Manipulation of plant architecture to enhance lignocellulosic biomass

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Abstract

Background Biofuels hold the promise to replace an appreciable proportion of fossil fuels. Not only do they emit significantly lower amounts of greenhouse gases, they are much closer to being ‘carbon neutral’, since the source plants utilize carbon dioxide for their growth. In particular, second-generation lignocellulosic biofuels from agricultural wastes and non-food crops such as switchgrass promise sustainability and avoid diverting food crops to fuel. Currently, available lignocellulosic biomass could yield sufficient bioethanol to replace \( \approx 10 \% \) of worldwide petroleum use. Increasing the biomass used for biofuel production and the yield of bioethanol will thus help meet global energy demands while significantly reducing greenhouse gas emissions.

Scope We discuss the advantages of various biotechnological approaches to improve crops and highlight the contribution of genomics and functional genomics in this field. Current knowledge concerning plant hormones and their intermediates involved in the regulation of plant architecture is presented with a special focus on gibberellins and cytokinins, and their signalling intermediates. We highlight the potential of information gained from model plants such as \textit{Arabidopsis thaliana} and rice (\textit{Oryza sativa}) to accelerate improvement of fuel crops.

Introduction

Biofuel production and biomass enhancement

Renewable biofuels are expected to replace an increasing proportion of fossil fuels consumed in the coming decades. Their appeal hinges principally on their low overall emission of carbon dioxide (CO\textsubscript{2}) because their utilization only emits CO\textsubscript{2} previously taken up during recent growth. Biofuel crops are commonly converted into bioethanol before use. Strategies to increase the amount of biofuel can target all steps between photosynthetic capture of solar energy and ethanol production itself. None of these steps is especially efficient. For example, photon use efficiency of plants lies between 4 and 6 \%. An increase here would significantly contribute to higher yields, while improving the efficiency of converting biomass into bioethanol would also be highly beneficial. Most of the energy is derived from

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cell wall components, i.e. lignocellulosic biomass. However, the lignin content of cell walls severely impedes saccharification—the break-down into simple sugars, which are converted microbially to ethanol. Limiting the amount of lignin present in cell walls may therefore help to improve the recovery of energy stored in cell walls (Abramson et al. 2010). However, in this review, we focus on biomass build-up, rather than biofuel production itself.

First-generation biofuels are produced mostly from corn, sugarcane and beets. However, these are essentially food crops grown on cultivable land and thus require the diversion of food crops. This is likely to increase commodity prices and threaten food security for the rapidly growing world population (Stamm et al. 2011). Second-generation biofuels promise greater sustainability and avoid the socioeconomic conflict of ‘food versus fuel’. These biofuels are mainly cellulose-rich agricultural wastes such as corn stover, rice and wheat straw. Non-food crops such as Miscanthus and switchgrass (Panicum virgatum) are also gaining in importance. Owing to its high perennial productivity and low maintenance cost, switchgrass is a particularly promising biomass source (Young et al. 2010). The net energy derived from switchgrass biomass is $\sim 13$ times higher than the input energy; furthermore, emissions of greenhouse gases from switchgrass-derived biofuel are considered to be up to 94 % lower than those from fossil fuels (Schmer et al. 2008).

Currently, an estimated 3 billion tons of lignocellulosic biomass are generated worldwide in the form of corn stover, rice and wheat straw annually (Engel et al. 2005; Binod et al. 2010). Assuming a 40 % efficiency of biomass to ethanol conversion, over a trillion litres of ethanol per year could be generated from the lignocellulosic biomass of these three common crops. This is equivalent to the annual petroleum demands of the USA, or $\sim 25$ % of the annual world petroleum oil needs. Furthermore, petroleum constitutes $\sim 34$ % of the world energy usage, with coal and natural gas forming another 50 % (Table 1). According to this estimate, the contribution by biofuels in 2006 was <0.5 % of the total primary energy forms. With additional lignocellulosic mass from perennial grass crops and forest by-products, etc., the total amount of renewable bioethanol could readily approach $\sim 10$ % of current petroleum use. If additional research can improve ethanol yield from plant biomass feedstock (as has been shown in recent years, see Fu et al. 2011), overall yield could be increased still further.

In summary, a major challenge is to garner extensive and sustainable amounts of biomass without affecting food production. Routes to achieving this include manipulating food crops to increase the biomass of waste straw and stover for biofuel production without negatively affecting grain yield for food production, or using non-food crops that can be grown on land sub-optimal for food crops and manipulating them for maximum biomass production. Both approaches will require combining expertise in breeding, molecular genetics and biotechnology.

