown in transgenic plants with 100μM DEX treatment (Table 1). For transgenic plants of line 2, neither 50μM nor 100μM DEX did cause growth reduction compared to the mock treatment, suggesting that DEX does not reproducibly work to induce the activity of the d8-GR fusion protein.

Considering that DEX might not be well suited for the activation of GR fusion proteins in maize, another glucocorticoid analog, estradiol, was also tested. Since estradiol could also be dissolved in ethanol, ethanol was consistently used as the solvent. In contrast to DEX, significant reductions of LER were observed when plants were treated with estradiol compared to the mock treatment (50μM estradiol treatment in line 1 resulted in a reduction of -24.9%, P=0.03 and 100μM estradiol in line 2 resulted in a reduction of -37.2%, P=0.045) (Table 1). The estradiol mediated growth reductions were mainly the consequence of a reduced LER during the steady-state growth (Figure 4B-C). However, growth reductions induced by estradiol were not consistent for all concentrations, as neither line 1 with 100μM nor line 2 with 50μM estradiol treatment showed a significant growth effect compared to the mocks (Table 1). To confirm that estradiol is able to activate the d8-GR fusion protein, the 50μM estradiol treatment was repeated on both line 1 and line 2. Line 1 again exhibited a significantly reduced LER of leaf 3 upon exposure to estradiol (P=0.016), while line 2 was not affected.
by 50µM estradiol treatment (P=0.88) (Supplementary Figure 3). As the activated d8 effect was only observed in certain lines with certain concentrations of estradiol, it seems that the use of estradiol to induce the GR is not very efficient.

Table 1. The LER differences between treatments in *EF1α:d8-GR* transgenic line 1 and line 2.

<table>
<thead>
<tr>
<th></th>
<th>control versus mock_50µM</th>
<th>control versus mock_100µM</th>
<th>mock_50µM versus DEX_50µM</th>
<th>mock_100µM versus DEX_100µM</th>
<th>mock_50µM versus EST_50µM</th>
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<td></td>
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<tr>
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</tr>
<tr>
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<tr>
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</table>

Control means plants were grown in Hoagland solution, mock means plants were grown in Hoagland solution with corresponding ethanol content (indicated in µM). The pair comparisons are carried out on LER of leaf 3 from each treatment. Statistics in treatment comparisons are calculated by two-way ANOVA (n>3). The segregating transgenic seeds for each experiment were randomly grouped to each treatment, no non-transgenic seeds from line1 was grouped to 50µM DEX containing medium in this experiment. DEX: dexamethasone; EST: estradiol; NT: non-transgenic plants from the segregating line; T: transgenic plants from the segregating line.

We next examined two non-leaky lines (line 3 and line 4) to see whether estradiol has any effect on the phenotype of these lines. Again the ethanol effects persisted in the non-leaky lines as was previously observed (Supplementary Figure 4). Different estradiol concentrations did not affect the growth of line 3 and line 4 (Supplementary Figure 4).
Figure 4. The growth reduction caused by the d8 induction from glucocorticoid analogs in EF1α::d8-GR. A. LER of leaf 3 of transgenic plants in line 1 with 50µM DEX treatment. B. LER of leaf 3 of transgenic plants in line 1 with 50µM estradiol treatment. C. LER of leaf 3 of transgenic plants in line 2 with 100µM estradiol treatment. Error bars indicate standard error (n>3 in all panels). T, transgenic plants. NT, non-transgenic plants. EST, estradiol.
Estradiol mediated activation of d8-GR represses CDKB1;1 expression

To test whether the estradiol mediated activation of d8 could also be observed molecularly, we initiated a new experiment to investigate the expression level of a putative DELLA target gene upon estradiol induction in leaky line 1. In Arabidopsis, DELLAs trigger mitotic exit by inhibiting expression of cell cycle genes, such as CYCLINE DEPENDENT KINASE 1;1 (CDKB1;1) (Claeys et al., 2012). CDKB1;1 expression levels were monitored in the growing leaf 4 during steady state growth. Similar to the reported expression profile in a wild-type growing maize leaf (Nelissen et al., 2012), CDKB1;1 was highly expressed in the basal division zone of the growing maize leaf and decreased to a basic expression level in the expansion zone in non-transgenic plants (Supplementary Figure 5). In the transgenic plants of leaky line 1, CDKB1;1 expression was also high at the basal division zone, but the overall expression level was lower than in non-transgenic plants (Supplementary Figure 5), suggesting that the dwarf phenotype in leaky plants without induction was due to the decreased mitosis.

The first 2 centimeter samples of steady state growth leaf 4 in leaky line 1 following treatment with 50μM estradiol were taken for quantitative PCR analysis. CDKB1;1 expression was followed at 0, 4 and 24 hours after the onset of estradiol treatment. The transcriptional repression of CDKB1;1 started 4 hours after d8 activation, especially in the division zone of the growing maize leaf (Figure 5A-B). At the basal division zone (the first half centimeter leaf samples), the expression of CDKB1;1 was downregulated to 75% 4 hours after d8 activation, and to 70% after 24 hours induction, indicating that cell division was continuously inhibited by expressing the dominant d8 DELLA protein (Figure 5C). The repression of the cell cycle gene was in accordance with previously observed growth inhibition in leaky line 1 after estradiol induction.

It will be of interest to analyze the expression of maize homologs of other genes known to be negatively regulated by DELLAs in Arabidopsis, such as KLUH, ANGUSTIFOLIA3 (AN3), GROWTH-REGULATING FACTOR 5 (GRF5), and several TEOSINTEBRANCHED1-CYCLOIDEA-PCF (TCP)-type transcription factors (Gonzalez et al., 2012; Claeys et al., 2014). Eventually, RNA-sequencing could be used to identify at a genome wide level genes that are putative DELLA targets and to compare these with the putative DELLA targets identified by a similar approach in Arabidopsis (Claeys et al., 2014).
Figure 5. Expression levels of CDKB1;1 in transgenic plants of EF1α::d8-GR leaky line 1 after 50μM estradiol induction. CDKB1;1 expression in the maize leaf 4 during steady state growth 4 hours (A) and 24 hours (B) after 50μM estradiol induction. (C) The relative induction of CDKB1;1 at the basal 0.5 centimeter of EF1α::d8-GR leaves 4 and 24 hours after 50μM estradiol induction. Data are presented as expression levels from samples subjected to ethanol or estradiol relative to the basal 0.5 centimeter leaf sample with ethanol treatment. Error bars indicate standard error (n = 3 in all panels). EST: estradiol.

