Review

Cytokinin dehydrogenase: a genetic target for yield improvement in wheat

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Summary

The plant hormone group, the cytokinins, is implicated in both qualitative and quantitative components of yield. Cytokinin has opposing actions in shoot and root growth—actions shown to involve cytokinin dehydrogenase (CKX), the enzyme that inactivates cytokinin. We revise and provide unambiguous names for the CKX gene family members in wheat, based on the most recently released wheat genome database, IWGSC RefSeq v1.0 & v2.0. We review expression data of CKX gene family members in wheat, revealing tissue-specific gene family member expression as well as sub-genome-specific expression. Manipulation of CKX in cereals shows clear impacts on yield, root growth and orientation, and Zn nutrition, but this also emphasizes the necessity to unlink promotive effects on grain yield from negative effects of cytokinin on root growth and uptake of mineral nutrients, particularly Zn and Fe. Wheat is the most widely grown cereal crop globally, yet is under-research compared with rice and maize. We highlight gaps in our knowledge of the involvement of CKX for wheat. We also highlight the necessity for accurate analysis of endogenous cytokinins, acknowledging why this is challenging, and provide examples where inadequate analyses of endogenous cytokinins have led to unjustified conclusions. We acknowledge that the allohexaploid nature of bread wheat poses challenges in terms of uncovering useful mutations. However, we predict TILLING followed by whole-exome sequencing will uncover informative mutations and we indicate the potential for stacking mutations within the three genomes to modify yield components. We model a wheat ideotype based on CKX manipulation.

Keywords: CKX, cytokinin oxidase/dehydrogenase, cytokinin, wheat, yield, TILLING, whole-exome sequencing, root growth, mineral nutrition.

Introduction

Wheat is the most widely grown crop around the globe and ranks second in importance to rice for food (FAOSTAT, 2018). It is the second most important food crop in China (UD Dowla et al., 2018) and yet, in comparison with rice and maize, wheat is under-explored (Schnurbusch, 2019). Clearly, there is a need to increase yield in the face of a world population increasing from the current 7.5 billion to 9 billion by 2050, and this is with the unprecedented challenges posed by climate change with its increased risk of drought (Hochholdinger, 2016; Hochman et al., 2017; UD Dowla et al., 2018), concerns regarding the need to reduce N-fertilizer use (Zörrb et al., 2018) and increasing disease risk (Gautam et al., 2003; Zhang et al., 2015). However, quality must also be considered, not only if the end product is for baking and processing (Zörrb et al., 2018) but also for when wheat is the staple food, consumed as the major source of carbohydrate and providing basic nutrition. Wheat can, for instance, be lacking in Zn and Fe, leading to micronutrient malnutrition (the ‘hidden hunger’) (Mayer et al., 2018) in developing countries (see Beasley et al., 2019; Ramireddy et al., 2018a, b).

The Green Revolution cereals were developed at a time when nitrogen fertilizer was increasing. The crops, selected for their semi-dwarf, strong straw characteristics, yielded highly when supplied with N, water and treated with pesticides. Subsequently, it was shown that semi-dwarf wheats and maize were gibberellin-insensitive mutants, while the semi-dwarf rice was a gibberellin biosynthentic mutant (Hedden, 2003). Significantly, these crops were not specifically selected for increased grain number or size—increased yield is considered due to reduced competition for assimilates from the shortened stem and reallocation of resources to the spikes increasing grain number (Fischer and Stockman, 1986).

The plant hormone group, the cytokinins, has been strongly implicated in many aspects affecting yield, particularly grain number and size (Jameson and Song, 2016; Yamburenko et al., 2017), but including response to biotic and abiotic stressors (Cortleven et al., 2019; Pavlů et al., 2018), mineral status (Gao et al., 2019; Guo et al., 2017) and leaf senescence (Höning et al., 2018; Zwack et al., 2013). The cytokinins, which are positive regulators of shoot growth and negative regulators of root growth (Werner et al., 2003), are implicated in the control of both shoot architecture (Bartrina et al., 2011; Han et al., 2014) and root system architecture (Waidmann et al., 2019; Werner and Schmulling, 2009), and crown root formation (Gao et al., 2014). Recent research indicates a significant negative role in the control of micronutrient uptake (Gao et al., 2019; Nehnevajova et al., 2019; Ramireddy et al., 2018a).

