Will cytokinins underpin the second ‘Green Revolution’?

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A substantial body of knowledge relating to plant growth and development has derived from research on model plants—particularly Arabidopsis thaliana (Arabidopsis). It is imperative that this knowledge is used to develop crop plants that are not only higher yielding but also tolerant of environmental stressors, under the joint scenario of limitations of productive land and global climate change. Schwarz et al. (2020) provide a prime example of translational research, where knowledge from earlier Arabidopsis research with the cytokinins conducted in Thomas Schmülling’s lab (Bartrina et al., 2011) has now been directly applied to increasing yield traits in Brassica napus L., oil seed rape.

The gibberellins are the plant hormone that underpin the semi-dwarf cereals of the Green Revolution, with the crop plants showing either reduced response to gibberellin (wheat and maize) or reduced ability to synthesize gibberellin (rice) (Hedden, 2003). However, at the time, selections within these crops were based on high-yielding varieties with a semi-dwarf phenotype which, alongside improved resistance to lodging, had increased seed yield due to a greater proportion of assimilate being reallocated to the grain (Fischer and Stockman, 1986). In other words, seed yield per se was not directly selected for. Not only have the cytokinins been shown to directly increase seed yield (e.g. Ashikari et al., 2005; Bartrina et al., 2011; Schwarz et al., 2020), they are also a significant component of the response of plants to environmental stressors (Cortleven et al., 2019). Because of their recognized effects on increasing seed number and seed size and our ability to moderate internal levels of cytokinins to ameliorate the effects of stress and mineral nutrient deficiencies (reviewed in Chen et al., 2020), the cytokinins may well be the hormone that underpins the second ‘Green Revolution’ (Lynch, 2007). For a brief summary of the cytokinins, please refer to Box 1.

While the cytokinins had, for many years, been implicated in regulating seed yield (reviewed in Jameson and Song, 2016), it was the seminal work of Ashikari et al. (2005) that cemented their importance in crop production. They showed that rice cultivars with mutations in CKX2, which codes for the enzyme that destroys cytokinin, had increased seed number. The work with rice was followed by that of Bartrina et al. (2011) showing that double ckx3,5 mutants of Arabidopsis had increased seed number. Subsequently, numerous approaches have supported a role for CKX in negatively controlling seed number and seed size (reviewed in Chen et al., 2020).

Amelioration of drought stress

One of the most significant challenges when working with the cytokinins is that they have opposite effects on shoot and root growth—promoting shoot growth while inhibiting root growth (Werner et al., 2003)—so that a generic increase in cytokinin levels may well promote shoot growth while inhibiting root growth. Cytokinins also delay senescence, but too much cytokinin may lead to a competitive interaction between source leaves and the sinks (pods and seeds) (e.g. Sýkorová et al., 2008), or even cause excessive branching through the release of apical dominance (McKenzie et al., 1998). Increased cytokinin also leads to an increase in tillering in cereals, which may in fact not be ideal if the tillers mature at different stages (Chen et al., 2020). Significantly, however, modest enhancement of cytokinin levels, through careful selection of senescence-, maturation-, or stress-responsive promoters to drive the biosynthetic isopentenyl transferase (IPT) gene, has been a successful strategy to ameliorate the impacts of drought stress on yield of both dicots and monocots, including under field conditions (reviewed in Jameson and Song, 2016; Joshi et al., 2019).

The alternative approach to manipulating cytokinin levels in planta has been either to increase the expression of CKX in the roots or to decrease the expression of CKX in the shoots. By targeting the expression of specific CKX gene family members...
Box 1. The isoprenoid cytokinins

The naturally occurring cytokinins, which are adenine derivatives, fall into two classes—those with an isoprenoid side chain and those with an aromatic side chain. The isoprenoid cytokinins are ubiquitous in the plant kingdom and are considered to be the predominant cytokinin type. Cytokinin levels within the plant are controlled by biosynthesis, translocation, destruction, and inactivation (Sakakibara, 2006). Additionally, responses to the active forms require functional receptors and signal transduction components. The cytokinins are biosynthesized either directly by ADP/ATP-isopentenyl transferases (IPTs) or indirectly by tRNA-IPTs with subsequent turnover of the isoprenylated tRNA molecules (Miyawaki et al., 2006). The cytokinins exist in free base, riboside, and nucleotide forms. The nucleotides are the first formed cytokinins which can be converted by Lonely Guy (LOG) to the free base forms. The four free base forms—trans-zeatin (tZ), cis-zeatin (cZ), dihydrozeatin (DHZ), and isopentenyl adenine (iP)—are considered the active forms detected by two-component receptors. iP carries an unmodified isopentenyl side chain, DHZ has a saturated side chain, whereas tZ and cZ carry hydroxylated side chains. Cytokinins oxidase/dehydrogenase destroys cytokinin activity by removing the side chain of the tZ-, cZ-, and iP-type cytokinins. Inactivation occurs through O-glucosylation of the side chains of tZ, cZ, and DHZ, and their ribosides and nucleotides. As these forms can be reactivated by β-glucosidases, they are considered to be storage forms. Further inactivation can occur by glucosylation at positions 7 and 9 of the adenine moiety. Whether cytokinin levels are reduced principally through destruction by CKX or inactivation by O-glucosylation or N-glucosylation (or all three) can be species and organ specific (reviewed in Jameson, 2017).