The estradiol induction failed in soil grown plants

Since a growth reduction was already caused by ethanol in liquid solution and thus making the intended growth reduction by d8 DELLA activation, we analyzed the effect of estradiol on soil grown plants. Higher concentrations of estradiol (250μM and 500μM) than in liquid medium were applied.
to soil grown plants of leaky line 1. Only mock solution with the corresponding amount of ethanol necessary to dissolve 500μM estradiol was taken as control. Maize seeds were initially sown in dry soil, and subsequently watered daily with the ethanol solutions containing no; 250μM or 500μM estradiol. The LER of leaf 3 and leaf 4 was measured to determine the effect of estradiol.

The LER profiles of both leaf 3 and leaf 4 were similar between the mock and two concentrations of estradiol (P>0.05, two-way ANOVA) (Figure 6). The difference between the transgenic and non-transgenic lines, due to leakiness of the d8-GR construct was however maintained (P=0.00 for leaf 3 and P=0.00 for leaf 4, two-way ANOVA) (Figure 6). These data might suggest that the amount of estradiol we applied is not effective for soil grown plants.

Figure 6. LER of segregating transgenic EF1α::d8-GR line 1 grown in soil with estradiol treatments. T, transgenic plants; NT, non-transgenic plants; EST, estradiol.

Discussion

Leakiness when using the Glucocorticoid-receptor based inducible system is observed in Arabidopsis and maize

As the key intracellular repressors of GA responses, DELLA proteins and their homologs are widely distributed in angiosperms (Yasumura et al., 2007). GA INSENSITIVE (GAI), one of the DELLA proteins in Arabidopsis, has been shown to regulate vegetative growth (e.g. leaf development, stem elongation and adaxial trichrome development) (Dill et al., 2001). Maize DWARF8 (D8) is the ortholog of the Arabidopsis GAI gene (Peng et al., 1999), controlling maize vegetative development (Cassani et
Mutations within the conserved N-terminal DELLA domain of DELLA proteins have been identified for the two genes, as *gai* and *d8* (Willige et al., 2007). Both *gai* and *d8* proteins cannot be degraded by a GA mediated proteolysis pathway and thereby act as dominant negative DELLA proteins. Overexpression of the two GA-insensitive genes (*gai* and *d8*) in Arabidopsis results in similar dwarf plant phenotype, indicating that they might exert similar functions during vegetative growth (Willige et al., 2007). Since a similar strategy was applied by introducing *gai* and *d8* to glucocorticoid-receptor (GR) based inducible gene expression system in a previous study in Arabidopsis (Claeys et al., 2012) and in our study in maize, we therefore can compare the inducible system in the two species.

Previously, dwarf plants resembling *gai* mutant phenotypes were found in transgenic Arabidopsis plants expressing the GR-based inducible gene expression construct (35S::*gai*-GR) in the absence of either DEX or estradiol (Claeys et al., 2012). Theoretically, GR fusion proteins would be expected to be retained in the cytoplasm in the absence of a steroid, while in the presence of glucocorticoid analogs, the fusion protein would relocate to the nucleus and become active (Padidam, 2003), and thus, in the case of dominant negative DELLA-GR fusion proteins, activate growth inhibition. Here, we show that similar *d8* mutant phenotypes are also observed in some maize *EF1α::d8*-GR transgenic lines without any steroids induction, suggesting that the GR system is possibly less tight than original thought. The dwarf phenotype only occurred in transgenic maize with very high *d8*-GR overexpression level, indicating that there might be a threshold for the maintenance of GR in cytoplasm.

DELLA proteins have been shown to specifically localize in the nucleus (Gallego-Bartolome et al., 2010; Locascio et al., 2013), and exerting their functions mainly through interacting with other transcription factors (Daviere and Achard, 2013; Park et al., 2013; Sarnowska et al., 2013; Claeys et al., 2014). For this reason, it is likely that some of the *gai*-GR (in case of Arabidopsis) or *d8*-GR (in case of maize) already can be found in the nucleus in the absence of any steroid hormone. However, we cannot exclude a cytoplasmic function of D8. The GA receptor, GID1, which binds GA and DELLA protein in GA-DELLA signaling pathway, localizes mainly to the nucleus but also appears to localize to the cytoplasm (Ueguchi-Tanaka et al., 2005; Willige et al., 2007). Interestingly, only the leaky lines can be induced in Arabidopsis (Claeys et al., 2012) and maize (our study), meaning that the leakiness is somehow showing that the construct is working.
The glucocorticoid receptor based inducible gene expression system shows limited induction in maize

Inducible gene expression in a chemical dependent manner can be achieved by an inducible gene expression system containing glucocorticoid receptor (GR) domain (Borghi, 2010). The efficiency of the GR domain has been examined by luciferase expression in tobacco (Aoyama and Chua, 1997) and GUS activity in rice (Ouwerkerk et al., 2001). In agar medium, the luciferase activities are correlated with the DEX concentration (1µM to 10µM) in tobacco, and the activity of GUS can be induced by a range of DEX concentration (1µM to 10µM) in rice. In Arabidopsis, the external steroids also successfully induced the GR fused genes that regulate leaf morphology (Aoyama et al., 1995), flowering time (Simon et al., 1996) and plant defense system (McNellis et al., 1998). The concentrations of DEX in agar medium to activate genes in Arabidopsis seedlings range from a few nM to 30µM, and daily applied 10µM DEX was also sufficient to induce gene activities in soil. In our study, we tested several concentrations in liquid medium but only in three experiments (50µM estradiol and 50µM DEX in leaky line 1 and 100µM estradiol in leaky line 2), a significant effect of DEX or estradiol was found. Compared with other species, our results suggested that maize needs high levels of glucocorticoids to obtain induction, that is still not completely efficient. Nevertheless, the induction in soil was still not working by using higher estradiol concentrations (250µM and 500µM). One possibility of the high concentration of steroids needed and the low penetrance could be that the efficiency of steroids absorption by maize roots is relatively low. A study examined the efficiency of maize take estradiol through a hydroponic system, which is similar to the hydroponic system in our study: the germinated maize seedling were grown in a 9 mL glass vial with 2µM estradiol dissolved in methanol (Card et al., 2012, 2013). The estradiol absorption was evaluated by the estradiol content in the hydroponic system and maize root and shoot tissue over time. Although the 2µM estradiol could be fully taken by maize root after several days, much lower concentrations of estradiol was detected in maize shoot tissues compared to root tissues, which might be attributed to limited translocation or irreversible binding of estradiol in root tissues (Card et al., 2012, 2013). Therefore, the low d8 activity by estradiol in leaves in our study might because of the low estradiol content in maize leaves. Although not conclusive we have the impression that estradiol works better than DEX. DEX has a higher molecular mass than estradiol, with an additional fluorine atom and extra carbon atoms. Since both chemicals need to be absorbed by the maize root and travel to maize leaves to be functional, the higher molecular weight may explain why DEX gave more strict induction in maize. Therefore, direct application of estradiol or DEX to maize leaf might be a solution. As shown in another study, through foliar application (leaf spraying), steroids (brassinosteroid and ecdysteroid) with structures close to estradiol could be absorbed by maize (Rothová et al., 2014). Moreover, our study showed that the high levels of ethanol, needed to dissolve the steroids, caused severe growth...
inhibition, making the GR-based inducible system suboptimal to study growth. As an alternative, other solvents to dissolve the steroids (for instance, DMSO or methanol) should be tested. Moreover, higher concentration of the steroid stock should be tried to reduce the solvent content of each treatment.