In this review, we emphasize aspects of cytokinin biology in cereals before focusing on the cytokinin catabolism gene family,
cytokinin oxidase/dehydrogenase (CKX). We provide a brief introduction to the cytokinins and briefly introduce the components of yield in cereals and changes in endogenous cytokinins during grain development before revising the naming of the CKX gene families in wheat. As there are recent reviews covering the cytokinins in biotic and abiotic stress, and disease (Cortleven et al., 2019), in nitrogen nutrition (Gu et al., 2018) and senescence (Hönig et al., 2018; Zwack and Rashotte, 2013), we focus on research implicating CKX in controlling grain yield in rice, barley and wheat, and in root growth and the uptake of micronutrients, and reveal gaps in our knowledge concerning wheat. Having identified these gaps, we foresee the use of mutants of specific TaCKX gene family members, identified through Targeting Induced Local Lesions IN Genomes (TILLING) followed by whole-exome sequencing, in the creation of a wheat ideotype.

**Cytokinins**

The committed step in the synthesis of the cytokinins occurs in two ways: either by an isopentenyl transferase (IPT) attaching an isoprenoid side chain to an ATP/ADP leading to the formation of the nucleotides of isopentenyl adenine (ip) and trans-zeatin (tz), or by a trna-IPT leading, indirectly, to the cisZ-type cytokinins. LONELY GUY (LOG) activates the cytokinin by releasing the free bases from the nucleotide forms, while destruction of cytokinin is carried out by cytokinin oxidase/dehydrogenase (CKX). Inactivation can also occur via glycosylation—either for storage by O-glucosylation or for inactivation via N-glucosylation. The active free base forms (trans-zeatin (tz), isopentenyl adenine (ip), dihydrozeatin (DHZ) and cis-zeatin (cz)) are detected by histidine kinase receptors, followed by a multistep phosphorelay to activate type A and type B response regulators (RRs). Type A RRs operate as a negative feedback system, whereas type B RRs are transcription factors that target primary cytokinin-responsive genes. For more detail on cytokinin forms, function and signal transduction, see Jameson (2017), Kieber and Schaller (2018), Romanov et al. (2018) and Worthen et al. (2019).

It is critically important that analysis of cytokinin content is performed appropriately. There are over 20 different forms of cytokinins in wheat alone (Sýkorová et al., 2008), most of which can interconvert to release the active free bases. Comprehensive analysis requires a variety of purification steps, separation, and identification and quantitation via MS/MS using appropriate internal standards (e.g. Dobrev and Vankova, 2012; Novák et al., 2017; Powell et al., 2013). It has never been acceptable to attempt to quantify cytokinins using UV absorbance following HPLC/UHPLC of partially purified extracts (e.g. as in Geng et al., 2017; Nayyar et al., 2013), because the cytokinins are usually present in low ng to pg/g FW quantities, and UV-absorbing impurities will mask the cytokinin. ELISA or RIA of partially purified but not chromatographically separated extracts is also unacceptable (see Sayavedra-Soto et al., 1988 for an example of the pre-purification and separations steps required prior to immunoassay). Including an identification of kinetin, which has never been identified as naturally occurring, and adenosine (usually present at 1000 times greater quantity than the cytokinins) is indicative, not only of unacceptable technique, but also of a lack of understanding of the complexity of the cytokinins (e.g. as evidenced in Joshi et al., 2018). Basunia and Nonhebel (2019) also emphasized this issue of inadequate analyses with regard to auxin and cytokinin analyses.

