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REVIEW PAPER

Cytokinin: a key driver of seed yield

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Abstract

The cytokinins have been implicated in many facets of plant growth and development including cell division and differentiation, shoot and root growth, apical dominance, senescence, fruit and seed development, and the response to biotic and abiotic stressors. Cytokinin levels are regulated by a balance between biosynthesis [isopentenyl transferase (IPT)], activation [Lonely Guy (LOG)], inactivation (*O*-glucosyl transferase), re-activation (β-glucosidase), and degradation [cytokinin oxidase/dehydrogenase (CKX)]. During senescence, the levels of active cytokinins decrease, with premature senescence leading to a decrease in yield. During the early stages of fruit and seed development, cytokinin levels are transiently elevated, and coincide with nuclear and cell divisions which are a determinant of final seed size. Exogenous application of cytokinin, ectopic expression of *IPT*, or down-regulation of *CKX* have, on occasions, led to increased seed yield, leading to the suggestion that cytokinin may be limiting yield. However, manipulation of cytokinins is complex, not only because of their pleiotropic nature but also because the genes coding for biosynthesis and metabolism belong to multigene families, the members of which are themselves spatially and temporally differentiated. Previous research on yield of rice showed that plant breeders could directly target the cytokinins. Modern genome editing tools could be employed to target and manipulate cytokinin levels to increase seed yield with the concurrent aim of maintaining quality. However, how the cytokinin level is modified and whether *IPT* or *CKX* is targeted may depend on whether the plant is considered to be in a source-limiting environment or to be sink limited.

Key words: Cell division, CKX, CRISPR, cytokinin oxidase/dehydrogenase, isopentenyl transferase, IPT, senescence, sequence-specific nucleases, sink, source.

Introduction

A major consequence of an increasing global population is the requirement to provide food, fibre, and fuel in ever-increasing amounts while recognizing the need for sustainable production methods operating within a changing global environment. The seed production industry is of fundamental importance to this provision as it provides the starting material (the seed) for feed, fibre, and fuel. However, it has been recognized that there is often a conflict between the production of the seed and the final end-product. Optimizing seed yield must be done against a background of maintaining quality of the food (cereal, vegetable, forage), fibre, or fuel. The cytokinins have been implicated in seed development since the high levels in developing seeds facilitated the identification of the first naturally occurring cytokinin, zeatin, from *Zea mays* (Letham, 1963*a*). Four decades later, the seminal work by Ashikari *et al.* (2005) on yield of rice showed that plant breeders could directly target the cytokinins. This review provides a historical basis to our current knowledge of the cytokinins and seed yield, and an introduction to the techniques that are being, or could be, used to manipulate

cytokinins towards a sustainable future. Also highlighted is the choice of whether to enhance cytokinin biosynthesis via isopentenyl transferase (IPT) or decrease cytokinin degradation via cytokinin oxidase/dehydrogenase (CKX), as these alternative strategies may depend on whether the plant is perceived to be in a source-limiting situation or sink limited, respectively.

A brief overview of cytokinin biology

Cytokinins are key hormones that regulate many developmental and physiological processes in plants. They play a crucial role in regulating the proliferation and differentiation of plant cells, and also the control of various processes in plant growth and development, including promotion of shoot growth, inhibition of root development, fruit and seed development, delay of senescence, the transduction of nutritional signals, as well as a role in response to both abiotic and biotic stress. Cytokinin homeostasis is maintained through biosynthesis, activation, degradation, and conjugation of the bioactive molecules. Cytokinins exist as nucleotide, nucleoside, and nucleobase forms. The nucleobase forms [*trans*-, *cis*-, and dihydro-zeatin (Z)], and isopentenyl adenine (iP) are considered to be the active forms that bind to cytokinin receptors. Transport of Z-type cytokinins via the transpiration stream and iP types via the phloem is considered to provide root-to-shoot-to-root communication. At the molecular level, cytokinin homeostasis is maintained by enzymes for cytokinin biosynthesis (IPT), activation [Lonely Guy (LOG)], degradation (CKX), reversible inactivation through conjugation by zeatin *O*-glucosyl transferases (ZOG), reactivation by β-glucosidases (GLUs), and irreversible *N*-glycosylation by UDP glycosyltransferases (UGTs). These genes exist in multigene families, and the individual gene family members are spatially and temporally differentially expressed.

The cytokinins are grouped into three categories: the isoprenoid cytokinins, exemplified by Z and iP, are the most abundant type; the naturally occurring adenine derivatives with aromatic substituents, the topolins, are considered less abundant; and the highly active, synthetic diphenylureas, such as CPPU (4-chlorfenuron), are the third category. For a more extensive but general overview of the cytokinins, see Jameson (2016). For more detailed reviews, refer to Zwack and Rashotte (2015), Schaller *et al.* (2014), Spichal *et al.* (2012), Kudo *et al.* (2010), and Werner and Schmülling (2009).

Seed development

Seed development begins with double fertilization, which leads to the development of the embryo and endosperm. However, the progress of development differs between monocots and dicots: in monocots the endosperm constitutes the major part of the mature seed, whereas in many eudicots the endosperm grows rapidly initially but is eventually consumed by the cotyledons of the developing embryo, which occupies most of the mature seed (Sundaresan, 2005). The seed coat arises from maternal integument tissue, whereas both the triploid endosperm and diploid embryo are filial tissues. In both cases, the number of cells determined during the phase of free nuclear divisions in the developing syncytial endosperm is a determinant of final seed size, as the size of the seed is primarily associated with the initial growth of the endosperm, and not with the later growth of the embryo (Sundaresan, 2005; Mizutami *et al.*, 2010).

Seed development is often considered to occur in two distinct phases: the first, during which endosperm development, cell divisions, and embryo and cotyledon differentiation occurs, is referred to as the morphogenesis phase. The second phase, referred to as maturation, includes embryo growth by expansion, the absorption of the endosperm by the embryo, and dry matter accumulation (Locascio *et al.*, 2014). In eudicots, the number of fruits and the number and size of seeds within the fruits may determine yield. In monocots, the number of spikelets and fertile florets within spikelets and final caryopsis (grain/seed) size determine yield.