*Other chemical inducible systems could be explored in maize*

So far, several chemical inducible systems have been developed in plants (Gatz, 1997; Corrado and Karali, 2009). Beside the glucocorticoid receptor based transcriptional activation system, two other inducible systems have been reported based on transcriptional activation mediated by the human estrogen receptor (ER) (Bruce et al., 2000; Zuo et al., 2000) and an insect ecdysone receptor (Martinez et al., 1999). The ER system can activate gene expression by estradiol in maize Black Mexican Sweet (BMS) cell cultures, showing a good correlation between luciferase reporter protein activities with the estradiol concentrations (Bruce et al., 2000). However, since the low efficiency of estradiol induced protein activity observed in our study might be due to the poor absorptivity of maize roots, it is unlikely this system could work in planta. In addition to the ER system, the insect ecdysone receptor system has been reported to work in tobacco, with tebufenozide as the inducer (Martinez et al., 1999). Unlike steroids, tebufenozide is evaluated as an insecticide with very low mammalian toxicity, making it suitable for field application. Another study shows a successfully application of this system to maize by using the ligand-binding domain from the European corn borer (*Ostrinia nubilalis*) ecdysone receptor (Unger et al., 2002). The ecdysone receptor can bind to the non-steroidal inducer, methoxyfenozide, and enable transcriptional activation. They reported that methoxyfenozide can be absorbed by maize root, delivered either by watering or incorporating into potting soil. Moreover, methoxyfenozide exhibits high insecticidal efficacy against a wide range of important caterpillar pests and has an excellent margin of safety to non-target and beneficial insects (Carlson et al., 2001), making it a good inducer for field application.

Another concept of gene control is the promoter-repressing system. The repression principle is based on sterical interference of a repressor protein with proteins important for transcription. An example is the bacterial Tet inducible repression system applied in tobacco (Gatz et al., 1992). Transgenic tobacco contains one construct with a gene of interest under control of a modified cauliflower mosaic virus (CaMV) 35S promoter containing binding sites of the Tet repressor, and another construct placing the repressor protein under control of a constitutive promoter. When there is no tetracycline, the repressor will consistently repress the expression of gene of interest. Whereas when applying tetracycline, the repressor cannot bind the modified 35S promoter anymore, and therefore
the efficient de-repression can be achieved (Gatz et al., 1992). Although this system works well in tobacco and potato, applications in other plant species like Arabidopsis and tomato are not convincing (Gatz, 1997). But since it is shown that maize roots can take tetracycline related substance (oxytetracycline) from soil (Migliore et al., 2010), it is worth-while to test this system in maize.
Materials and methods

Gene cloning and transformation

Maize mutant dwarf8: d8 (2003-2154-4) in a W23xM14 wild-type background was provided by the Maize Genetics Cooperation Stock Center (www.maizegdb.org). The maize DELLAl dominant negative mutation gene d8 was cloned from the dwarf8 mutants, which contains one amino acid replacement and four amino acids deletion in DELLAl domain (Lawit et al., 2010). PCR primers for amplification d8 were designed with NCBI Primer-Blast (Forward primer: ATGAAGCGCGAGTACCAA and reverse primer: CGGAGCGGCGGCGGCCGAC). PCR condition for amplifying d8 was as followed: initial denaturation at 98°C for 3 min, followed by 10 cycles touchdown PCR of 98°C for 10 s, annealing at 68 °C for 30 s, extension at 72°C for 2 min, annealing temperature decrease 0.8°C per cycle, then a normal PCR of 98 °C for 10 s, annealing at 60°C for 30 s, extension at 72°C for 2 min and a final extension step of 5 min at 72°C. The EF1α::d8-GR construct was made based on a 35S::gai-GR construct in Arabidopsis (Claeys et al., 2012). EF1α::d8-GR construct was created using Gateway cloning by introducing T-DNA into pBbm42GW7 Gateway vector ((Karimi et al., 2013); https://gateway.psb.ugent.be/). Immature embryos of maize inbred line B104 were transformed by A. tumefaciens cocultivation containing transgene as described previously (Coussens et al., 2012). Transformed progenies were undergoing segregating and phenotypic analysis, transgenic plants were detected by an immunochromatographic assay detecting the PAT (Phosphinotrin Acetyltransferase) protein (AgroStrip, Romer) and a PCR-based method detecting the BAR selectable marker and the transgene, single locus lines were chosen for further analysis.

Plant growth and chemical treatments

For maize growing in hydroponic system, maize seeds were sterilized with bleach solution: 3 min in ethanol, and 15 min two times in 5% NaClO, followed by 3 min five times washing with sterilized water. The sterilized seeds were further wrapped by paper towels submerged with sterilized water for germination, and then transferred to 50ml focal tubes with Hoagland’s nutrient solution (Zhao et al., 2012). For chemical treatments in liquid solution, dexamethasone (Sigma-Aldrich) and estradiol (Sigma-Aldrich) were dissolved in 100% ethanol to make a stock solution (50mM). Dilutions were made to Hoagland solution from the stock solution immediately before use. The chemical treatment can start from maize germination or after germination. The solution in focal tubes was changed every
two days. For maize growing in soil, 25ml water was applied to maize plants every day. Estradiol was dissolved in ethanol and further diluted with water before adding into soil.

Plants were grown in growth chambers with controlled relative humidity (55%), temperature (24°C/18°C), and light intensity (170 mmol m-2 s-1 photosynthetically active radiation at plant level) provided by a combination of high-pressure sodium vapour (RNP-T/LR/400W/S/230/E40; Radium) and metal halide lamps with quartz burners (HRI-BT/400W/D230/E40; Radium) in a 16-h/8-h (day/night) cycle. Growth analysis was performed on the third and fourth leaf, leaf length was measured daily to determine leaf elongation rate (n=5).