**Morphological components contributing to grain yield in wheat, barley and rice**

In wheat and barley, the inflorescence is a spike, with the grain born within florets of the spikelets arranged on the spike. Yield is determined by 1000-grain weight (TGW), the number of grains per spike and the number of spikes per area (i.e. tiller number) (Feng et al., 2017). Tiller number is regulated genetically (Guo and Schnurbusch, 2015), but in the field, tiller number is determined to a greater or lesser extent by seed spacing at sowing. The spikelet of wheat is indeterminate and produces more than eight florets, whereas the inflorescence (i.e. the spike) is determinate (Feng et al., 2017), and the spikelet number is set at the end of the double ridge stage (Guo and Schnurbusch, 2015; Ochagavia et al., 2018). The terminal spikelet and floret differentiation have all occurred when the spike is around 2.0 mm in length (Gardner et al., 1985). Most of the florets abort, leaving three to five grains at harvest (Feng et al., 2017). Unlike wheat, barley has determinate spikelets (yielding two-rowed or six-rowed barley) but an indeterminate spike (refer Gauley and Boden, 2019).

In contrast to wheat and barley, the rice inflorescence is a panicle: yield is determined by TGW, the number of grains per panicle and the number of tillers (one panicle per tiller). Branching of panicles is set early and establishes the potential number of grains per tiller. Each spikelet bears one floret and, therefore, one grain (see Itoh et al., 2005).

When collecting material for detailed analysis, it is important to recognize that, in wheat, anthesis starts in the upper middle of the spike and moves in a wave up and down the spike. Grains in the two basal florets of a spikelet are the largest. Poor filling occurs in higher-level florets and in the last-developed spikelets at the tip of the spike. Consequently, for clear developmental analyses, dissection of ovules/grains from the two basal florets from the middle section of a spike is important, as analyses of whole spikes will contain ovules/grains at varying developmental stages. The development of the barley spike follows a similar pattern to that in wheat. In rice, anthesis starts from the apical region of the panicle, and it takes about six days to reach the basal region. Poor grain filling occurs in basal spikelets.

**Endogenous changes in cytokinins during grain development**

The endosperm of cereals is the storage organ and a critical component of yield. Development is often divided into four stages: early development (double fertilization and syncytium formation); cellularization; differentiation (delination of cell types, mitosis and endoreduplication, accumulation of storage materials); and finally, maturation (programmed cell death, dormancy and desiccation). All cereals, indeed all monocots and dicots, undergo a phase of free nuclear divisions resulting in a transitory coenocyte (Olsen, 2004). In wheat, barley, rice and maize, this is followed immediately by cellularization of the endosperm which is immediately followed by mitotic cell divisions, which have declined by about 12–14 days after anthesis (DAA) (Fig. 1). In cereals, cell number establishes final grain size. However, the transition from the syncytial stage to cellularization has been shown in rice to be critical. Precocious or delayed transition from the syncytium to cellularization in the endosperm is reported to cause abnormal seeds and, potentially, seed abortion in rice (Chen et al., 2016; Folsom et al., 2014).
The peak levels of cytokinin in barley, rice and maize are reported to occur around 10-12 DAA (Fig. 1), although Hluska et al. (2016) suggest a much later cytokinin peak in maize kernels. It is not well recognized that the timing of changes in endogenous cytokinins in wheat differs from that in barley, rice and maize. In wheat, rapid changes in cytokinins occur around and immediately following anthesis, with a sharp peak of zeatin around 3-4 DAA, at the onset of mitotic cell divisions (e.g. Hess et al., 2002; Jameson et al., 1982; Lenton and Appleford, 1986; Morris et al., 1993) (Fig. 1). However, there are over 20 different cytokinins present in wheat (Sýkorová et al., 2008), but a comprehensive analysis over close time frames during spike, spikelet, ovule and grain development has not yet been reported for wheat (or rice) using LC-MS/MS, nor have the unusual glycosides mentioned by Lenton and Appleford (1986) been identified. Most of the cytokinins have been ignored in recent papers, including the O-glucosides. Cytokinins are present in the ovule around fertilization (Jameson et al., 1982; Lenton and Appleford, 1986), but trans-zeatin is the predominant form after anthesis (unpublished data). There is rapid metabolism of cytokinins during early wheat grain development, and this is matched by marked changes in gene expression. It is clear from the expression of TaIPT, CKX, CZOG1 and B-glucosidase gene family members at 2 to 4 DAA that specific members of these four multigene families play key roles in determining the level of cytokinin during the phase of free nuclear and subsequent mitotic cell divisions (Song et al., 2012).