Cytokinins and seed development

Endogenous cytokinin changes correlate with the phase of nuclear and cell divisions and establishment of sink size

Following the identification of zeatin by Stuart Letham in *Zea mays* (Letham, 1963*a*), a tight positive correlation between cytokinin levels and the phase of cell division has been shown in developing fruits (e.g. Letham, 1963*b*; Letham and Williams, 1969; Bohner and Bangerth, 1988; Lewis *et al.*, 1996*a*), and seeds. Cereals (maize, wheat, rice, and barley) have sharp, transiently elevated cytokinin levels immediately after anthesis (Morris *et al.*, 1993). In wheat, for example, the sharp increase in endogenous cytokinins (Jameson *et al.*, 1982) occurs during the phase of rapid endosperm nuclear and cell division in the developing grain (Bennett *et al.*, 1973), a phase critical in establishing final sink size. Similar conclusions were reported for maize (Dietrich *et al.*, 1995; Brugière *et al.*, 2008) and rice (Yang *et al.*, 2003). In legumes, detailed GC-MS work on developing white lupin seed showed a high, transient peak of cytokinin in the liquid endosperm of developing seeds (Emery *et al.*, 2000), confirming early bioassay data of Davey and van Staden (1979). However, the cytokinins may be involved in sink activity in addition to, and independent of, cell division (Emery *et al.*, 2000; Brugière *et al.*, 2008; Hwang *et al.*, 2012).

Cytokinin applied to detached leaves not only delayed senescence but attracted radiolabelled amino acids to the site of cytokinin application (Mothes and Engelbrecht, 1963). A close link between cytokinin, cell wall invertase (CWINV), and sink activity was initially established in cell culture (Ehneß and Roitsch, 1997), and subsequently shown in senescing tobacco leaves (Balibrea *et al.*, 2004) and drought-stressed tomato leaves (Albacete *et al.*, 2015) from plants ectopically expressing a *CWINV* gene. Cytokinin-mediated senescence delay may be caused by increased sink activity via the direct activation of CWINV activity by cytokinin (Hwang *et al.*, 2012). However, cytokinin is also required for activation of specific cell cycle genes (Schaller *et al.*, 2014),

and this appears to be in concert with CWINV supplying hexose sugars (Riou-Khamlichi *et al.*, 1999, 2000; Wang and Ruan, 2012). It is clear that both cytokinin and CWINV are required for normal seed development (Cheng *et al.*, 1996; Rijavic *et al.*, 2009; Ruan *et al.*, 2010). The transition from cell division and expansion to storage activities in seeds is usually associated with a decrease in invertase expression and activity (Wang and Ruan, 2013), and both *IPT* and *CWINV* expression were recently shown to be restricted to the morphogenesis phase of seed development in forage brassica (*Brassica napus*) (Song *et al.*, 2015).

Origin of the cytokinin in reproductive tissues

Early work from the Letham lab tracked the movement and metabolism of cytokinin supplied to the xylem of lupins and soybeans (Letham, 1994). It was clear from this work that, while adenosine supplied to the xylem reached the developing embryo (Nooden and Letham, 1984), similarly supplied cytokinin did not. While limited quantities reached the pod wall (Jameson *et al.*, 1987) and even the seed coat (Singh *et al.*, 1988), the cytokinin did not cross the apoplastic space between the maternal seed coat and the embryo (Letham, 1994). Emery *et al.* (2000) estimated that the supply of cytokinin via phloem and xylem to developing fruit and seed of white lupin accounted for most of the cytokinin during early pod set but could not account for the bulk of the cytokinin in the seed. Consequently, in contrast to the dependence of the flower/ovule on maternally supplied cytokinin, the developing legume embryo would appear dependent on the filial tissues for cytokinin biosynthesis (Singh *et al.*, 1988; Emery *et al.*, 2000).

Cytokinin is limiting to flower/pod set/seed set

In both lupin and soybean, application of cytokinin prevented flower abortion/pod set and increased yield (e.g. Carlson *et al.*, 1987; Atkins and Pigeaire, 1993; Nagel *et al.*, 2001; Nonokawa *et al.*, 2012, and references therein). However, cytokinin application to legumes was less successful in the field (Dyer *et al.*, 1987; Nagel *et al.*, 2001). To be successful, repeated applications to flower traces as they develop successively in the field may be necessary (Atkins *et al.*, 2011; Nonokawa *et al.*, 2012), but are precluded presumably based on cost and impracticality. To overcome this problem, Atkins *et al.* (2011) coupled an *IPT* gene to a flower-specific promoter. However, the somewhat less than specific activity of this promoter led to enhanced branch formation and a consequent increase in the total number of pods formed (but not increased seed size) in lupin.

Compared with kiwifruit and grapes, which respond by marked increases in size to application of the diphenyl urea cytokinin, CPPU, under field conditions (e.g. Lewis *et al.*, 1996*b*; Ferrara *et al.*, 2014), field application of cytokinins, including CPPU, to cereals has had limited success (Karanov *et al.*, 1992; Hosseini *et al.*, 2008; PEJ, unpublished data) in increasing yield, whereas when Dietrich *et al.* (1995) supplied cytokinin to developing maize via the stem at pollination, kernel number, and in some cases total kernel weight per ear, was increased due to a reduction in apical kernel abortion. Inconsistent responses of cereals in the field may be because of the narrow window of opportunity associated with the limited phase of cell division [Yang *et al.* (2003) showed increased cell division and grain weight of rice following application of kinetin daily for 5 d, starting 2 d post-anthesis, to plants in pots in the field], variable stages of tiller development in the field, activation of cytokinin destruction (see below), or possibly suboptimal versus optimal field conditions. However, taken together, these results indicate that cytokinin is limiting yield in both eudicots and monocots but that field application is unlikely to be commercially viable.

The developing seed is a site of cytokinin biosynthesis

Early research attempting to show that seed tissue could biosynthesize cytokinin from applied substrates was unsuccessful (see Letham, 1994). However, with knowledge that the supply of cytokinin entering seed tissues from maternal sources was limited, and in support of the endogenous measurements, analysis of gene expression shows expression of specific *IPT* gene family members in seed of both monocots and dicots. For example, the rapid increase and almost equally rapid decrease in cytokinin post-anthesis in wheat (Jameson *et al.*, 1982) is mirrored by the expression of specific *TaIPT* gene family members (Song *et al.*, 2012). In maize, *ZmIPT2* expression occurred at the time when cytokinin levels were elevated and cell division was occurring (Brugière *et al.*, 2008).

In *Arabidopsis thaliana*, Miyawaki *et al.* (2004) showed, using *IPT::GUS* reporter gene constructs, that *AtIPT4* and *AtIPT8* are expressed in immature seeds, with the strongest expression located to the chalazal endosperm. Using laser-assisted microdissection of seed 4 d post-pollination, Day *et al.* (2008) showed enrichment in genes associated with the cell cycle and with cytokinin biosynthesis and signal transduction in the syncytial endosperm. Specifically, they showed the expression of *AtIPT8* in the chalazal region of the developing endosperm. Subsequently, Belmonte *et al.* (2013) assessed gene expression in the subregions of Arabidopsis seeds during development and showed strong expression of both *IPT4* and *IPT8* restricted to the chalazal endosperm during the morphogenesis phase of seed development.