Expression analysis

The forth leaf was harvested from plants at the indicated time points after transfer. Leaf 4 was dissected out and sampled into 0.5cm scale. Dissected leaves were transferred to a new tube, frozen in liquid nitrogen, and ground with 4-mm metal balls in a Retsch machine. Samples for one experiment include five biological replicates. Total RNA was isolated with the guanidinium thiocyanate-phenol-chloroform extraction method using TRI-reagent (Sigma-Aldrich). First-strand cDNA was synthesized from 1 µg total RNA using the iScript™ Advanced cDNA Synthesis Kit for qRT-PCR (Bio-Rad Laboratories). Primers for qRT-PCR were designed by Primer-Blast tool from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). qRT-PCR was performed on a LightCycler 480 (Roche Diagnostics) on 384-well plates with LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer’s instructions. PCR reactions were done in triplicate. For relative quantification, a threshold cycle was set at the same level for each reaction within the exponential amplification phase. For normalization, the transcript levels of the housekeeping gene, 18S rRNA were used as internal control.

Primer sequence:

<table>
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<th>Reverse primer</th>
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<td>GACTTGACCAAACATCTCAGGCAC</td>
</tr>
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<td>CDKB1;1</td>
<td>CCACAACACAACACTCCTCACC</td>
<td>ACCGTCGATGGGGAAGGG</td>
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Supplementary Data

Supplementary Figure 1. Expression levels of EF1α::d8-GR in leaf 4 of six transgenic lines. Line 1-6 are independent transgenic events, backcrossed to B104, rendering a segregating transgenic and non-transgenic progeny. Primer 1 is designed to amplify the C-terminal of d8 and N-terminal of GR, and primer 2 is against the C-terminal of GR. Data are presented as expression levels relative to 18S rRNA. Error bars indicate standard error (n=3 in all panels). T, transgenic siblings; NT, non-transgenic siblings.
Supplementary Figure 2. The leaf elongation duration (LED) of leaf 3 from non-transgenic (A) and transgenic (B) plants of segregating line 1 grown in control, DEX and corresponding ethanol solutions. Significance was calculated based on student T-test (n>3; *, **, and *** correspond to P-values of 0.05>p>0.01, 0.01>p>0.001, and p< 0.001, respectively). T refers to transgenic plants and NT refers to non-transgenic siblings.

Supplementary Figure 3. LER of leaf 3 in transgenic plants of EF1α::d8-GR leaky lines under estradiol treatment. T, transgenic plants.
Supplementary Figure 4. LER of leaf 3 in EF1α::d8-GR non-leaky lines with 50µM estradiol treatment. Control means plants were grown in liquid solution, mock means plants were grown in liquid solution with corresponding ethanol content. T, transgenic plants. NT, non-transgenic plants. EST, estradiol.

Supplementary Figure 5. Expression levels of CDKB1;1 in leaf 4 of segregating EF1α::d8-GR leaky line 1. Data are presented as expression levels relative to the basal 0.5 centimeter leaf sample from the transgenic plant. Error bars indicate standard error (n = 3). T, transgenic plants. NT, non-transgenic plants.
References


DELLA inducible system


DELLA inducible system


Chapter 6

Conclusions and perspectives
Conclusions and perspectives

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AUTHOR CONTRIBUTION

X.S. was the main author of this chapter. D.I. and H.N. contributed to the writing of this chapter.
Conclusions and perspectives

One of the biggest global challenges today is the production of sufficient food to meet the demand of the growing population. Reductions in crop yield caused by water scarcity and the variable weather patterns that are associated with climate change further aggravate the burden on agriculture. In this thesis, we focused on one of the most important crop plants, maize, to investigate the growth mechanisms contributing to yield. By using the maize leaf as a model, we explored the regulation of cell division and cell expansion at both the temporal and spatial level.

The expanding knowledge on growth regulation in the maize leaf

Leaf growth rate (leaf elongation rate, LER) has been identified to play an important role in determining final leaf length. When GA20-OXIDASE-1 (GA20OX-1), a rate limiting enzyme of GA biosynthesis, is constitutively overexpressed (UBIL::GA20OX), LER is increased resulting in larger maize leaves (Nelissen et al., 2012). Elevated LER, leading to increased final leaf length can be also observed when a miRNA396 resistant GROWTH-REGULATING FACTOR1 (GRF1) was overexpressed (Nelissen et al., 2015). In both cases, the enhanced LER is due to an increase in the size of the division zone during the steady state growth stage, suggesting that the spatial control of division zone size is crucial for maize leaf development.

Here we discovered that besides LER, the leaf growth duration (LED) also plays an important role in determining final leaf length. LED can be quantified by LEAF-E, based on daily measurements of leaf length (Voorwind et al., 2014). Prolonged LED is observed when maize plants were subjected to mild drought stress. Mild drought significantly reduces the division zone size during the steady state growth phase, resulting in a 28% decreased LER. However, the growth reduction by LER was partly compensated by a prolonged growth period (LED) and therefore only a 10% decrease of final leaf length is actually observed under mild drought. This compensatory growth is also observed, but not quantified, in another study on maize leaf growth under both mild and severe drought conditions (Avramova et al., 2015). Our transcriptome data showed that genes enriched in DNA replication and cell cycle GO categories were significantly upregulated at the transition zone and basal expansion zone under drought stress. Several of these genes are known targets of E2F/DP (Vandepoele et al., 2005), indicating that the prolonged duration of growth is associated with a prolonged potential in cell division, probably under the control of E2F/DP transcription factor. Interestingly, the prolonged LED was only observed when LER is reduced, suggesting that probably signaling occurs in dividing cells to trigger them to divide for a longer period of time. The nature of such signal is currently unknown.
The compensatory mechanism of prolonged growth duration was initially shown under mild drought conditions, and a maize cytochrome P450 gene, CYP78A1/ZmKLUH (KLUH), was identified as a key molecular player in the regulation of LED under normal and stress conditions. KLUH is the maize homolog of Arabidopsis CYP78A5/AtKLUH (AtKLUH) and rice CYP78A11/PLA1 (PLA1) (Miyoshi et al., 2004; Anastasiou et al., 2007; Nagasawa et al., 2013). AtKLUH and PLA1 positively promote growth and elongate the growth duration in Arabidopsis and rice, respectively (Miyoshi et al., 2004; Anastasiou et al., 2007). In maize, we showed that ectopically overexpressed KLUH by the GA2oxidase promoter (GA2ox::KLUH), resulted in plants with longer leaves due to an extended period of cell division and thus a prolonged LED. Interestingly, KLUH reinforces the prolonged LED under mild drought conditions, suggesting that the KLUH-mediated growth mechanism is also part of the compensatory growth mechanism in which a reduction of LER is compensated by a prolonged LED. In Arabidopsis, AtKLUH was shown to act non-cell autonomously in growth stimulation (Anastasiou et al., 2007). Although not yet conclusive, transcriptome and metabolite analysis in GA2ox::KLUH transgenic plants showed elevated levels of two possible growth promoting molecules auxin (Perrot-Rechenmann, 2010; Peer et al., 2011) and chlorogenic acid (CGA).