Breeding has an impressive track record for crop improvement and trait enhancement. The most iconic examples of its success are the ‘Green Revolution’ rice variety IR8 and the wheat varieties ‘Lerma Rojo 64’ and ‘Sonora 64’. The widespread adoption of these new varieties led to significant increases in grain yield in the 1960s and 1970s (Peng et al. 1999). In the biofuel crop switchgrass, forage nutritional quality has been significantly improved by breeding (Vogel et al. 1989; McLaughlin et al. 1999). Additional breeding programmes to improve the biofuel crops Miscanthus and switchgrass are in progress and draft linkage maps have been published (Atienza et al. 2002; Missaoui et al. 2005). However, higher density mapping and many more molecular markers are needed to achieve breeding goals such as improved biotic and abiotic stress tolerance (Jakob et al. 2009). Furthermore, variability in plant height, stem density and stem thickness among different species of biofuel crops can be utilized by plant breeders to genetically improve these crops. For example, plant height of different Miscanthus species varies from 1.5 to 5.0 m (Jakob et al. 2009), while switchgrass height can range from 2.4 m in upland varieties to 4.0 m in lowland tall varieties (Bouton 2007). Plant breeding can combine desirable traits such as these into one variety with enhanced

| Energy source | 1973 % | 2007 % |
|---------------|--------|--------|
| Oil           | 46.1   | 34.0   |
| Coal/peat     | 24.5   | 26.5   |
| Gas           | 16.0   | 20.9   |
| Combustible renewables and waste | 10.6 | 9.8 |
| Hydro         | 1.8    | 2.2    |
| Nuclear       | 0.9    | 5.9    |
| Others (e.g. bioethanol, geothermal, solar, wind, heat, etc.) | 0.1 | 0.7 |

Information based on statistics from the International Energy Agency (IEA; http://www.iea.org/textbase/nppdf/free/2009/key_stats_2009.pdf).
plant biomass production. However, traditional breeding has severe limitations. It is slow and several generations are needed to fix a trait. Furthermore, agronomic traits can only be introduced one by one, making the generation of one variety with a combination of desirable traits extremely lengthy. Lastly, cross-hybridization in some species can be difficult, if not impossible, due to hybridization incompatibilities that are hard to overcome in traditional breeding programmes. This is particularly true for switchgrass, a wind-facilitated cross-pollinated species with strong genetic self-incompatibility (Martinez-Reyna and Vogel 2002). Only recently has a self-compatible switchgrass genotype been identified using a simple sequence repeat (SSR) marker. This important step may lead to the development of inbred lines of switchgrass (Liu and Wu 2011). However, in recent years this has to be overcome in traditional breeding programmes. The availability of the complete genome sequence of one of the wild grass model species, Arabidopsis thaliana and rice (Oryza sativa) have significantly advanced our understanding of the genetic basis of plant growth and development. The availability of the complete genome sequence of Arabidopsis (Arabidopsis Genome Initiative 2000) and rice (International Rice Genome Sequencing Project 2005) and their synteny with other plants have facilitated the research to improve agronomically important traits. Genomes of many plant species have been, and are being, sequenced. Recently, the complete genome sequence of one of the wild grass model species, Brachypodium distachyon, has been released, with a genome size of 272 Mbp and predicted 25 532 protein-coding gene loci. The Brachypodium genome has 99.2 % synteny with rice, sorghum and wheat (The International Brachypodium Initiative 2010). Thus, homologous genes could be identified in this species, which in turn could serve as starting points to genetically modify other biofuel grasses such as Miscanthus and switchgrass. However, their different growth strategies (annual Brachypodium vs. perennial switchgrass) may mean that different signalling pathways regulating development are in place for these species. Therefore, thorough analysis of gene function is required for every species to be manipulated.