Most recently, Song *et al.* (2015) monitored *IPT* and *CKX* gene family members for both cytokinin biosynthesis and degradation in forage brassica, *B. napus* cv. Greenland. They concluded that the source leaves, developing floral buds, and the pods immediately after anthesis were likely to be dependent on cytokinin supplied from maternal sources, whereas *IPT* expression in the elongating silique and developing seeds indicated that these organs were capable of biosynthesizing their own cytokinin.

The problematic pleiotropic nature of the cytokinins

It might seem logical simply to express an *IPT* gene ectopically to enhance the endogenous cytokinin levels within the plant. However, the cytokinins are involved in multiple facets

of plant development in multiple organs, with contrasting effects on shoots and roots (Werner *et al.*, 2003; Brenner and Schmülling, 2012). Constitutive overexpression of *IPT*, or the use of leaky promoters, has led to bushy plants with reduced or inhibited root growth (e.g. McKenzie *et al.*, 1994, and references therein; McKenzie *et al.*, 1998; Guo and Gan, 2014, and references therein). Hence, a plant-wide increase in cytokinin levels through the use of constitutive promoters and inducible promoters such as heat shock (Medford *et al.*, 1989; Roeckel *et al.*, 1998), light-induced (Beinsberger *et al.*, 1991), or Cu-induced (McKenzie *et al.*, 1998) promoters coupled to an *IPT* gene have had no agronomic application, but have served to emphasize the importance of spatial and temporal manipulation of cytokinin. However, even tissue-specific expression of *IPT* genes may lead to the systemic spread of cytokinins (e.g. McKenzie *et al.*, 1998; Atkins *et al.*, 2011). Importantly, Roeckel *et al.*, (1998) noted that very slightly elevated cytokinin from a leaky heat shock promoter increased the number of seeds per silique and weight of seeds of *B. napus* (Table 1), and it has been shown subsequently that slightly increased cytokinin levels can enhance stress tolerance and impact yield in a wide variety of plants (detailed below).

Cytokinin is limiting to seed development

The elevated levels of cytokinins during fruit and seed development notwithstanding, seed yield responses to seed-specific increases in cytokinin indicate that cytokinin is limiting not only to pod/seed set but also to the developing seed (Table 1). For example, Ma *et al.* (1998) selected a vicilin promoter that specified embryo-specific expression to drive expression of an *IPT* gene. The cytokinin levels were increased only in the developing seeds, and the transgenic tobacco plants were morphologically normal. Both cell division and dry weight of the vicilin-IPT seeds were enhanced (Ma *et al.*, 2002). Subsequently, Ma *et al.* (2008) overexpressed an *IPT* gene fused to the promoter of a soybean seed-specific lectin. The transgenic tobacco showed increased seed weight, and increased carbohydrate and protein content by 7–8%, without any morphological abnormalities.

Daskalova *et al.* (2007) also transformed tobacco but chose a wheat high molecular weight (HMW) glutenin 12 promoter fused to an *IPT* gene. This endosperm-specific promoter was expressed at mid to late stages of seed development. Modest increases in cytokinin were linked to increased storage assimilation. The significant increase in yield was associated with increased seed weight but not seed number. These authors also noted that tobacco seed retains its endosperm to maturity and in this is more similar to cereal than to many other eudicot seeds (Daskalova *et al.*, 2007).

Cis-*cytokinins and embryogenesis*

The biologically active forms of cytokinins, *trans*-Z, dihydroZ, and iP, are derived through the activity of IPT using ATP/ADP as substrate. The fourth biologically active form, *cis*-Z, relates to the activity of a tRNA-IPT which prenylates certain tRNA forms which, upon degradation, release *cis*-Z (Miyawaki *et al.*, 2006). Initially, as it generally has low activity in the standard cytokinin bioassays, *cis*-Z was considered not to be a biologically relevant form of cytokinin (Kamínek *et al.*, 1979). However, maize receptors do recognize this form (Lomin *et al.*, 2015), and certain isoforms of AtCKX exhibit high affinity for the *cis*-Z isomer (Gajdošová *et al.*, 2011). Gajdošová *et al.* (2011) expressly focused on the *cis*-Z types, and found that they occurred ubiquitously in the plant kingdom. *Cis*-cytokinins are found in numerous plants but especially the Poaceae (Gajdošová *et al.*, 2011). They have also been reported in developing seeds of specific

Table 1. *Impact on yield attributes from enhancing cytokinin biosynthesis*

Target gene	Specificity control	Target	Yield component impacted	References
IPT	**Promoter**			
Ectopic expression (target plant)				
Embryo-specific (tobacco)	Pea vicillin	Seed	↑seed weight	Ma *et al.* (1998, 2002)
Seed-specific (tobacco)	Soybean lectin	Seed	↑Seed weight	Ma *et al.* (2008)
Endosperm-specific (tobacco)	Wheat glutenin-12	Mid to late stage seed development	↑Seed weight	Daskalova *et al.* (2007)
Flower-specific (lupin)	Tobacco TP12 (but non-specific)	Flower, but basal axillary branches developed	↑Pod number ↑Yield	Atkins *et al.* (2011)
Constitutive (*B. napus*)	Slightly leaky maize heat-shock (hsp70)	Plant body	↑Seed number ↑Seed weight	Roekel et al. (1998)
Senescence-induced (numerous plants)	P_{SAG12} from Arabidopsis	Senescing leaves	Variable effects on seed yield	Summarized by Guo and Gan (2014)
Stress/maturation	P_{SARK} from bean	Drought-stressed plant	Reduced yield penalty under drought stress	
Tobacco				Rivero *et al.* (2007)
Rice				Peleg *et al.* (2011)
Peanut				Qin *et al.* (2011)
Maturation-induced *B. napus* (Canola)	AtMYB32xs	Plant body excluding roots; ±stress	↑Flowers ↑Siliques ↑Seed yield (t ha^{-1})	Kant *et al.* (2015)

cultivars of chickpea at very high levels (Emery *et al.*, 1998; Lulsdorf *et al.*, 2013), and also in lupin (Emery *et al.*, 2000), lentil, and field pea (Quesnelle and Emery, 2007; Slater *et al.*, 2013). Although they found that the levels of the *cis*-isomers can vary throughout the plant life cycle, Gajdošová *et al.* (2011) noted that these forms are predominantly present in the developmental stages with limited growth, and that they might function to regulate cytokinin responses in plants under growth-limiting conditions. The transition from the accumulation of *cis*-forms to the accumulation of *trans*-Z-forms occurred following early embryo development in barley (Powell *et al.*, 2013). A similar transition occurred in lupin ovaries that were destined to set pods (Emery *et al.*, 2000). Quesnelle and Emery (2007) showed that pea embryos also contained a predominance of *cis*-isomers, and supported a role for *cis*-cytokinins in promoting embryogenesis. It should be noted that there is little current support for the functional activity of a *cis-trans* isomerase (Schäfer *et al.*, 2015, and references therein), with the origin of the *cis*-isomers likely to be tRNA.