Recently, by integrating the transcriptome and phenotypic data from a H99×B73 recombinant inbred line (RIL) population and a MAGIC (multiparent advanced generation intercross) population, 517 genes have been identified of which the expression levels in the division zone (anti)-correlated with LED in at least one of the populations (Baute et al., 2015; Baute et al., 2016). Among the 517 genes, 25 genes (of a total 533 genes) are also differentially expressed between the division zone of GA2ox::KLUH and wild-type plants after the steady-state growth stage. More importantly, their association with LED is consistent among GA2ox::KLUH transgenic plants and the RIL and MAGIC populations, making them strong candidate genes to be involved in the control of growth duration (Table 1). A putative auxin efflux carrier (ZmPIN1b) (Carraro et al., 2006) was negatively associated with LED in the H99×B73 RIL population and lower expressed in GA2ox::KLUH. Together with the higher auxin accumulation in GA2ox::KLUH, this observation leads us to postulate that alterations of auxin level and/or localized auxin content during leaf development are important determinants of LED. Moreover, altered auxin signaling pathway could also affect the growth duration. Overexpression of ARGOS1 (Auxin Regulated Gene involved in Organ Size), an auxin-inducible gene, results in plants with longer leaves in both maize and Arabidopsis (Hu et al., 2003; Guo et al., 2014; Shi et al., 2015). By following CycB1 directed GUS activity in Arabidopsis ARGOS overexpression plants, it was shown that the increase in leaf size is mainly attributable to prolonged cell division duration (Hu et al., 2003). Noticeably, chorismate synthase that catalyzes the biosynthesis of chorismate (Tzin and Galili, 2010), is also negatively associated with LED in H99×B73 RIL population.
and significantly downregulated in the KLUH overexpression lines. Chorismate is the precursor of auxin and many phenylpropanoids, including flavonoids and lignin monomers. Downregulation in phenylpropanoid metabolism has been shown to reduce lignin production and promote growth. Transgenic aspen (Populus tremuloides) tree with suppressed Pt4CL1 (4-coumarate:coenzyme A ligase) expression exhibited lignin reduction but cellulose increase, associated with enhanced root and stem growth, probably due to increased cell division (Hu et al., 1999). Defects in phenylpropanoid biosynthesis arising from deficiency in HCT (hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase) or C3'H (p-coumaroyl shikimate 3’hydroxylase) lead to growth inhibition due to reduced lignin (Li et al., 2010). Two trihelix transcription factors are also negatively associated with LED, and one of them is the homolog of Arabidopsis GT-2 LIKE 1 (GTL1), which was shown to repress the transcription of the activator of the anaphase-promoting complex/cyclosome (APC/C) and further arrest the endocycle and cell growth in both trichome and leaf epidermal cells (Breuer et al., 2009; Breuer et al., 2012). In contrast, two R2R3 MYB transcription factors (ZmMYB022, ZmMYB079) are positively correlated with LED: ZmMYB022 shares the highest phylogenetic identity with Arabidopsis AtMYB117, and ZmMYB022 is phylogenetic close to AtMYB17 (Du et al., 2012). AtMYB117 is also known as LATERAL ORGAN FUSION1 (LOF1) functioning in boundary specification, meristem initiation and maintenance during vegetative development (Lee et al., 2009), while AtMYB17 is known as LATE MERISTEM IDENTITY2 (LMI2) positively controlling the meristem identity transition during flower development (Pastore et al., 2011). Two components of the SWI/SNF chromatin remodeling complex, ANGUSTIFOLIA3 (AN3) and GROWTH-REGULATING FACTOR2 (GRF2) are positively associated with LED. The Arabidopsis AN3 was shown to regulate both rate and duration of cell division (Vercruyssen et al., 2014). In maize, plants overexpressing AN3 grew slower, although no significant effect on growth rate and final leaf size was observed (Nelissen et al., 2015). AN3 associates with distinct GRFs in a tissue and organ dependent manner and GRF2 was associated to the SWI/SNF complex in the developing ear (Nelissen et al., 2015).

LER and LED are shown to be uncorrelated in both the H99-B73 RIL population and in the MAGIC population, suggesting that the two mechanisms driving leaf growth might be independent (Baute et al., 2015; Baute et al., 2016). We demonstrated that the transgenic plants overexpressing both GA20OX and KLUH exhibit additive effects of LER and LED, further reinforcing the idea that the two processes drive growth independently. However, since prolonged LED is observed when LER is reduced in drought, there has to be a mechanism that connects LER and LED, at least under stress conditions.
Table 1. Candidate genes in the control of leaf elongation duration.

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Conclusions and perspectives

a): K6 and W6 are basal (0.5cm) leaf samples harvested at six days after their emergence in GA2ox::KLUH and its non-transgenic siblings, respectively. At this time point, the non-transgenic plants have passed through the steady-state growth, while GA2ox::KLUH plants are still in steady-state growth due to prolonged cell division. Therefore, genes that are differentially expressed in the GA2ox::KLUH and non-transgenic plants are postulated to be involved in the timing of growth. The expression level is shown as log fold change (\(|\logFC|>1\)) between GA2ox::KLUH and wild-type. K: GA2OX::KLUH; W: wild-type. The transcriptome data is adapted from Chapter 4 in this thesis.

b): “+” indicates the genes significantly correlate with LED. 2-way refers to the H99-B73 RIL population (Baute et al., 2015) and 8-way to the 8-parent MAGIC population (Baute et al., 2016). The correlation is adapted from Baute et al., 2016.

c): Gene annotation from PLAZA3.0 (Proost et al., 2015).

**Hypothetical basis for mild drought causing prolonged growth duration**

The reduction in final leaf length resulting from a diminished number of dividing cells and consequently reduced LER was partly counteracted by an increased LED in maize plants under drought stress. This phenomenon is reminiscent of what is known as “compensation” in Arabidopsis. Compensation is suggestive of a co-ordination between cell division and cell expansion, defined as enhanced post-mitotic cell expansion associated with a decrease in cell number during lateral organ development (Horiguchi et al., 2006; Hisanaga et al., 2015).