**Phylogenetic analysis and renaming of CKX gene family members in bread wheat**

Cytokinin oxidase/dehydrogenase has been depicted as a key enzyme regulating the cytokinin levels in cereals including maize (Brugière et al., 2003), rice (Ashikari et al., 2005), barley (Zalewski et al., 2014) and wheat (Ogonowska et al., 2019; Song et al., 2012; Zhang et al., 2012b). CKX belongs to a small gene family. *Arabidopsis thaliana* (arabidopsis) has seven homologues and 11, while in bread wheat 11 to 14 gene family members have been proposed (Mameaux et al., 2012; Ogonowska et al., 2019; Shoib et al., 2019; Song et al., 2012). Recently, Ogonowska et al. (2019) and Shoib et al. (2019) have suggested different numbering of the TaCKX2.2, 3, 6, 9, 10 and 11 gene family members and allocation to chromosomes. Ogonowska et al. (2019) mainly followed the gene nomenclature suggested by former researchers (Lei et al., 2008; Lu et al., 2015; Mameaux et al., 2012; Song et al., 2012; Zhang et al., 2012b), and allocated the TaCKX2 family to two subfamilies TaCKX2.1 and TaCKX2.2 (Table 1). Shoib et al. (2019) renamed most of the TaCKX gene family members based on a comprehensive phylogenetic analysis. In detail, TaCKX3, TaCKX6, TaCKX9, TaCKX10 and TaCKX11 that had been named by former researchers were...
To avoid further confusion, we mined the most recent wheat genome feature format annotation file (gff3 file). By comparing the recent numbering of the just released, updated genome sequence IWGSC RefSeq v2.0 genomes (Fig. S1). In addition, Shoaib et al. (2019) suggested that five genes, that is TaesCS3A01G311100, TaesCS3D01G143500, TaesCS3D01G143300 and TaesCS3D01G143200, be given new sub-family names TaesCS3A14A, TaesCS3X14B, TaesCS3X12D, TaesCS3X14D and TaesCS3X13D, respectively (Table 1). Notably, all of these five genes were classified as the TaesCS3X2.2 subfamily by Ogonowska et al. (2019).

To avoid further confusion, we mined the most recent wheat genome feature format annotation file (gff3 file), as well as the just released, updated genome sequence IWGSC RefSeq v2.0. By comparing the recent numbering of CKX homologues in closely related monocot species including rice, maize, barley, Aegilops tauschii, Setaria italica and Brachypodium distachyon, we suggest renumbering the family members as TaesCS3A14A, TaesCS3X14B, TaesCS3X12D, TaesCS3X14D and TaesCS3X13D, respectively (Table 1). Notably, all of these five genes were classified as the TaesCS3X2.2 subfamily by Ogonowska et al. (2019).

Additionally, we mapped all of the TaesCSX family genes onto their corresponding positions on each chromosome (Fig. 2). Notably, one of the TaesCSX11 subfamily, TaesCSX11-ChrUn, had not been mapped to a position on any one of the 21 chromosomes in the IWGSC RefSeq v1.0 genome sequence. However, we mapped it onto the 7D chromosome position 622521325 in our local BLAST database, which was developed using the IWGSC RefSeq v2.0 genome sequence. Thus, all 35 TaesCSX gene family members can now be allocated to one of the three chromosomes associated with the A, B and D genomes (Fig. 2). Finally, to indicate on which chromosome the gene family member is located, the renumbered gene names have also been marked by their allocated chromosome number (Table 1; Fig. S1).