All organisms contain *tRNA-IPT* genes, and all higher plants have at least two copies (Spichal *et al.*, 2012; Schäfer *et al.*, 2015). These genes were generally considered to be expressed more or less constitutively during development (Miyawaki *et al.*, 2004). Liu *et al.* (2013) showed low constitutive expression of both *tRNA-IPT* genes in Chinese cabbage (*Brassica rapa*), as did Song *et al.* (2015) for *BnIPT2* in forage rape (*B. napus*). However, Song *et al.* (2015) reported slightly increased expression during early seed development, in agreement with Miyawaki *et al.* (2004) who noted slightly higher expression in proliferating tissue of Arabidopsis. In contrast, the *tRNA-IPT* genes in maize are strongly, constitutively expressed throughout development and in response to stress (Vyroubalová *et al.*, 2009). Expression of genes capable of the *O*-glucosylation of *cis*-Z derivatives was detected both in maize (Vyroubalová *et al.*, 2009) in the spike, and pre- and post-anthesis in developing wheat grains (Song *et al.*, 2012). Consequently, the role of the *cis*-Z-type cytokinins during the early stages of pod set and embryo development warrants further investigation.

Enhancing yield and abiotic stress tolerance by delaying senescence

Historically, yield increases in cereals have been a consequence of increased harvest index (HI), rather than an increase in total biomass. However, the HI of many cereals is considered by some to be reaching a plateau, so further increases in yield may need to come from increases in crop biomass through increases in total net photosynthesis (e.g. Spano *et al.*, 2003). This could be achieved by extending the duration of photosynthesis by delaying the age-related senescence of source leaves by extending the period of carbon capture and delaying nitrogen remobilization—a feature captured in 'stay-green' plants (Thomas and Ougham, 2014). However, there are conflicting reports in the literature where, in some cases, the delayed senescence 'stay-green' plants have increased yield, but in other cases this has retarded the reallocation of assimilates to the seeds and reduced yield (Gepstein and Glick, 2013; Gregersen *et al.*, 2013; Guo and Gan, 2014). In contrast, in rice, rapid senescing cultivars have been shown to have higher yield compared with slower senescing counterparts (Rubia *et al.*, 2014, and references therein).

For many years it has been known that application of cytokinin or ectopic expression of *IPT* in leaves can delay senescence (Guo and Gan, 2014, and references therein). Guo and Gan (2014) summarize a wealth of experiments where an *IPT* gene has been linked to a promoter (P_{SAG12}) that is activated predominantly in the lower leaves as the plant begins to senesce and then is negatively autoregulated. The *SAG-IPT* constructs cause small increases in endogenous cytokinin levels. In the original experiments in tobacco, *SAG12-IPT* plants showed retarded senescence but no other developmental abnormalities. Due to the increased life span, transgenic plants produced 80% more flowers, and seed yield was increased by 40% under controlled conditions (Gan and Amasino, 1995). Guo and Gan (2014) list the numerous species subsequently transformed with *SAG-IPT* constructs, including both monocot and dicot plants. Under field conditions, increases in yield have been recorded for, *inter alia*, *SAG-IPT* rice, *B. napus* lines, and tomato fruit (cited in Guo and Gan, 2014).

However, as mentioned above, delaying senescence as a means to increase the longevity of the source can be problematic as this can establish competition between source and sink. Jordi *et al.* (2000) describe such a situation when *SAG12-IPT* tobacco was grown under N-limiting conditions. Senescence of the older leaves at the base of the plant was clearly delayed. However, N translocation to younger non-senescing leaves was progressively reduced, an effect attributed to the greater sink activity of the lower leaves of the *SAG-IPT* plants (Jordi *et al.*, 2000). This effect was also noted by Robson *et al.* (2004) in maize transformed with a maize senescence-enhanced promoter, P_{SEE}::IPT. The extended greenness was associated with functional C capture, but in some lines there was reduced internal N recycling to younger leaves. While Guo and Gan (2014) suggest that this effect may be ameliorated by addition of N, this is a feature worth noting as moves towards sustainable agriculture target reducing N application. In wheat, the use of a *SAG-IPT* construct clearly delayed senescence but had no impact on yield, which was interpreted as the delayed senescence delaying the translocation of metabolites from leaves to developing grains (Sýkorová *et al.*, 2008). Similarly, in cassava, delayed leaf senescence did not ultimately lead to increase yield of roots (Zhang *et al.*, 2010).

Increasing endogenous cytokinin via enhanced *IPT* expression has been shown convincingly to impact positively on yield, but this is dependent on the specificity of the promoter and the timing in terms of development and environmental conditions when this is activated (Table 1; Fig. 1). For instance, premature plant senescence caused by abiotic stress negatively impacts on crop yield (Gepstein and Glick, 2013), but transgenic *IPT* plants with relatively small to essentially undetectable increases in cytokinin showed tolerance to a variety of abiotic stresses (cited in Guo and Gan, 2014). Rivero *et al.* (2007) showed remarkable drought tolerance