Three different modes of action for the compensation phenomenon have been postulated based on the analysis of various compensation exhibiting mutant plants: i) increased cell expansion rate, ii) extended cell expansion duration, iii) increased cell size in both the division and expansion period (Ferjani et al., 2007; Hisanaga et al., 2015). However, the observed compensation in maize under drought stress seems not to belong to any of the compensation modes of action. As the mature cell size in mild drought treated leaves are smaller than well-watered leaves, the compensated prolonged growth period is not from cell expansion but from a longer period of cell division.

Previously, two interpretations of this compensation mechanism have been proposed, the organismal theory and the cell theory. The organismal theory postulates that the size of an organism is predetermined by its genetic information rather than determined by individual cell division (Kaplan and Hagemann, 1991; Hemerly et al., 1995), while the cell theory proposes that cells are the unit of morphogenesis and that the sum of the behavior of each cell controls multicellular morphogenesis (Tsukaya, 2002). Recently, compensation in Arabidopsis is proposed to act through intercellular
signals, which is in accordance to the cell theory (Kawade et al., 2010; Hisanaga et al., 2015). Two genes (*ANGUSTIFOLIA3* (AN3) and *KIP RELATED PROTEIN2* (KRP2)) have been identified to produce different signals in regulating compensation. Loss-of-function mutant *an3* and *KRP2* overexpression plants show similar phenotypes with a decreased number of leaf cells but with a larger cell size due to the compensation (Horiguchi et al., 2006; Ferjani et al., 2007). Kawade et al. (2010) investigated the signal directing the compensation by analyzing chimeric leaves for *AN3* and *KRP2* expression using Cre/lox system. When *AN3* expression was chimerically induced in an *an3* mutant background, all cells in the chimeric leaf showed typically compensation at the same level as the *an3* mutant leaves, suggesting that *an3* cells generate a non-cell autonomous signal. In contrast, compensation by *KRP2* was found to be a cell-autonomous process, as only cells with induced expression of *KRP2* exhibit compensation (Kawade et al., 2010). However, the nature of both *AN3* and *KRP2* mediated signal is currently unknown. The compensation phenomenon observed in maize leaves under mild drought stress might be triggered by a reduced activity of cell division, possibly resulting in a yet unknown signal that extends the duration of the cell division machinery.

Additionally, the increased duration to compensate for a reduced cell division activity might not be maize specific. In Arabidopsis, genes such as *AN3* and *CYCLINE D3* (CYCD3) were shown to positively regulate cell division duration, whereas *DA1* and *ENHANCER OF DA1* (EOD) negatively regulate the timing of cell division (Gonzalez et al., 2012; Gonzalez and Inzé, 2015). But to our knowledge, no such compensation has been reported in Arabidopsis leaves subjected to mild drought stress. The small size of growing Arabidopsis leaves make it technically difficult to study this phenomenon. In Arabidopsis, cell division is frequently monitored by the activity of the cell cycle gene *CYCLIN B1;1* (Kazama et al., 2010). Using this tool, a careful study could be conducted to understand how mild drought stress affects the timing of leaf growth.

**Maize and Arabidopsis leaf growth**

The initial and long term growth effects mediated by mild osmotic stress, which causes a dehydration effect resembling drought stress, have been characterized in Arabidopsis. In actively growing Arabidopsis leaves, ethylene and genes encoding ETHYLENE RESPONSE FACTORS act on cell cycle progression and cause a very quick and reversible pause in the cell cycle in young proliferating leaves under mild osmotic stress (Skirycz et al., 2011; Dubois et al., 2013). When the stress persists, the pausing of the cell cycle will become irreversible and cells will enter the differentiation process. The shutdown of the cell cycle could be compensated by additional cell divisions from meristemoid development when the stress is released (Skirycz et al., 2011). However, the strategy of maize plants
for water deficit tolerance is different from that of Arabidopsis. Instead of completely shutting down cell division, maize plants tend to maintain cell division at a lower pace but for a longer period. Therefore, the maintenance of cell division for a longer period might allow the LER to rise again when drought stress is removed. In this way, the maize leaf growth could resume again, a hypothesis that is currently being tested in our research group.

In Arabidopsis at least five different parameters are proposed to affect final leaf length: the number of cells incorporated in leaf primordia, the rate of cell division, the developmental window of cell division, the timing of meristemoid division, and the extent of cell expansion (Gonzalez et al., 2012). At least four of these mechanisms appear to be present in maize, with the exception of the mechanism that relies on meristemoid divisions. In maize, the guard mother cell forms in one asymmetric, transverse division of an epidermal cell (Howell, 1998). As no self-renewing cells are produced in the stomatal lineage, the contribution to leaf size from stomatal formation is limited. The absence of PEAPOD (PPD) proteins, that are known to negatively regulate meristemoid division (White, 2006), also indicates the absence of this process in monocots.

With the exception of meristemoids, there are some parallels between growth regulatory mechanisms in Arabidopsis and maize, despite their evolutionary distance. Upregulation of GA biosynthesis (GA20OX1 overexpression) can promote both cell division and cell expansion during Arabidopsis leaf growth (Huang et al., 1998; Gonzalez et al., 2010), while in maize, high GA levels as seen in GA20OX1 overexpression plants spatially shifted the transition zone between cell division to cell expansion more distally from the leaf basis resulting in an enlarged division zone size, containing more cells (Nelissen et al., 2012). In Arabidopsis leaves, also brassinosteroid (BR) has been shown to impact both cell number and cell size and mutants in BR signaling are usually dwarfed (Azpiroz et al., 1998; Oh et al., 2011; Zhiponova et al., 2013). In maize, interfering with the BR signaling pathway significantly decreased the number of dividing cells (Kir et al., 2015). The components of AN3 associated SWI/SNF chromatin remodeling complex are conserved between maize and Arabidopsis (Debernardi et al., 2014; Vercruyssen et al., 2014; Nelissen et al., 2015). However, overexpression of AN3 in Arabidopsis results in bigger leaves (Vercruyssen et al., 2014), but leaf size is barely affected by constitutive expressed AN3 in maize (Nelissen et al., 2015). On the other hand overexpressions of GRFs were shown to increase leaf size both in Arabidopsis and maize (Nelissen et al., 2015). In this thesis, we are able to show that the ability of KLUH to stimulate cell division is conserved both in maize and Arabidopsis. Atkluh rosettes contained more leaves, but they were small due to a reduction in cell number (Anastasiou et al., 2007)(Claeys et al., unpublished data). Conversely, AtKLUH overexpression led to increased cell number, but opposite growth effects were observed depending on the strength of the promoter. When AtKLUH is overexpressed under its endogenous
promoter, larger rosettes and leaves were observed, while use of the constitutive promoter 35S led to a reduced leaf size due to a dramatic reduction in cell size (Claeys et al., unpublished data). However, in maize, both mild and constitutively overexpression KLUH stimulate cell division and generate bigger leaves. Taken together, Arabidopsis and maize share similar, but not exactly the same mechanisms that govern leaf growth.