There are many genomic studies on switchgrass. For example, a complete linkage map has been constructed using 238 full-sibling populations obtained from a cross between the two tetraploid lowland varieties Kanlow and Alamo (Okada et al. 2010). The ploidy levels of switchgrass plants range from diploid (2x = 18) to dodecaploid (12x = 108), the lowland switchgrass varieties Alamo and Kanlow being tetraploids (4x = 36) with an estimated genome size of 1600 Mbp (Lu et al. 1998; Saski et al. 2011). Owing to the complex polyploidy and large genome size of tetraploid switchgrass varieties, these plants are not amenable to molecular genetic studies. To overcome this, research has focused on the alternative switchgrass Panicum hallii. This species has a small and simple diploid genome of ~500 Mbp and is a model plant for genomic and transcriptomic analyses (http://openwetware.org/wiki/Texas_Switchgrass_Collaborative). In addition to this, several resources have been developed to facilitate genomic and functional genomic studies in biofuel crops. A large number (~11.5 million) of expressed sequence tags have been isolated to map and identify genes using RNA isolated from switchgrass grown under optimal and stressed conditions. Also, several cDNA libraries are available derived from RNA isolated from P. virgatum under optimal and stressed conditions. Lastly, microarray chips to facilitate gene expression analysis of switchgrass have been produced by Affymetrix Inc. (Zhang et al. 2011a). Genetic variation between lowland and upland switchgrass genotypes throughout the USA has been assessed using amplified fragment length polymorphisms (AFLP) and 658 markers (Todd et al. 2011). Morris et al. (2011) also studied genomic differences between switchgrass ecotypes using high-throughput sequencing. As a consequence, the complete chloroplast genome sequence as well as > 10 000 nuclear loci of P. virgatum are now available. The authors furthermore identified 203 variable single-nucleotide polymorphism sites (SNPs), classified into eight haplogroups with 4–27 differentiated SNPs.

To use this vast amount of genomic information now available, genes related to specific traits need to be identified and their exact functions determined. To ascribe functions to genes identified through genomics, researchers use functional genomics approaches, inducing mutations in the genome with various mutagens such as X-rays, ethyl methanesulfate or by insertion mutagenesis. Disrupting a gene by insertion mutagenesis is a direct way to identifying its functions through analysing the resulting mutant phenotype. For this, T-DNA and transposons have been shown to be useful. Large-scale collections of mutants have been generated 

Genomics and functional genomics approaches to enhance plant biomass

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using T-DNA insertional mutagenesis in *A. thaliana* (Krysan et al. 1999) and rice (Jeon et al. 2000). On the other hand, large collections of mutants have been generated using a transposon tagging system for the maize Activator/Dissociation transposon system (Hirochika et al. 2004; Kolesnik et al. 2004; Pernisova et al. 2009). All these resources serve as important tools to help identify and understand gene function. In crop plant species where appropriate tools have yet to be developed, bio-informatic tools can significantly aid in functional annotation of genes. The recently developed ‘Expressolog Tree Viewer’ for seven species (*Arabidopsis*, soybean, *Medicago truncatula*, poplar, barley, maize and rice) (Patel et al. 2012), for example, promises to be a major advance for identifying homologous genes, ranking them by similarity in both sequence and expression profile.

Once a candidate gene has been identified, its targeted manipulation can occur by ectopic expression (tissue specific or constitutive) or by gene suppression. Apart from gene knock-out by T-DNA or transposon insertion, both of which are non-directed, knock-down approaches using RNAi tools provide great accuracy and precision in down-regulating specific genes (Jagtap et al. 2011). Gene suppression by antisense or double-stranded RNA has been used in the genetic manipulation of plants for a long time. More recently, the use of microRNA (miR), either intrinsic or artificial, has emerged as a new tool to down-regulate specific genes. Thus, miR technology is a promising tool for crop improvement (Liu and Chen 2010). One recent example of a successful application of this technology is the overexpression of miR156 from rice in switchgrass (*P. virgatum*), leading to an increase in biomass between 58 and 101%, which is the result of increased tillering (Fu et al. 2012). In addition, solubilized sugar yield was improved in these transgenics, albeit for unidentified reasons. The ectopic expression of a maize miR in poplar (*Populus tremula* × *P. alba*) led to similar results; transgenics exhibited a significant increase in branches, shorter internodes and a reduced lignin content in the stem (Rubinelli et al. 2012). The use of miR is therefore an appealing approach to genetic manipulation of crops.

The molecular strategies discussed above are potentially useful for improving biofuel crops for which molecular data and manipulation tools are yet to be generated. They can serve as starting points for identifying candidate genes, generating mutants and analysing gene function, with the aim of identifying genes whose manipulation would enhance plant biomass and/or other important traits.