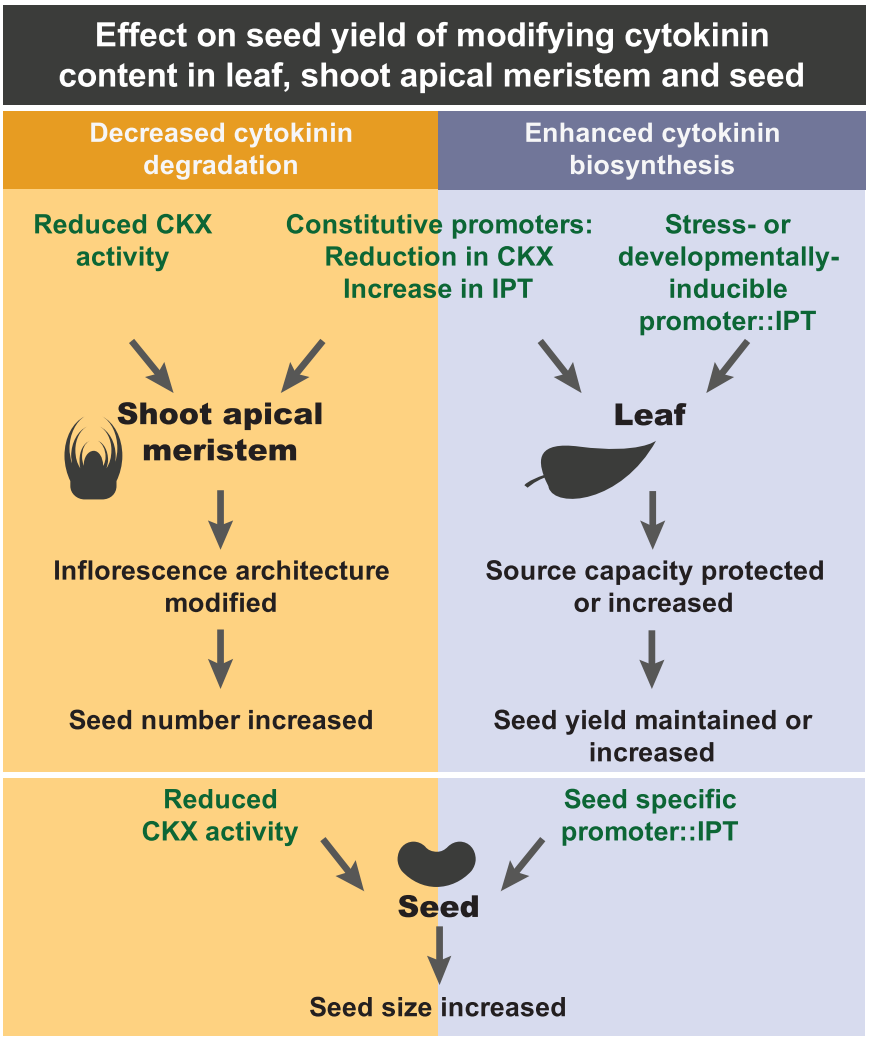

Fig. 1. Effect on seed yield of modifying cytokinin content in leaf, shoot apical meristem, and seed. IPT: isopentenyl transferase is the key cytokinin biosynthetic enzyme. CKX: cytokinin oxidase/dehydrogenase causes cytokinin degradation.

and significantly reduced yield penalty in tobacco transformed with a promoter linked to *IPT* that was both stress and maturation/senescence inducible (Table 1). The promoter derived from a bean leaf senescence-associated gene, the senescence-associated receptor kinase (*SARK*). P_{SARK} was expressed in all leaves during drought, with the additional cytokinin providing protection of photosynthesis during drought stress (Rivero *et al.*, 2009). Similarly, increased yield relative to water-stressed controls was reported for P_{SARK}-*IPT* rice grown under glasshouse conditions, although yield was still less than well-watered plants. The increased yield was due to the enhanced sink capacity in the flag leaf as a consequence of cytokinin maintaining both photosynthetic capacity and nitrate assimilation during stress (Reguera *et al.*, 2013). Under well-watered conditions, there was no difference in yield between the transgenic rice and controls (Peleg *et al.*, 2011). Similar results were reported for field-grown transgenic peanuts (Qin *et al.*, 2011). Additionally, Qin *et al.* (2011) showed that there was no change in the quality of the oil extracted from the transgenic peanuts versus control plants grown in well-watered conditions.

Because P_{SAG} and P_{SARK} are senescence and/or stress induced, they did not provide beneficial effects under well-watered scenarios, a situation outlined above for rice and peanuts. Kant *et al.* (2015) chose to use a promoter from a developmentally regulated transcription factor gene, *AtMYB32*, modified to remove the root expression motif. Under field conditions, the timing of budding, flowering, and physiological maturity of the transgenic canola (*B. napus*) was not significantly different from controls. However, the transgenic lines exhibited delayed senescence and produced significantly greater numbers of flowers and siliques, and an overall increased seed yield (t ha^{-1}) not only under rain-fed (stressed) and but also under irrigated field conditions. Seed quality parameters were either maintained or improved in the transgenics.

Increased cytokinin levels are associated with increased cytokinin degradation

A feature that needs to be considered when targeting an increase in cytokinin is the possibility of increasing cytokinin

degradation, as the plant reacts to the increased cytokinin by activating homeostatic mechanisms. Key to this is CKX which catalyses the irreversible degradation of many cytokinin forms (Werner *et al.*, 2006; Gajdošová *et al.*, 2011). Both *IPT* and *CKX* gene family members are expressed in numerous tissues, as shown by promoter::GUS reporter analysis for *AtIPT* (Miyawaki *et al.*, 2004) and for *AtCKX* (Werner *et al.*, 2003; Galis *et al.*, 2005). There are now numerous reports showing that whenever cytokinin levels are elevated, developmentally or by exogenous application of cytokinin or by ectopic expression of an *IPT* gene, increased expression of *CKX* gene family members and/or CKX activity occurs (e.g. Brugière *et al.*, 2003; Motyka *et al.*, 2003; Liu *et al.*, 2013), with Brugière *et al.* (2003) suggesting that endogenous cytokinin levels 'dictate' *CKX1* expression in maize and, more specifically, that *ZmCKX1* and *ZmIPT2* are the most likely gene family members controlling cytokinin homoeostasis in developing maize kernels (Brugière *et al.*, 2008). Positive correlations have also been observed between expression of *IPT* and *CKX* gene family members in rapid cycling *B. rapa* (O'Keefe *et al.*, 2011), wheat (Song *et al.*, 2012), and forage brassica, *B. napus* (Song *et al.*, 2015).

Directly targeting CKX

The developing seed was not a direct target of the plant breeders whose semi-dwarf varieties led to the Green Revolution crops. Increased yield was a direct consequence of nitrogen fertilizer application and reduced lodging, and an indirect consequence of altered assimilate partitioning from the shortened stem, with an overall increase in the HI. Subsequently, the semi-dwarf character was shown to be due to reduced perception of gibberellin in maize and wheat, and reduced gibberellin biosynthesis in rice in vegetative tissues (Hedden, 2003).

Ashikari *et al.* (2005) showed that cytokinins could be directly targeted by plant breeders. In naturally occurring rice cultivars, a quantitative trait locus (QTL) for increased grain number was identified as coding for *OsCKX2*. Increased cytokinin accumulation in the inflorescence meristem was associated with a yield increase of >20% in the loss-of-function *Osckx2* mutant. Ashikari *et al.* (2005) suggested that the expression of *OsCKX2* in inflorescence meristems regulated the cytokinin level and thus controlled the number of flowers. Additionally, they showed that transgenic rice overexpressing *OsCKX2* had reduced grain numbers, whereas transgenic rice with antisense *OsCKX2* cDNA had reduced expression of endogenous *OsCKX2* and developed more grains (Table 2).