Translating basic research into applications

Following the identification of the novel mechanism triggered by KLUH by which leaf growth can be stimulated in maize, we wondered whether this mechanism could improve maize biomass and yield. In Arabidopsis, constitutive overexpressing of AtKLUH produces plants with an altered morphology. However, when AtKLUH is expressed with its endogenous promoter, the plants show normal stature and increased vegetative and reproductive growth (Claeys et al., unpublished data), suggesting the importance of promoter selection and the strength of KLUH transgene expression. Similarly, in maize, strong overexpression of ZmKLUH leads to altered plant architecture and abortion of reproductive organs, while increased stover biomass and grain yield are obtained when ZmKLUH is mildly overexpressed under control of the GA2 oxidase promoter. The fact that the choice of a milder promoter rather than a strong constitutive promoter could generate agricultural advantages is also shown by another example. Organ size was negatively affected by strong expression of CCS52A under the control of the 3SS promoter of cauliflower mosaic virus (CaMV), whereas milder overexpression enhances organ size (Baloban et al., 2013). Moreover, the precise spatiotemporal regulation of a transgene can also significantly impact phenotypic outcome. Trehalose is a non-reducing disaccharide of glucose that functions as a compatible solute in the stabilization of biological structures under abiotic stress (Garg et al., 2002). Ectopically overexpressing the trehalose biosynthetic gene (trehalose-6-phosphate phosphatase, TPP) by the OsMADS6 promoter improves grain yield under drought conditions, whereas overexpression of TPP by OsMADS13 negatively influenced yield (Nuccio et al., 2015; Smeekens, 2015). The OsMADS13 expression cassette showed activity in similar tissues as OsMADS6, including the ear vasculature and ear spikelet, but OsMADS13 has a more restricted expression pattern in ovules and is less active in the vasculature (Nuccio et al., 2015). Currently, many growth promoting genes have been identified and expressing these genes under the control of promoters with specific activity in organs, during development or in response to specific environments could avoid undesired pleiotropic effects, and further enhance yield.

As KLUH and GA promote leaf growth by two mechanisms, LED and LER respectively, it is interesting to explore whether growth could be further increased by a combination of both growth promoting
mechanisms. Indeed, the combination of LED and LER shows an additive effect and further drives vegetative growth, suggesting that this strategy is working in maize. However, it might not be always the case for any two gene or two mechanism combinations. The genetic interactions between 13 genes that positively regulate leaf growth in a gain- or loss-of-function situation have been recently studied in Arabidopsis. Among the analyzed 61 binary combinations of genes of which each individually promotes leaf growth, 16 combinations resulted from a synergistic effect, and 8 were the result of an additive effect (Vanhaeren et al., 2014). The gene staking strategy to promote organ growth can be further expanded to more genes. A triple combination of three loss-of-functions genes, which each on their own have been demonstrated to enhance leaf growth, results in a remarkable increase in leaf, root and flower size as compared to wild-type and all parental lines (Vanhaeren et al., 2015). Although gene stacking has been shown to be a successful way to tackle a complex trait such as plant growth by simulating multiple mechanisms underlying the process, negative combined effects were observed in some gene combinations suggesting that the interactions among different genes are currently not predictable (Vanhaeren et al., 2014; Vanhaeren et al., 2015). Thus, large-scale studies on the genetic interactions could provide much information about the growth network and create opportunities to further promote yield. Currently, several established phenotyping platforms, mostly with automated weighing, irrigation and imaging (Granier et al., 2006; Tisné et al., 2013; Granier and Vile, 2014), have allowed large-scale experiments that are challenging to carry out manually. In the future, with the establishment of the phenotyping platforms in the field, we could directly select the plants with the desired performance in natural conditions.

Hybrid vigor or heterosis has a large effect on biomass and yield and is the consequence of a large scale interaction of the two parental genomes (Duvick, 1999). Because of the higher performance of the hybrids as compared to the inbreds, most commercialized maize varieties are hybrids generated by crossing elite inbreds. In this study, we analyzed whether the growth promoting effect of KLUH is also visible when the transgene is expressed in a hybrid background. To this end, we introduced the ZmKLUH transgene in a hybrid by crossing GA2ox::KLUH (B104 inbred background) with three other inbreds (CML91, H99, W153R). An overall increased leaf growth in the hybrid transgenics was observed compared to the non-transgenic hybrid in the greenhouse. Similarly, a better performance of the transgenic hybrid has been observed in the hybrid overexpressing the maize ARGOS transgene (Guo et al., 2014).

Although several strategies are proposed to increase growth, the inherent variability and heterogeneity of the field environment makes it difficult to translate elite traits from laboratory to field. In this study, in order to test the performance of KLUH, field experiments were carried out with GA2ox::KLUH inbred plants and GA2ox::KLUH × CML91 hybrid plants. The GA2ox::KLUH transgenic
The vegetative traits that contributed to stover biomass including leaf length, leaf width and plant height were significantly increased in the hybrid transgenics in both locations, and in the GA2ox::KLUH in US, suggesting a robust effect of KLUH stimulating vegetative growth. The grain yield seems to vary between years and locations; nevertheless, the overall yield is still increased in the hybrid transgenics. At both locations, the hybrid transgenics produce significantly longer ears, moderate but consistent increased kernel number, kernel dry weight and kernel volume compared to non-transgenic hybrid plants. Also in other species KLUH or related CYP78As were shown to promote organ growth. It is reported that upregulation of a KLUH homologue in tomato, SIKLUH, enhances cell division in both fruit and seed (Chakrabarti et al., 2013). However, overexpressing CYP78A5 or CYP78A9 in Arabidopsis produces larger seeds through increased cell division, but wider and shorter siliques, similar to overexpression of wheat TaCYP78A3 in Arabidopsis (Adamski et al., 2009; Sotelo-Silveira et al., 2013; Ma et al., 2015). Thus, CYP78As functions are conserved, but probably have different effects at the organ level among different plant species. Nevertheless, due to the conserved function of CYP78A family members, the performance of KLUH transgene in maize under field conditions could be translated to other crops, such as rice, wheat, by moderate overexpression KLUH or KLUH homologs.