Genetic manipulation of plants further requires established tissue culture and genetic transformation systems. This is routinely carried out using *Agrobacterium*-mediated transformation, a system that has also been established in biofuel crops. In 2004, successful transformation of *Miscanthus* was reported (Zili et al. 2004), and efforts to culture and genetically manipulate switchgrass tissues have been described (Somleva et al. 2002; Xi et al. 2009; Chen et al. 2010; Fu et al. 2011; Li and Qu 2011). Tissue culture micropropagation methods for switchgrass now allow efficient establishment of embryogenic callus as well as plant regeneration (Burris et al. 2009). Recently, a simplified method of generating transgenic switchgrass has been established in our laboratory (Ramamoorthy and Kumar 2012). Using these methods, several examples of genetic modifications to switchgrass have been reported. A recent report shows that suppression of the lignin biosynthetic gene *caffeic acid 3-O-methyltransferase* (*COMT*) in *P. virgatum* reduces lignin content slightly without altering overall plant phenotype (Fu et al. 2011). This reduced lignin content in turn increased bioethanol yield from 30 to 38% using conventional fungal fermentation processes. Another gene, *PvMYB4*, encoding an R2R3-MYB transcription factor, has been cloned and functionally characterized. Ectopic expression of *PvMYB4* in switchgrass reduces lignin content and increases sugar release from cell wall residues (Shen et al. 2012). In addition, the recent cloning and characterization of two polyubiquitin promoters (*PvUbi1* and *PvUbi2*) will be useful to drive desired candidate gene constitutive expression in switchgrass (Mann et al. 2011). These examples clearly indicate that with sufficiently intensive research, more genes will be identified whose manipulation will enhance biomass and ethanol yield.

To identify more candidate genes that may regulate plant biomass, it will be essential to understand the molecular mechanisms underlying plant growth and development. In the following sections, we focus on genes that influence plant architecture, since the architecture of a crop plant can have a substantial influence on achievable yield. An enhanced branching phenotype with an increased biomass, for example, would be highly desirable for biofuel crops. The architecture of a plant is determined by its meristems, the shoot apical meristem (SAM), axillary buds and the root apical meristem (RAM). They give rise to all organs and tissues, and therefore establish both number and position of branches, leaves and flowers of the mature plant. Apart from the influence on plant architecture of external factors (e.g. light, temperature, humidity, nutrients, plant density, etc.), a plant’s genome is the main determinant of its architecture. Phytohormones including auxins, gibberellins, cytokinins, ethylene, abscisic acid, brassinosteroids, salicylic acid, jasmonates and strigolactones are key players in the regulation of growth and
developmental responses. Thus, phytohormones are key determinants of plant architecture, and manipulating plant architecture has the potential to increase biomass in biofuel crops (Jakob et al. 2009). We will review in brief the roles of gibberellins and cytokinins in modifying different aspects of plant architecture to indicate potential points of manipulation in their biosynthesis or signalling pathways likely to enhance plant biomass production.

Gibberellins and plant height

Gibberellins play important roles in the regulation of numerous aspects of plant growth and development, such as seed germination, leaf expansion, induction of flowering, and flower and seed development (Sun and Gubler 1999). Gibberellins and plant height are regarded as the main factors influencing plant height. Changing plant architecture by merely manipulating the height can have indirect but significant effects on crop yield. The best-known example of this are the mutant wheat and rice plants of the Green Revolution (Peng et al. 1999). Those semi-dwarf varieties are now known to carry mutations in gibberellin signalling intermediates, such as wheat Reduced height1 (Rht1) (Peng et al. 1999) or gibberellin biosynthesis genes, such as rice semi-dwarf1 (sd1) (Sasaki et al. 2002). Their decreased height renders them resistant to lodging, thus leading to a substantial increase in world wheat and rice grain production. The manipulation of gibberellin metabolism may thus be an effective strategy for generating high-yielding semi-dwarf crop plants of various species (Sakamoto 2006). Another successful example of the generation of a semi-dwarf transgenic rice plant comes from Sakamoto et al. (2003). The authors reported transgenic plants that over-express the gibberellin catabolic enzyme OsGA2ox1 in a tissuespecific manner. Using the promoter of a gibberellin biosynthetic gene, OsGA3ox2 (D18) (Itoh et al. 2001), the authors were able to limit its expression to vegetative tissues. This example also demonstrates the importance for agricultural applications of a detailed knowledge of the molecular basis of phytohormone action, since a tissue-independent, constitutive overexpression of the gibberellic acid (GA) catabolic enzyme GA2ox in rice, wheat or Arabidopsis leads to a severe dwarfism with strong defects in flower and grain development (Hedden and Phillips 2000; Sakamoto et al. 2001).