In the model eudicot, overexpression of *AtCKX3* (the Arabidopsis orthologue of rice *OsCKX2*) reduced the number of flowers because of a decreased rate of primordium formation in the floral meristem of Arabidopsis (Werner *et al.*, 2003). Subsequently, Bartrina *et al.* (2011) recorded elevated cytokinin in the inflorescences of a double *Atckx3ckx5* mutant and a 55% increase in yield due to enhanced primordia formation, leading to larger inflorescences and more floral meristems, which in turn led to an increase in flower size, flower number, and silique number. Additionally, an increase in ovule number resulted in increased seed set and seed number per silique. Seed size *per se* was not reported. This work

Table 2. *Effects of increased or decreased CKX expression/activity on yield attributes*

Target gene	Gene family member	Organs impacted	Yield component impacted	References
CKX				
Naturally occurring rice mutants	↓*Osckx2*	Inflorescence meristems	↑Seed number	Ashikari *et al.* (2005)
Antisense	↓*OsCKX2*		↑Seed number	
Ectopic expression	↑*OsCKX2*		↓Seed number	
Naturally occurring wheat variants	↓*TaCKX6*-D1	Seed	↑1000 grain weight	Zhang *et al.* (2012)
Ectopic expression (Arabidopsis)	↑*AtCKX3*	Decreased rate of primordium formation in floral meristems	↓Flower number ↑Seed size	Werner *et al.* (2003)
Induced mutations (Arabidopsis)	↓*Atckx3ckx5*	Inflorescence meristems Ovule formation	↑Flower number ↑Silique number ↑Seed number	Bartrina *et al.* (2011)
Targeted down-regulation using RNAi (barley)	*35S::HvCKX1-RNAi* or *35S::HvCKX9-RNAi*	Plant body	↑Spike number ↑Seed number and/or size	Zalewski *et al.* (2010, 2012, 2014)
Constitutive down-regulation using RNAi (cotton)	*35S::GhCKX-RNAi*; moderate suppression	Delayed leaf senescence, more fruiting branches, greater yield of lint and seed	↑Seed number ↑100 seed weight	Zhao *et al.* (2015)
Mutations in upstream regulators				
Atrock1	↓*AtCKX1*	SAM activity enhanced	↑Flower number ↑Silique number	Niemann *et al.* (2015)
OsDST^{reg1}	↓*OsCKX2*	Inflorescence meristem	↑Grain number ↑1000 grain weight	Li *et al.* (2013b)
OsDST^{REG1}-RNAi	↓*OsCKX2*	More panicle branches	↑Grain number	
Ectopic expression *OsDST*^{REG1}	↑*OsCKX2*	Fewer panicle branches	↓Grain number	
Oslarger panicle	↓*OsCKX2*	More inflorescence branches	↑Grain number ↑1000 grain weight	Li *et al.* (2011)
Unknown regulator (finger millet)	↓*EcCKX1* and *EcCKX2*	Inflorescence meristems	↑Grain number	Radchuk *et al.* (2012)

confirmed the earlier reports that cytokinin was limiting to flower development, pod set, and seed set.

Cytokinin is considered to have a major regulatory role in the shoot apical meristem (SAM) in both monocots and eudicots (Werner and Schmülling, 2009; Han *et al.*, 2014). LOG (the enzyme shown to release active cytokinins directly from cytokinin nucleotides) was shown to be located in the most apical region of the SAM in rice, and reduced meristem activity occurred in *log* mutants (Kurakawa *et al.*, 2007). Subsequently, in eudicots, Kuroha *et al.* (2009) showed strong activity of *AtLOG1* and *AtLOG4* in the SAM of Arabidopsis. Enhancing the level of cytokinin in the SAM through a reduction in CKX activity is expected to lead to change in inflorescence complexity, potentially leading to increased seed number and possibly yield (Han *et al.*, 2014). Increased grain number is a target for plant breeders of both rice (e.g. Li *et al.*, 2013b, and references therein) and wheat (e.g. Gregersen *et al.*, 2013).

The effect of reduced *CKX* expression on seed number or seed weight may depend on whether the SAM or the developing seed is the target (Table 2; Fig. 1). In contrast to rice, variants of *TaCKX6*-D1, a wheat orthologue of *OsCKX2*, were significantly associated with grain weight but not grain number. An inverse correlation was shown between *CKX* expression and 1000 grain weight across seven wheat varieties (Zhang *et al.*, 2012). Ongoing work with barley lines expressing RNAi targeted to *HvCKX1* and *HvCKX9* showed that the more significant effect on yield was when the gene family member showing the greater expression in the developing grain was the target. In hindsight, *HvCKX4* might prove to be an even better target owing to its greater expression in developing grain (Zalewski *et al.*, 2010, 2012, 2014).

In contrast, rather than spatio-temporal down-regulation of specific *CKX* family members, Zhao *et al.* (2015) showed that moderate enhancement of cytokinin throughout the plant body may lead to increased seed yield along with the other positive attributes of cytokinin, including delaying leaf senescence, enhancing photosynthesis, increased branching, and enhanced tolerance of abiotic and biotic stress (Zhao *et al.*, 2015). They used the constitutively expressed 35S *Cauliflower mosaic virus* (CaMV) promoter to drive an RNAi *CKX* construct (*CaMV35S::GhCKXRNAi*) in cotton. Severely down-regulated lines showed the expected morphological abnormalities of cytokinin overproduction, but more moderately down-regulated lines showed normal growth and development, delayed leaf senescence, more fruiting branches and bolls, larger seed size, and, overall, a higher yield of both seed and fibre including in the field. They suggested, in terms of manipulating cytokinin levels, that 'manipulation of CKX is more likely a softer regulator than IPT' (Zhao *et al.*, 2015).

Interestingly, in several examples, the classic inverse correlation between seed number and seed size (Sadras, 2007; Paul-Victor and Turnbull, 2009; Van Daele *et al.*, 2012) has been over-ridden, through enhancing the cytokinin level (Tables 1 and 2). The reduction or breakage of the inverse correlation between seed number and seed size was also reported in model and crop plant species using QTL molecular markers (Alonso-Blanco *et al.*, 1999; Gnan *et al.*, 2014; Griffiths *et al.*, 2015). This makes both the seed size and seed number attractive targets for marker-assisted selection (MAS) to develop high-yielding crop varieties (Griffiths *et al.*, 2015). Moreover, the considerable increase in seed number in the *Atckx3ckx5* mutant supports the notion that strong sink activity influences carbon capture by the source (Bartrina *et al.*, 2011).