Besides KLUH, several other growth regulators have been found to consistently regulate both vegetative and reproductive organ growth in maize. The maize ARGOS1 (ZAR1) enhances maize organ growth, causing the formation of larger leaves and longer ears with increased kernel numbers per ear (Guo et al., 2014; Shi et al., 2015). Down-regulation of ACC synthases (ACSm; enzymes that catalyze the rate-limiting step in ethylene biosynthesis) delays leaf senescence under normal growth conditions and inhibits drought-induced senescence (Young et al., 2004), and increases kernel numbers per ear under drought conditions (Habben et al., 2014). In addition, some other proteins are controlling leaf and ear or kernel initiation, such as genes in the CLAVATA-WUSCHEL signaling pathway regulating both leaf and ear initiation (Bommert et al., 2005; Stahl and Simon, 2010; Bommert et al., 2013), and the SQUAMOSA PROMOTER BINDING (SBP)-box transcription factors regulating leaf and kernel initiation (Chuck et al., 2007; Chuck et al., 2014). Future development of methods to analyze ear development at cellular level over time might allow for comparing the growth processes between leaf and ear. As a consequence, knowledge on leaf growth might become applicable to stimulate growth of maize ear resulting in further yield increases.

Our research provides a successful example of translating basic knowledge into field applications. Based on the existing data about the mode of action of growth-regulating genes, several strategies...
can be exploited for future selection of plants with optimal traits: specific promoter-gene combinations; gene stacking strategies; and the combination of transgene and heterosis effect. By analyzing the effect in field conditions, novel insights can be obtained that can be further analyzed in the lab. This ‘lab-to-field-to-lab’ innovation cycle will speed to generation of novel climate adapted crops (Nelissen et al., 2014).

**Tool development in maize**

In this thesis, we contributed to the development a new bioinformatics tool (Leaf Growth Viewer, LGV) to visualize with high resolution transcriptome data along the developing maize leaf. LGV has some feature in common with the eFP Browser, which contains the expression profiles of transcripts specifically involved in maize leaf development and other developmental stages (Wang et al., 2014). However, compared to the eFP Browser, more features have been installed. Despite the transcriptome data of maize leaf under well-watered condition, LGV also contains the transcriptome of leaf under mild drought treatment, and with GA perturbation. Moreover, LGV enables users to select the genes of interest based on their expression profiles in relation to position in a growing leaf and/or growth conditions, and consequently visualize them as heat map images. The link of LGV with PLAZA3.0 (Proost et al., 2015) provides more information about gene ontology and enables GO enrichment analysis for the selected genes. In the future LGV can be further complemented with other experimental datasets and additional bioinformatics features. For instance, LGV currently only contains transcriptome information, but by installing other datasets, such as the dynamic changes in protein complex along the developing leaf (Nelissen et al., 2015), and protein-protein interactions as already present in the bioinformatics tool CORNET (De Bodt et al., 2012), LGV could be developed into a powerful tool for expanding the existing knowledge of the network of genes involved in maize leaf growth.

We also attempted to develop a GR inducible system in maize, with the aim to identify genes that are directly influenced by DELLA and to compare those with the DELLA targets previously identified in Arabidopsis. In Arabidopsis, through the use of a glucocorticoid receptor (GR) based inducible gene expression system, downstream targets of DELLA proteins were identified in proliferating leaf cells, including several growth regulators known to control cell division (Claeys et al., 2014). Especially, *AtKLUH* is among the early response genes, suggesting an interaction between GA-DELLA signaling and *AtKLUH*. To also explore the ZmKLUH regulating network, a dominant DELLA variant of maize (d8) was fused to the GR receptor and introduced in transgenic maize. Due to the leakiness of the construct or a cytoplasmic function of d8, this system in maize led to dwarf plants with a defect in
growth. Several experiments showed that only these leaky lines can be induced by steroid application in maize, but nevertheless, the induction in maize occurs only occasionally and requires very high steroid content in liquid solution and even higher steroid dosages are expected to be required to induce plants grown in soil. However, since the high amount of solvent (ethanol) used to dissolve a high dose of steroids severely affects maize growth, soil applications of high dosages of steroids become impractical. Using hydroponic cultures it was found that estradiol application to roots only causes small increases in estradiol in shoots, suggesting a problem with the root to shoot translocation (Card et al., 2012). In contrast, successful chemical absorption was observed using directly spraying chemicals on maize leaf (da Silva Messias et al., 2013; Rothová et al., 2014), a strategy that requires further testing on the DELLA-GR transgenics. Currently, the Department of Plant Systems Biology is building a safe spraying cabinet to be used for steroid hormones and other chemicals.

The mechanisms/signals that regulated the temporal expression of KLUH expression are unknown. In Arabidopsis, down-regulation of AtKLUH expression precedes the cell cycle arrest front during early leaf development (Kazama et al., 2010). Recently, the transcription factors SOD7/NGAL2 and DPA4/NGAL3 were shown to redundantly repress AtKLUH expression in Arabidopsis seeds (Zhang et al., 2015). One of the NGAL homologs in maize shows a constitutively high expression level (as shown in LGV) in both dividing and expanding tissue of growing maize leaf. Therefore, it is interesting to explore whether NGALs could regulate KLUH expression in maize leaf. The interaction between NGAL and KLUH could be examined by studying the expression level of KLUH in transgenic plants with disturbed NGAL expression using the maize leaf as a model.

Moreover and as discussed above, the substrate and end-product of KLUH remain uncertain. The metabolomics analysis on the dividing leaf tissues shows that CGA is significantly more abundant in KLUH overexpression plants and is decreased in kluh mutants, suggesting that CGA might be the product of KLUH. Cell division in Arabidopsis rosette leaves was highly stimulated by CGA treatment, however not to the same extent as observed in Arabidopsis strongly overexpressing AtKLUH, meaning there might be other molecules produced by KLUH. As KLUH is highly expressed only at the basal division zone of maize growing leaf, we expect that the KLUH end-product would be higher at the leaf basis than more distal from the leaf basis, a hypothesis that requires further testing. The identification of the KLUH end-product can possibly result in the development of growth promoting substances that can be used to boost crop yield. As no effect was observed in maize seedling upon CGA treatment in soil, direct application to maize leaves might enhance growth. Moreover, applying CGA to husk leaves during reproductive phase of maize growth might also promote ear development with higher grain yield. In summary, our work uncovered the fundamental knowledge on maize leaf
size control in well-watered and water deficit conditions, which could be beneficial for agricultural applications.
References


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