External factors such as light and temperature as well as internal factors like auxins regulate the actual level of active gibberellins in a plant (Ross et al. 2000; Yamaguchi and Kamiya 2000; Garcia-Martinez and Gil 2001; Frigerio et al. 2006). Upon biosynthesis, gibberellins are perceived by their soluble receptor GID1 (Ueguchi-Tanaka et al. 2005; Hartweck and Olszewski 2006), of which there are three isoforms in Arabidopsis: GID1a, GID1b and GID1c (Nakajima et al. 2006; Ueguchi-Tanaka et al. 2007). Within the gibberellin signalling pathway, DELLA proteins are the key negative regulators of gibberellin responses (reviewed in Hartweck 2008; Schwechheimer and Willige 2009). Arabidopsis contains five genes coding for DELLA proteins with both overlapping and distinct functions, while rice, wheat and barley contain only one DELLA protein (Peng et al. 1999; Cheng et al. 2004; Cao et al. 2005). DELLA proteins repress elongation growth mostly by sequestering transcription factors, thus inhibiting transcription (Davière et al. 2008; de Lucas et al. 2008). This repression is relieved by the GA-mediated degradation of DELLLAs via the 26S proteasome pathway (Dill et al. 2004; Sun and Gubler 2004). Upon recognition and binding of GA, the receptor GID1 changes its conformation, which leads to the binding of DELLA proteins (Murase et al. 2008; Shimada et al. 2008). This in turn recruits the SCF33 E3 ubiquitin ligase complex, resulting in the degradation of DELLA proteins (Achard and Genschik 2008). Recent findings have expanded the DELLA-repression model. Hou et al. (2010) were able to show that DELLA proteins directly interact with JAZ proteins, major repressors of jasmonic acid signalling. JAZ proteins sequester MYC2, a transcriptional activator of jasmonic acid responses; thus, by being bound to DELLA proteins, MYC2-mediated transcription can occur. Therefore, the model of DELLA function now incorporates both transcriptional repressions by sequestration of transcription factors, as well as transcriptional activation by sequestering transcriptional repressors (Gao et al. 2011). This is in line with studies to identify direct target genes of DELLA proteins. Both DELLA-repressed and DELLA-induced genes have been detected (Zentella et al. 2007; Hou et al. 2008).

DELLA proteins have been proposed to be favourable targets for crop improvement on several occasions (Peng et al. 1999; Ikeda et al. 2001). Apart from being single dominant dwarfing genes whose genetic manipulation can generate semi-dwarf transgenics (Sakamoto 2006), DELLLAs have also been shown to act as key integrators of several environmental as well as endogenous cues (e.g. Achard et al. 2003, 2006; Fu and Harberd 2003; de Lucas et al. 2008; Feng et al. 2008). Signalling pathways of several other phytohormones appear to integrate into appropriate responses at the level of DELLA proteins (Fig. 1). Auxins were shown to be required for the gibberellin-mediated degradation of DELLLAs in roots (Fu and Harberd 2003), whereas both ethylene
and abscisic acid repress root growth by stabilizing DELLA proteins (Achard et al. 2003, 2006). In the SAM, on the other hand, cytokinins appear to induce the expression of DELLA genes to achieve the required low levels of gibberellin signalling or responses (Brenner et al. 2005). Furthermore, DELLAs directly integrate the response to light quality by physically interacting with the light-responsive transcription factors PHYTOCHROME INTERACTING FACTOR3 (PIF3) and PIF4 (de Lucas et al. 2008; Feng et al. 2008). This interaction sequesters PIF3 and PIF4 in inactive complexes, thus inhibiting transcriptional activation of downstream targets. DELLA stability, on the other hand, is regulated by gibberellins, auxins, ethylene and abscisic acid (Achard et al. 2003, 2006; Fu and Harberd 2003). It has therefore been hypothesized that DELLAs function as main regulators of light-regulated gene expression since they integrate both hormonal and light-mediated growth regulation (Stamm and Kumar 2010).

Taken together, DELLA proteins probably play key roles in the complex network that integrates environmental cues and phytohormone signalling responses. Their manipulation in crop plants is likely to have a wide range of effects ranging far beyond the mere reduction in plant height. For example, DELLAs were shown to promote the survival of plants under salt stress by increasing reactive oxygen species-detoxifying enzymes (Achard et al. 2008). Other studies show that DELLAs contribute to freezing tolerance (Achard et al. 2008), and also mediate plant growth arrest induced by the flagellar peptide flg22 (Navarro et al. 2008).