Indirectly targeting CKX

Mutations in three recently identified genes each led to decreased expression of *CKX,* and highlight the positive effect of reduced CKX activity on seed yield (Table 2). The *ROCK1* (*REPRESSOR OF CYTOKININ DEFICIENCY*) gene in Arabidopsis is a putative endoplasmic reticulum-located transporter of *N*-glycosylation components (Niemann *et al.*, 2015). Most CKX enzymes require glycosylation (Morris *et al.*, 1999; Schmülling *et al.*, 2003), a role that ROCK1 could be facilitating. Notably the activity of the reproductive meristem was strongly increased in a *rock1* mutant, with increased flower and silique numbers in a similar manner to that seen by Bartrina *et al.* (2011) in the double *ckx3ckx5* mutants, implicating ROCK1 as a positive regulator of CKX proteins in the SAM (Niemann *et al.*, 2015).

The zinc finger transcription factor, DROUGHT AND SALT TOLERANCE (DST) in rice, was shown by Li *et al.* (2013b) to regulate *OsCKX2* directly in the SAM. Using both RNA *in situ* hybridization and quantitative real-time PCR (RT-qPCR), tissue with a mutant form of *DST* (*reg1*) was shown to have reduced expression of *OsCKX2* (as well as other *CKX* gene family members) and increased cytokinin levels relative to the wild type. Additionally, overexpression of *DST* led to plants with reduced stature, fewer panicle branches, and decreased grain number, whereas plants with reduced DST (RNAi targeted to *DST*) had increased panicle branches and enhanced grain number and grain size (Li *et al.*, 2013b). Furthermore, transgenic wheat expressing DST^{REG1} and DST^{reg1} showed decreased ear length and reduced spikelet number, and increased ear length and increased spikelet number, respectively (Li *et al.*, 2013). It is likely that DST-directed expression of *CKX* regulated the level of cytokinin in the SAM and directly controlled the number of reproductive organs and, consequently, grain number (Li *et al.*, 2013b).

Earlier, Li *et al.* (2011) characterized two allelic larger panicle mutants (*lp*), derived through mutagenesis of rice, which had more inflorescence branches and produced more grains, and increased grain yield. LP was shown to be an F-box protein. While *LP-GUS* analysis showed that LP was produced in many tissues, *in situ* hybridization showed *LP* to be highly expressed in the rachis/branch primordial region. *OsCKX2* was shown to be down-regulated in the *lp* mutant, implicating LP in some way, either directly or indirectly, in the expression of *OsCKX2* (Li *et al.*, 2011).

Interestingly, a somaclonal line of finger millet (*Eleusine coracana*) with increased grain yield, resulting from increased numbers of inflorescences, flowers, and seeds relative to the wild type, displayed decreased *EcCKX1* and *EcCKX2* expression in young leaves, seedlings, and initiating inflorescences (Radchuk *et al.*, 2012). An increase in iP was detected during early inflorescence development. As the sequences of the *CKX*

genes were similar to those of the wild type, down-regulation of the *CKX* genes must have occurred at the transcriptional level (Radchuk *et al.*, 2012). This example, and the preceding three examples, all lend support to CKX functioning to limit yield through effects within the SAM (Table 2), and providing targets for breeders interested in increasing seed number.

When compared with the above examples, where decreased CKX in the SAM led to positive effects on yield, the situation seems more complex within the seed. In contrast to those publications showing decreased CKX expression/activity correlating with enhancing seed size (Zhang *et al.*, 2012; Li *et al.*, 2013b; Zhao *et al.*, 2015), transgenic cytokinin-deficient plants in which CKX activity was increased (Werner *et al.*, 2003), and receptor mutant plants that lack perception of cytokinins (Riefler *et al.*, 2006) were both shown to have increased seed size. Triple receptor mutants exhibited very limited growth and developed few seeds, but the volume of these seeds was three times that of the controls, greater than could be accounted for by seed size compensating for reduced seed number (Riefler *et al.*, 2006). Lack of cytokinin perception led to increased endogenous cytokinin in seedlings. Potentially, the same may have occurred in the developing seeds, where any enhanced cytokinin may have interacted directly with enzymes involved in the syncytial nuclear divisions. In the case of the cytokinin-deficient plants, fewer seeds were produced per silique, some aborted, but the few that developed were approximately twice the wild-type weight (Werner *et al.*, 2003). While data from both these publications support a key role for cytokinins in SAM development and seed set, the large seed size could be indicative of delayed cellularization (Garcia *et al.*, 2003) and a loss of tightly regulated control of seed development, as evidenced below from the IKU pathway.

Somewhat unexpectedly, but in alignment with the above two examples, the expression of *AtCKX2* was shown to be strongly down-regulated in *iku1* and *iku2* mutants of Arabidopsis (Li *et al.*, 2013a), both of which have reduced endosperm growth, precocious cellularization, and reduced seed size (Garcia *et al.*, 2003). *AtCKX2* was identified as a transcriptional target of the IKU pathway (Li *et al.*, 2013a). *IKU1* and *IKU2* have both been shown to be regulators of seed size in Arabidopsis (Garcia *et al.*, 2003), operating in a pathway activated by SHORT HYPOCOTYL UNDER BLUE1 (Zhou *et al.*, 2009; Kang *et al.*, 2013) and repressed by ABI5 (Cheng *et al.*, 2014). While *AtIPT4* and *AtIPT8* are expressed in the chalazal region (Belmonte *et al.*, 2013), Li *et al.* (2013a) proposed that the IKU pathway restricted AtCKX2 activity to the micropylar endosperm and limited cytokinin function to the syncytial and chalazal endosperm. Additionally, epigenetic methylation of *AtCKX2* by PRC2 may have inhibited AtCKX2 activation by IKU signalling during early endosperm growth. They concluded that the IKU pathway maintains the gradient of CKX2 whereas PRC2 modulates the level of expression of *CKX2*, describing *AtCKX2* as an integrator of genetic and epigenetic regulation of endosperm growth in Arabidopsis (Li *et al.*, 2013a). Notably, the *iku* pathway can be rescued by cytokinin receptor mutants and by overexpression of *CKX2*, with the seeds significantly larger than those of the *iku* mutant, but not larger than the parental ecotype (Li *et al.*, 2013a). Their model (Li *et al.*, 2013a, Supplementary Fig. S9), shows cytokinin inhibiting endosperm growth. The enhanced seed size of the *35S::CKX* transgenics (Werner *et al.*, 2003) may indicate that the additional *CKX* was not repressed by PRC2.

However, how data that implicate decreased expression/activity of CKX and enhanced IPT expression/activity in enhancing seed size (Tables 1 and 2) can be reconciled with the IKU pathway and the model presented by Li *et al.* (2013a) remains to be determined, although it may be relevant that *AtCKX5* is expressed in the chalazal region (Bartrina *et al.*, 2011). Importantly, for future work a key target should be to extend the duration of syncytial nuclear proliferation and defer cellularization, this being a critical determinant of seed size (Mizutani *et al.*, 2010).