Additionally, TaesCSX2 has undergone gene duplication (Mameaux et al., 2012), with Lu et al. (2015) suggesting that the TaesCSX2s on chromosome 3D could be subdivided into two groups based on their homology, as shown by Ogonowska et al. (2019). The phylogenetic tree shown in Figure 3 separates the paralogues into two sub-gene families (CKX2.1 and 2.2). Notably,

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Table 1 Revised naming of the CKX gene family members in wheat

| Current suggestion | Gene ID (RefSeq v1.0) | Ogonowska et al. (2019) | Shoabi et al. (2019) | Song et al. (2012) | Others (from Ogonowska et al., 2019) |
|--------------------|-----------------------|------------------------|---------------------|--------------------|-------------------------------------|
| TaesCSX1-3A        | TaesCS3A01G1095000    | TaesCSX1               | TaesCSX1A           | TaesCSX1           |
| TaesCSX1-3B        | TaesCS3B01G1287000    | TaesCSX1B              |                     |                    |
| TaesCSX1-3D        | TaesCS3D01G111300     | TaesCSX1D              |                     |                    |
| TaesCSX2.1-3A      | TaesCS3A01G311000     | TaesCSX2.1             | TaesCSX2A           | HM195292           |
| TaesCSX3-1B        | TaesCS3B01G1611000    | TaesCSX2B              |                     | TaesCSX2.5         |
| TaesCSX3-1D        | TaesCS3D01G143600     | TaesCSX2D              |                     | TaesCSX2.3         |
| TaesCSX5-3A        | TaesCS3A01G311100     | TaesCSX2.2             | TaesCSX2.1A         | TaesCSX6a2         |
| TaesCSX5-3B        | TaesCS3B01G161000     | TaesCSX2.2B            |                     | TaesCSX2.4         |
| TaesCSX5-3D        | TaesCS3D01G143500     | TaesCSX2.2D            |                     | TaesCSX2.1         |
| TaesCSX6D1         | TaesCS3D01G143300     | TaesCSX2.2C            |                     | TaesCSX6D1         |
| TaesCSX6D2         | TaesCS3D01G143200     | TaesCSX2.2D            |                     | TaesCSX2.1         |
| TaesCSX3-1A        | TaesCS3A01G159600     | TaesCSX3A              | TaesCSX3A           | TaesCSX6           |
| TaesCSX3-1B        | TaesCS3B01G176000     | TaesCSX3B              |                     |                    |
| TaesCSX3-1D        | TaesCS3D01G157000     | TaesCSX3D              |                     |                    |
| TaesCSX4-3A        | TaesCS3A01G481000     | TaesCSX4A              | TaesCSX4A           | TaesCSX4           |
| TaesCSX4-3B        | TaesCS3B01G525300     | TaesCSX4B              |                     |                    |
| TaesCSX4-3D        | TaesCS3D01G475800     | TaesCSX4D              |                     |                    |
| TaesCSX5-3A        | TaesCS3A01G321100     | TaesCSX5A              | TaesCSX5A           |                    |
| TaesCSX5-3B        | TaesCS3B01G344600     | TaesCSX5B              |                     |                    |
| TaesCSX5-3D        | TaesCS3D01G310200     | TaesCSX5D              |                     |                    |
| TaesCSX6-6A        | TaesCS3A01G185800     | TaesCSX6A              | TaesCSX7A           | TaesCSX7B          |
| TaesCSX6-6B        | TaesCS3B01G214700     | TaesCSX6B              |                     |                    |
| TaesCSX7-6D        | TaesCS3D02G172900     | TaesCSX6D              |                     |                    |
| TaesCSX8-2A        | TaesCS3A01G378300     | TaesCSX8A              | TaesCSX11           |                    |
| TaesCSX8-2B        | TaesCS3B01G395200     | TaesCSX8B              |                     |                    |
| TaesCSX8-2D        | TaesCS3D01G374600     | TaesCSX8D              |                     |                    |
| TaesCSX9-1A        | TaesCS3A01G234800     | TaesCSX9A              | TaesCSX10           |                    |
| TaesCSX9-1B        | TaesCS3B01G248700     | TaesCSX9B              |                     |                    |
| TaesCSX9-1D        | TaesCS3D01G237200     | TaesCSX9D              |                     |                    |
| TaesCSX10-7A       | TaesCS3A01G363400     | TaesCSX10A             | TaesCSX9            |                    |
| TaesCSX10-7B       | TaesCS3B01G264400     | TaesCSX10B             |                     |                    |
| TaesCSX10-7D       | TaesCS3D01G359700     | TaesCSX10D             |                     |                    |
| TaesCSX11-7A       | TaesCS3A01G536800     | TaesCSX11A             | TaesCSX3            |                    |
| TaesCSX11-7B       | TaesCS3B01G455000     | TaesCSX11B             |                     |                    |
| TaesCSX11-7D       | TaesCS3Un01G106300    | TaesCSX11D             |                     |                    |