With respect to plant architecture, stabilized DELLAs will only lead to dwarf or semi-dwarf plants. However, this small change in architecture comes with a whole range of possible benefits. Apart from the resistance to lodging, plants with stabilized DELLA proteins also exhibit increased resistance to a variety of abiotic as well as biotic stresses. This in turn can increase farmers’ yield by significantly reducing losses both from lodging and from a variety of stresses. Interestingly, a recent study in poplar (Populus sp.) showed that manipulation of gibberellin metabolism and signalling can indeed have effects on plant architecture in addition to reduced height (Zawaski et al. 2011). The authors showed that plants overexpressing PcGA2ox, coding for a pea gibberellin catabolic enzyme, exhibit a reduced apical dominance, resulting in short trees with wide crowns. Overexpression of the poplar orthologue, PtGA2ox, on the other hand, showed a much stronger height reduction as well as a reduction in size of all aerial organs. This study reiterates the importance of studying gene functions and molecular mechanisms in various different systems of interest, since the physiology of different crop plants can differ substantially. These differences are not unexpected, since there are five DELLA genes in Arabidopsis, but only one in rice.

Fig. 1 Schematic representation of the effects of gibberellic acid and DELLA proteins on plant height in Arabidopsis and rice. DELLA proteins are key repressors of gibberellin-mediated plant growth. Their destruction is brought about by the gibberellin-mediated recruitment of the SCFG1 complex, and leads to a relief of growth repression. Auxins promote DELLA destruction, and thus stimulate growth, whereas ethylene, abscisic acid and cytokinin stabilize DELLA proteins, making them repressors of growth. Arrows indicate an induction or activation (positive effect); diamonds suggest inhibition or repression (negative effect). This model is based on current knowledge obtained mainly from work on Arabidopsis and rice.
and other monocotyledonous species examined. It is therefore important to determine the function of homologous genes in each crop species. However, knowledge gained from basic research in model species can serve as a basis to verify hypotheses, thus potentially accelerating progress in biofuel crops.

**Cytokinins and meristems**

Cytokinins are another major class of phytohormones with key roles in shoot and root development. They regulate a variety of developmental processes such as cell division, cell differentiation, release of auxiliary bud dormancy and delay of senescence. Most of these functions affect plant architecture either directly or indirectly. Cytokinins are produced by the interplay of synthesizing and degrading enzymes that maintain a dynamic equilibrium between synthesis and catabolism of cytokinins. The rate-limiting step of cytokinin biosynthesis is catalysed by ISOPENTENYL TRANSFERASEs (IPTs) (Kakimoto 2001; Takei 2001). Recently, a LONELY GUY (LOG) gene has been identified in rice, which encodes a cytokinin-activating enzyme involved in converting inactive cytokinin nucleotides to biologically active free-base forms (Kurakawa et al. 2007). Homologues of rice LOG have also been identified in Arabidopsis (AtLOG), participating in a direct pathway for regulating cytokinin activity (Kuroha et al. 2009). Cytokinin degradation is catalysed by the CYTOKININ OXIDASE/DEHYDROGENASE (CKX) family of enzymes. In Arabidopsis, CKX2, CKX4 and CKX6 have shown significantly high activity with different types of cytokinins (Galuszka et al. 2007).

The cytokinin signalling cascade is derived from the two-component signalling system found in bacteria and yeast. In Arabidopsis, cytokinin receptors belong to the Arabidopsis Histidine Kinase (AHK) gene family consisting of AHK2, AHK3 and AHK4/CYTOKININ RESPONSE 1 (CRE1)/WOODEN LEG 1 (WOL1) (Nishimura 2004). Arabidopsis histidine kinases autophosphorylate upon perceiving the cytokinin signal and the phosphoryl group is subsequently transferred to ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEINs (AHPs). Arabidopsis contains six AHP proteins of which AHP1 to AHP5 function as positive regulators of cytokinin signalling (Hutchison et al. 2006). AHP6, on the other hand, is a pseudo-AHP that lacks the conserved histidine residue (Mähönen 2006). Hence, it negatively regulates cytokinin signalling by interfering with the phosphotransfer. Phosphorylated AHPs translocate to the nucleus and facilitate phosphorylation of the protein, severely affects SAM functions resulting in a nearly complete blockage of organ formation, similar to the wus mutant. This clearly indicates that repression of genes involved in the negative regulation of cytokinin signalling is essential for normal development of the SAM. Taken together, the above-mentioned studies provide compelling evidence that cytokinins are indispensable in the shoot meristem region (Fig. 2A). However, cytokinin function is not limited to SAM maintenance; it plays an equally important role in the development of lateral organs.