Strategies for enhancing endogenous cytokinin to increase yield

Le *et al.* (2012) suggest that 'Tissue-specific and developmental stage-related expression data are useful in the identification of genes that are involved in defining the precise nature of individual tissues in a given developmental stage'. Selecting target genes for breeding requires knowledge of the expression of individual gene family members in the target species. Of particular note is the fact that a QTL for grain number in rice was identified as *OsCKX2* (Ashikari *et al.*, 2005), whereas the rice orthologue in wheat (*TaCKX6*-D1) was associated with grain size (Zhang *et al.*, 2012). In wheat, there is a complex regulation of cytokinin levels in leaves, spikes, and developing grains with differential input of the various cytokinin gene family members (Song *et al.*, 2012). For example, *TaCKX1* is strongly up-regulated during seed development, but also during leaf senescence, so a targeted down-regulation of *TaCKX1* may also delay leaf senescence, thus setting up a source/sink competition. In contrast, although expressed to a lesser extent, *TaCKX2* and *TaCKX6* show a similar pattern during seed development to *TaCKX1* but are barely expressed during leaf senescence (Song *et al.*, 2012). As mentioned above, *TaCKX6*-D1 (recent phylogenetic alignment is to *TaCKX2* in Song *et al.*, 2012) is significantly associated with grain weight (Zhang *et al.*, 2012).

However, identifying an appropriate target can be problematic as many crop species are allopolyploids, with a concomitant increase in the number of gene family members. For example, *B. napus* not only has the A genome from *B. rapa* and the C genome from *B. oleraceace*, but there has also been a whole-genome triplication of the *Brassica* genomes since divergence from Arabidopsis (Lysak *et al.*, 2005), followed by genome shrinkage (Mun *et al.*, 2009; Wang *et al.*, 2011). In Chinese cabbage (*B. rapa*), genome shrinkage was evident for both *IPT* and *CKX* gene families (Liu *et al.*, 2013), and *IPT4* and *IPT6* were missing altogether from *B. rapa* (Ando *et al.*, 2005; Liu *et al.*, 2013), rapid cycling *B. rapa* (O'Keefe *et al.*, 2011), and from forage brassica *B. napus* (Song *et al.*, 2015). Notable is the fact that *AtIPT4*, along with *AtIPT8*, is

highlighted as a key gene family member in Arabidopsis seed development (Miyawaki *et al.*, 2004; Belmonte *et al.*, 2013). In contrast, in forage brassica, *BnIPT1* and *BnIPT8* are the most highly expressed in developing seeds, providing a salient lesson that individual gene family members should be identified in the target species (Song *et al.*, 2015).

An additional complexity occurs in polyploid crops, as homoeologous gene silencing and/or differential expression of the homoeologues may occur (e.g. Bottley *et al.*, 2006). This has been shown for *IPT* and *CKX* gene family members in tetraploid forage brassica seeds (Song *et al.*, 2015) and for *TacZOG* and *TaGLU* in hexaploid bread wheat grains (Song *et al.*, 2012)

Once a target gene family member has been identified, various plant breeding strategies can be invoked which tend to separate into those not involving genetic modification [e.g. MAS, TILLING (targeting induced local lesions in genomes), and EcoTILLING] and those using various genetic modification techniques such as RNAi and the recent targeted genome editing techniques (Gaj *et al.*, 2013). While EcoTILLING seeks mutations in naturally occurring accessions or cultivars of crop species, TILLING relies on populations developed following standard mutagenesis techniques. Both then rely on a sensitive DNA screening technique that identifies sequence mutations in a target gene. In the case of cytokinins, it is likely that a mutated structural gene such as a *CKX* gene is more likely to be detected than an overexpressed *IPT* gene. Ashikari *et al.* (2005) showed that reduced function and non-functional *CKX* genes exist in cultivars of rice.

The use of RNAi to enhance yield by knocking down specific *CKX* gene family members has been attempted in barley (also described above) but not necessarily with consistent effects over time (Zalewski *et al.*, 2014). In their review, Gaj *et al.* (2013) noted that targeted gene knockdown by RNAi may be incomplete, has variable success, has unpredictable off-target effects, and provides only temporary inhibition of gene function.

The most recent advances utilize sequence-specific nucleases (SSNs)—techniques commonly referred to as 'genome editing' (Gaj *et al.*, 2013). An excellent review by Chen and Gao (2014) summarizes the three approaches that utilize either zinc-finger nucleases (ZFNs), transcription activator-like nucleases (TALENS), or the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas-mediated RNA-guided DNA endonucleases. In contrast to classical genetic engineering, the precise manipulation of genomes by SSN techniques may overcome some of the constraints associated with transgene-based plant breeding (Chen and Gao, 2014). Of the three techniques, the CRISPR/Cas9 technique is rapidly gaining a reputation as being relatively cheap and easy to implement, with relatively low off-target activity according to Belhaj *et al.* (2015). However, although the end-product is precise manipulation of a specific genome sequence, the underlying technology still requires an initial transformation event, and the resulting plant may still be considered to have been genetically modified in some jurisdictions.

Conclusion

Different multigene families, whose individual gene family members provide precise co-ordinated control of organ development, regulate cytokinin homeostasis within an organ. Other plant hormones notwithstanding, seed yield can be directly or indirectly affected by disturbing the co-ordinate regulatory network of cytokinins (Fig. 1), but whether *IPT* or *CKX* is the target may depend on whether the crop is source or sink limited. Under stress conditions, the source may be limiting yield. Under such conditions, disturbance of the regulatory network may be manipulated by the judicious selection of promoters linked to an *IPT* gene, to ameliorate the impacts of stress on yield by modestly enhancing the endogenous cytokinin levels. Under sink-limiting conditions, a more direct approach, focused on *CKX*, can be used to disturb cytokinin homeostasis either in the SAM, with the aim of enhancing seed number, or targeted to the seed itself to manipulate nuclear and cell division to increase seed size. Irrespective of whether the crop is monocot or dicot, reduced expression of *CKX* has led to increased yield. Indeed, Bartrina *et al.* (2011) concluded that 'the role of *CKX* genes in determining yield has been evolutionarily conserved and is of functional significance for all or most flowering plants' (Bartrina *et al.*, 2011).

To achieve fine control, it appears necessary to identify the spatio-temporal expression patterns of *CKX* gene family members expressed in the SAM and/or during seed development. Such information will be useful in the identification of gene-specific, functionally associated markers for MAS or for inducing and/or detecting valuable mutations using a TILLING strategy. Even the CRISPR technique will require such detailed knowledge. However, the techniques are now available to target the down-regulation of specific *CKX* gene family members, with the potential of higher seed yield, although, in parallel and irrespective of technique, quality traits will need to be monitored (Kant *et al.*, 2015; Song *et al.*, 2015).

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