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HvCKX2.1 and HvCKX2.2 from *Hordeum vulgare* grouped with the TaCKX2.1 and TaCKX2.2 sub-clusters, respectively. However, the TaCKX2.2 sub-gene family has expanded and has more members compared with HvCKX2.2. Functional investigation of these new members is critical as they could be of considerable importance for genetic improvement.

After locating all the duplicated paralogues on their respective chromosomes, we also noted that the location of TaCKX2.1 and TaCKX2.2.1 on the 3A long arm contrasts to their location on the short arm of 3B and 3D (Fig. 2). We suggest that this may be a non-reserved transposition event from the 3A short arm to the 3A long arm, and this also resulted in the reversal of the position of TaCKX2.1 relative to TaCKX2.2.1 compared with that of 3B and 3D (Fig. 2). Similarly, we suggest that the TaCKX2.2.2 and TaCKX2.2.3 paralogues located on the 3D chromosome may also be a result of another transposition event, in this case a replicative transposition which produced two paralogues.

We suggest the following naming for these paralogues: TaCKX2.1-3A and TaCKX2.2.1-3A, and TaCKX2.1-3B and TaCKX2.2.1-3B on chromosomes 3A and 3B, respectively, and four TaCKX2 paralogues, TaCKX2.1-3D, TaCKX2.2.1-3D, TaCKX2.2.2-3D and TaCKX2.2.3-3D on chromosome 3D (Table 1). Shoaib et al. (2019) named these paralogues individually as 2, 12, 13 and 14, whereas Ogonowska et al. (2019) had allocated these paralogues to two distinct gene families—CKX2.1 and CKX2.2.

Individual CKX gene family members are expressed in different tissues (Werner et al., 2006). For wheat, this has been shown through gene expression analyses (Ogonowska et al., 2019; Song et al., 2012) and can be found in the comprehensive RNA-seq data set from the IWGSC (2018), where expression data were
obtained from hundreds of RNA-seq samples. By abstracting the TaCKX gene family members from data on roots, leaves, spike and grain, tissue specificity in wheat is clear, as is differential expression between sub-genomes (Fig. 4; Fig. S2). TaCKX1 is most expressed during grain development, with sub-genome D more highly expressed in the stigma + ovary at anthesis, sub-genome B in the grain at the milk stage and sub-genome A at the soft dough stage (Fig. 4 and Fig. S2A). TaCKX2 gene family members also expressed during grain development, with TaCKX2.1 more highly expressed than the TaCKX2.2 sub-gene family (Fig. S2B–F). Combining all eight TaCKX2 gene family members, it is clear that expression is targeted to the developing grain, with somewhat greater expression at the milk grain stage than in the ovary + stigma at anthesis (Fig. S2F).