The degree and pattern of shoot lateral organs, mainly leaves and branches, are another key determinant of...
plant architecture. They originate from the axillary meristem (AM) of shoots, and cytokinins are known to promote their outgrowth. Axillary meristems are usually dormant due to inhibitory effects of auxins produced in the primary shoot apex, a phenomenon known as apical dominance (Booker 2003). Additional experiments have elucidated that the underlying mechanism involves repression of local biosynthesis of cytokinins in the nodes, limiting the amount of cytokinins in the AMs (Tanaka et al. 2006). Cytokinins, on the other hand, oppose auxin action, and thus appear to break axillary bud dormancy and promote their outgrowth. This role in controlling axillary bud growth was further shown in the bushy and dwarf 2 (bud2) mutant in Arabidopsis (Cui et al. 2010); mutant plants exhibit a bushy phenotype caused by an increased axillary bud outgrowth, and showed elevated levels of endogenous cytokinins. Interestingly, bud2 plants were shown to be affected both in cytokinin homeostasis and sensitivities to auxins and cytokinins. In rice, manipulation of cytokinin levels was shown to affect panicle patterning as well.

Rice log mutants show premature termination of the inflorescence and panicle branch meristem, leading to panicle size reduction, abnormal branching patterns and decreased floral organs (Kurakawa et al. 2007). Similarly, an increase in expression levels of OsCKX2 in the vascular system of developing culms lowers cytokinin levels in the inflorescence meristems, leading to a reduction in reproductive organs, and thus fewer grains (Ashikari 2005). In contrast, suppression of OsCKX2 increases cytokinin levels in inflorescence meristems and increases the number of reproductive organs, resulting in a significantly higher grain yield. This demonstrates an important, direct role for cytokinins in yield enhancement. Another example for this function was reported recently in barley, where the silencing of barley HvCKX1 led to a significantly higher grain yield (Zalewski et al. 2010). Reports have also shown that cytokinins play a role in phyllotaxy, the geometric arrangement of leaves and flowers. A maize cytokinin-inducible gene ABPH1 encoding a type-A response regulator was shown to negatively regulate SAM expansion, thus
controlling leaf patterning by limiting the space for primordium initiation (Giulini et al. 2004). Thus, cytokinins appear to be intricately involved in maintenance of plant architecture, controlling growth and development of apical and lateral organs at various levels (Fig. 2B).

Root systems may not affect plant architecture directly, yet their influence on overall plant architecture cannot be underestimated. In contrast to the positive regulation of SAM, RAM is negatively controlled by cytokinins. Exogenous application of cytokinins, or ectopic expression of a bacterial IPT gene, reduces root growth and meristem size (Kuderova et al. 2008), whereas over-expression of CKX resulted in a larger RAM and rapid growth of roots due to reduced levels of cytokinins (Werner 2003). Cytokinins induce cell differentiation at the transition zone, a process that is mediated by the receptor AHK3 and type-B response regulators ARR1 and ARR12 (Dello Ioio et al. 2007). Consequently, ahk2 ahk3 receptor double mutants and type-B arr mutants show enhanced primary root formation (Mason 2005; Riefler 2006). Apart from being regulated by cytokinins, the RAM is also under tight control of the antagonistic interaction between cytokinins and auxins. SHY2/IAA3, a member of the Aux/IAA gene family, is responsible for maintaining root meristem size, and is under the control of ARR1 (Taniguchi et al. 2006). It encodes an auxin-response inhibitor, hence gain-of-function mutants show reduced meristem size, while loss-of-function mutants exhibit larger RAMs. Other auxin-related regulators of RAM size are PINFORMED (PIN) genes, encoding polar auxin transporters. PIN proteins regulate the direction of auxin movement in the plant by localizing asymmetrically in plant cells. This localization is itself controlled by the serine–threonine kinase PINOID (PID). Normal expression of PID in shoots locates PIN1 on the apical membrane of cells, whereas in roots PIN1, PIN2 and PIN4 are located on the basal membrane of cells, as PID is not normally expressed here (Friml 2004). Thus, it is evident that PID and PIN are major determinants of auxin transport, which in turn controls root meristem growth. Cytokinins, on the other hand, can control root meristem size by regulating the expression of multiple PIN genes, thus affecting polar auxin transport (Chen et al. 1998; Pernisova et al. 2009; Ruzicka et al. 2009). A recent report showing the role of type-A ARRs in regulating RAM function by increasing PIN protein levels further validates these previous studies (Zhang et al. 2011b) (Fig. 2C).

Conclusions and forward look

From the foregoing, it is clear that phytohormones have a significant influence on plant architecture. Hence, manipulation of their biosynthesis or signalling intermediates will offer valuable points of control for altering crop biomass and architecture to our advantage. Furthermore, hormonal control of plant growth is achieved by means of synergistic effects of several phytohormones, rather than the result of independent regulation of growth aspects by individual hormones. Signalling pathways of various hormones are intricately interlinked, as was shown on several occasions for gibberellins and cytokinins (Swain et al. 2001; Greenboim-Wainberg et al. 2005). This further re-emphasizes the importance of basic research to gain complete understanding of signalling pathways and their interactions, such that undesirable side-effects of the manipulation of single genes can be avoided. At the same time, such information gained from model plants helps one to identify corresponding pathways and intermediates that can be candidate genes for precise manipulation of crop plant growth.