It is the manipulation of the isoprenoid cytokinins that is the focus of much of the recent interest in the cytokinins. However, manipulation of cytokinins can activate strong internal homeostatic mechanisms, making interpretation of cytokinin levels in such plants at any developmental point in time challenging (Gasparis et al., 2019; Schwarz et al., 2020). Most higher plants will have more than two dozen cytokinin forms, many of which are capable of interconversion. There are relatively few laboratories equipped with both the knowledge and instrumentation required for comprehensive cytokinin analyses. There are no shortcuts: measuring and reporting on cytokinins detected in crude or even partially purified extracts by immunoassay, as exemplified in a recently published paper, in which the ‘cytokinin’ detected was not even named (Tsago et al., 2020), is simply unacceptable.

Targeted manipulation of seed yield

As mentioned above, the OsCKX2 mutant of rice (Ashikari et al., 2005), and the double dckx3,5 mutants of Arabidopsis (Bartrina et al., 2011) and oil seed rape (Schwarz et al., 2020), had increased yield traits (see also Chen et al., 2020). In contrast, targeted reduction through using RNAi and, more recently, gene editing, has not always yielded the results that experiments with mutants might have led us to expect. For instance, Gasparis et al. (2019) used gene editing to knock out barley CKX1 or CKX3. The results obtained were not straightforward and indicated that strong cytokinin homeostatic mechanisms came into effect, leading to different morphological and somewhat unanticipated responses for each of the two gene-edited lines. They concluded that the effect of full knockout, compared with RNAi knockdown (e.g. Zalewski et al. 2014), may have led to a stronger homeostatic response. They suggested that knockdown of two CKX gene family members may be required, as was required in both Arabidopsis and oil seed rape, although not in rice (Ashikari et al., 2005).

Combining the old and new: mutagenesis and next-generation sequencing

The controlled expression of IPT and the targeting of CKX via RNAi or gene editing all require transformation and cannot be readily scaled up. Furthermore, transgenic plants require regulatory clearance in many countries, and cannot be commercially grown in some jurisdictions (Fritsche et al., 2018). On the other hand, mutagenesis has been an aid to plant breeding for >80 years and plants derived through mutagenesis are free from regulatory hurdles. Traditionally, mutagenesis breeding has been carried out on a large scale through painstaking phenotypic selection and backcrossing over multiple generations. Now, the combination of TILLING (Targeting Induced Local Lesions in Genomes), which provides a non-transgenic method of inducing point mutations into a genome using ethyl
methanesulfonate (EMS) as the mutagen, with whole-genome or exome sequencing, enables the identification of mutations in multiple lines (Uauy et al., 2017; Chen et al., 2020). Once a specific mutation is detected, it is important to recognize that four to five generations of backcrossing to the parent is still required to provide a resource enriched in that mutation. However, with the use of the ‘speed breeding’ strategy, several generations per year can be accommodated (Ghosh et al., 2018), which is similar to the number of generations that can be achieved with model plants such as Arabidopsis—making working with non-model species that much more accessible.

Based on the previous work in Thomas Schmülling’s lab on Arabidopsis (Bartrina et al., 2011), Schwarz et al. (2020) focused on the CKX gene family members 3 and 5. However, oil seed rape is tetraploid, making it necessary to identify the EMS-induced mutations in each of the four copies of cdx3 and the two copies of cdx5 in the B. napus genome. Four generations of backcrosses were done for each of the six individually identified mutations, before the lines were crossed to achieve the quadruple cdx3 mutant, the double cdx5 mutant, and, finally, the sextuple cdx3,5 mutant which has a combination of all six of the individual loss-of-function alleles. Only following this could Schwarz et al. (2020) characterize the sextuple mutant which they did comprehensively both phenotypically and microscopically, along with its transcriptome and changes in cytokinins. While some differences between the Arabidopsis cdx3,5 and the sextuple cdx3,5 oil seed rape mutants were apparent, the translation of the information from the model plant to the crop plant strongly indicated that CKX has an evolutionarily conserved role in controlling seed yield (Bartrina et al., 2011; Schwarz et al., 2020). Further, they suggest that simultaneous enhancement of source strength (possibly via controlled IPT expression) with the increased sink capacity of the cdx3,5 mutant may provide resources to fill the additional ovules that were formed but did not mature.

**Box 2. Effect on yield traits of modifying cytokinin content in leaf, shoot apical meristem, seed, and root**

![Diagram showing the effect on yield traits of modifying cytokinin content in leaf, shoot apical meristem, seed, and root.]

IPT, isopentenyl transferase; CKX, cytokinin oxidase/dehydrogenase.

Figure adapted from Jameson and Song. 2016.
The challenges that faced Thomas Schmülling’s group with their tretraploid B. napus are exacerbated in crops including bread wheat, which is hexaploid. Here, the cytokinins, and the CKX gene family members in particular, are key targets (Chen et al., 2020; Jabłoński et al., 2020). Utilizing TILLING, speed breeding, and whole-exome sequencing, Song et al. (unpublished data) have identified multiple point mutations for all of the CKX gene family members from the A, B, and D subgenomes in the bread wheat genome (IWGSC RefSeq 2.0). The phenotype and contribution to seed yield of each family member will be evaluated after the triple ABD mutants are stacked and serial backcrossing to the wild type has been undertaken.

With our ability to enhance both source (leaf) and sink (pods and seeds) activities, as well as to ameliorate the impacts of stress (roots, leaves), and enhance nutrient uptake, through moderating cytokinin biosynthesis (CKX) and destruction (CKX), the cytokinins are indeed poised to drive the second ‘Green Revolution’ (Box 2).

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Keywords: Crop, cytokinin, cytokinin oxidase/dehydrogenase (CKX), seed, isopentenyl transferase (IPT), yield.

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