TaCKX3 was one of the more highly expressed gene family members with expression restricted to vegetative tissues: the roots at the tillering, flag leaf and the 30% spike stages, shoot apical meristem (SAM) at the seedling and tillering stages, and the shoot axis at the milk grain stage and full boot stages (Fig. 4; Fig. S2G). TaCKX4-3D did not express in the grain either, but in the lemma at the milk stage and at low levels in roots (Fig. 4; Fig. S2H). TaCKX5-3B and TaCKX5-3D expressed in most leaf blades including the flag leaf blade, with only low expression in the ovary at anthesis (Fig. 4; Fig. S2I).

TaCKX8 expressed mostly in the first leaf blade at tillering, the internode at 30% spike and the senescing flag leaf (Fig. 4; Fig. S2K). TaCKX11, and particularly TaCKX11-7D, had low expression at early stages of development, but expressed in leaves at the tillering stage and in the flag leaf blade at full boot, ear emergence and 30% spike stage. TaCKX11-7D was the most highly expressed TaCKX gene family member during leaf senescence (Fig. 4; Fig. S2N). TaCKX9 expressed strongly in anthers at anthesis, while TaCKX9-1A expressed in leaf blades and in the flag leaf (Fig. 4; Fig. S2L).

Both TaCKX7 and 10 were expressed at very low levels, TaCKX10 principally during early root growth (Fig. S2M), whereas TaCKX7-6B expressed later in roots at the flag leaf and 30% spike stages (Fig. S2L). Overall, these data are broadly similar to the RT-qPCR data published by Ogonowska et al. (2019). However, an important time window of seed development, between anthesis and milk stage, was not included in the RNA-seq data set:

TaCKX1 and TaCKX2 gene family members are particularly highly expressed at 1-14 DAA (Ogonowska et al., 2019; Song et al., 2012; Zhang et al., 2012b).

**Associations between CKX and seed yield components**

Bread wheat is a recent polyploid species, so there has been a relatively short time for diploidization—the process that dilutes and gradually erases the previous duplication (Uauy et al., 2017). Consequently, most genes are expected to have overlapping functions, although divergence amongst the three diploid genomes since their last common ancestor is to be expected (Uauy et al., 2017). Further, these authors suggest the effects of gene mutations in recent polyploid species will more frequently be masked by the other genomes. However, if a unique allele is identified in a landrace or modern variety but is absent from the wild progenitors, it is likely to have been selected during domestication. Both forward and reverse genetic approaches have been utilized that show that CKX gene family members are implicated in yield in rice, barley and wheat.

**CKX and yield in rice**

There are a number of publications linking poor grain filling in rice to lower cytokinin content, and particularly to greater levels of OsCKX expression/enzyme activity in different cultivars or in apical versus basal spikelets (see Panda et al., 2018 and references therein).

Since Ashikari’s seminal work with rice (Ashikari et al., 2005), CKX has been identified as having a key role in yield determination (summarized in Table 2). Ashikari et al. (2005) showed that the rice Gln1a QTL on chromosome 1 linked to increased yield is a gene for CKX2. Overexpressing or reducing activity of Gln1a reduced or increased grain number, respectively. A null mutation of OsCKX2 increased grain number in line 5150, a high yielding Chinese variety. When expression of all 11 rice CKX genes was compared, OsCKX2 showed greatest expression in the cv Koshishikari (which had the lowest grain number) and none of the other 10 CKXs was differentially expressed amongst the lines assessed pointing to OsCKX2 having a preeminent role in controlling yield.

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More recently, Li et al. (2016) used gene editing to target mutations to Gn1a of rice cultivar Zhonghua 11, a widely grown modern japonica cultivar in China. They found by editing in a mutation that led to a frameshift in OsCKX2, plant height increased, as did panicle size and number of flowers per panicle, but they did not report on grain number or 1000-grain weight (Table 2).

Investigating the association between grain number and grain size, Guo et al. (2018) suggest that GRAIN SIZE AND NUMBER (GSN1) controls the trade-off between grain size and number in rice, at least partly through CKX2. They showed that CKX2 was up-regulated in the gsn1 mutant and cytokinin levels reduced during young panicle development, with a consequent increase in grain size but a less branched panicle and overall fewer grains than the wild type. This supports a role for CKX2/cytokinin in the control of panicle architecture and, consequently, grain number in rice (Ashikari et al., 2005; Li et al., 2016).