It is clear that evolution has imposed the optimum biomass gain over reproductive growth to succeed in the natural habitats of various species. However, under intensive agricultural practices currently in place, environmental constraints imposed by natural selection no longer need to define the final architecture or biomass gain. These factors can be adjusted to suit the requirements of the different uses of the cultivated species, which was the basis of the hybrid crop plants of the Green Revolution.

The burning of fossil fuels is known to generate around 21.3 billion tons (21.3 gigatons) of CO2 per year, but nature can only absorb about half of that amount. Thus, use of fossil fuels causes a net increase of ~10 billion tons of atmospheric CO2 per year (http://www.iea.org/textbase/nppdf/free/2009/key_stats_2009.pdf). Therefore, if we can replace even 10 % of world petroleum use by bioethanol it can significantly reduce the net increase in atmospheric CO2. Based on these statistics, it is clear that the sceptic viewpoint of biofuels not making a significant dent in reducing the annual petroleum energy demands is adequately addressed.

According to a US Department of Agriculture report, biomass available in the USA for biofuel generation would reach about one billion tons by the year 2030, with ~50 % of this coming from annual crops. The three major annual crops of the USA are corn, soybean and wheat (Bomford 2009). These crops have shown remarkable increases in yield historically—corn yield doubled from 1950 to 1977, and almost doubled again by 2005 (Bomford 2009). This was in part due to the intensive research into crop breeding (harnessing heterosis and production of hybrid seeds) and adoption of modern agricultural practices (use of synthetic fertilizers, agrochemicals, irrigation, etc.). Similarly, soybean and
wheat yields doubled between 1950 and 2005, all of which are contributors of the Green Revolution. These spectacular levels of increase in yields are levelling off and may result in the failure to achieve the assumed 50% increase in grain yields by 2030 (Bomford 2009). Some of the factors contributing to this include the lack of sustained investment in agricultural research in the last decade. Several other environmental factors such as the anticipated depletion of water resources and soil fertility, as well as reduced access to fertilizers, will probably exacerbate the failure to meet the targeted crop yield increase. According to one projection based on historical yield data from 1950 to 2005, the yield increase may amount to ~30% each for corn and soybean, but the increase in wheat yield may only be ~2% by 2030 compared with the 2005 yield, which is far short of the target (Bomford 2009). The yield of hay from perennial crops including switchgrass and Miscanthus is the other major contributor for the billion ton biomass projected for 2030. However, the projection of hay yield by Bomford (2009) is one of a decline by ~22% with the current declining trend, owing to fertilizers becoming more expensive. Such a gloomy forecast highlights the urgent need to increase the investment in agricultural research immediately. If the major nations of the world, such as the G20 group of countries, implement such policies and proactively provide the financial backing, the targeted 50% increase in crop yield may well be achievable. This is illustrated by the fact that relatively short-term research on suppression of a single gene in the lignin biosynthetic pathway could result in up to 38% higher ethanol yield from switchgrass (Fu et al. 2011). The lack of intensive improvement efforts (genetic or otherwise) in some of these lesser used crop species means that concerted research efforts with judicious use of modern technologies could lead to the much needed spectacular improvements in yield. We should have an open mind on the use of modern molecular tools and biotechnology to achieve the desired yield increase. Altering plant architecture appears to be a promising strategy to accomplish higher biomass yield (Jakob et al. 2009). In the case of biofuel crops, vegetative biomass is the main source of bioenergy. Thus, increasing biomass by enhancing secondary branching or tillering will probably result in the desired increase in biomass per unit area.

Adapting the knowledge gained from model plant species such as A. thaliana or rice (O. sativa) will greatly accelerate research into biofuel crops. Further information from the Brachypodium genome sequence and the diploid switchgrass P. hallii will facilitate identification and characterization of candidate genes in other grass species like Miscanthus and switchgrass. Tools and techniques will have to be developed for most of the biofuel crop species; however, currently available methods can serve as starting points, and can be adapted or modified to suit specific needs. We hope that our discussion helps to point out some of the possible areas for research focus towards achieving this common goal.

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**Contributions by the authors**

P.S. and V.V. contributed equally to this work.

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**Conflict of interest statement**

None declared.

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