Mutations in the rice F-box gene, LARGER PANICLE (LP), a component of the ubiquitin-mediated pathway, enhanced the yield of rice (Li et al., 2011), through changes in panicle size and increased grain number. The plants were slightly taller and more resistant to lodging, with stronger culms and more vascular bundles. OsLP was shown to express in several tissues with in situ hybridization locating expression in primary and secondary branch primordia. Expression analysis in two allelic mutants revealed strong down-regulation of OsCKX2. Li et al. (2011) concluded that LP might be involved in moderating the cytokinin level through direct or indirect control of the OsCKX2 expression. However, a route between the reduced expression of CKX and the ubiquitin–proteasome system has yet to be shown.

An interesting interaction between strigolactones (SL) and cytokinins in rice was recently revealed, in which SL-regulated tiller development was shown to operate through transcriptional activation of OsCKX9 (Duan et al., 2019). OsCKX9 expressed in all tissues at the heading stage with greatest expression at the shoot bases. Both a gene-edited mutant, Osckx9, and overexpression of OsCKX9 increased tiller number, reduced plant height and decreased panicle size and grain number (Duan et al., 2019). Strigolactones are a group of carotenoid-derived plant hormones known to inhibit branching (Dun et al., 2012), while a SL signalling mutant of rice has increased tillering and panicle branching. Notably, OsCKX9 was shown to be unresponsive to cytokinin but to respond specifically to SL. Duan et al. (2019) showed that SL acts through elevation of OsCKX9 expression, decreasing both cytokinin level and OsRR5 expression. Earlier, Tsai et al. (2012) had shown expression of OsCKX9 to be shoot-

Figure 4 Heat map depicting expression of TaCKX gene family members based on RNA-seq data. The raw data sets were collected from IWGSC (Science 361, 2018) and calculated by Wheat Omics of China. We extracted the TaCKX gene expression data set using awk, python and perl programs. The figure was drawn using the expression mean value in R language.

More recently, Li et al. (2016) used gene editing to target mutations to Gn1a of rice cultivar Zhonghua 11, a widely grown modern japonica cultivar in China. They found by editing in a mutation that led to a frameshift in OsCKX2, plant height increased, as did panicle size and number of flowers per panicle, but they did not report on grain number or 1000-grain weight (Table 2).

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specific, and while showing other OsCKXs to be responsive to cytokinin, they did not show data for OsCKX9. However, it is important to recognize that increased tiller number is not likely to be beneficial to yield. Indeed, the aim of the new plant-type rice ideotype was to have low tillering capacity and no unproductive tillers (Rubia et al., 2014). The recently published paper by Zhao et al. (2019) implicated wheat TaD27-B (an orthologue of arabidopsis and rice D27, a SL biosynthetic enzyme) in mediating tiller number in wheat through modifying the size of the axillary buds. These authors suggest yield in wheat can be increased by manipulating SL biosynthesis (decreasing it) to increase tiller number. Indeed, a barley line carrying a mutation in strigolactone signalling (hvd14.d) was previously shown to produce a greater number of tillers (Marzec et al., 2016). However, multiple small tillers are considered undesirable as they redirect resources away from the main tillers and ultimately decrease yield (Hendriks et al., 2016; Kebrom et al., 2012).

While the focus has been on OsCKX2 for obvious reasons, the effect of targeting the more highly expressed OsCKX family members such as OsCKX11, which is the most highly expressed gene family member in the early rice panicles (Yamburenko et al., 2017), is yet to be investigated. These authors also point out that there is greater variation amongst the genes for cytokinin metabolism and type A RRs, than for genes in the primary signalling pathways, so that regulation of cytokinin levels is more

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**Table 2 Impacts on yield components of CKX manipulations**

| Plant | CKX family member | Alteration | Effect | References |
|-------|-------------------|------------|--------|------------|
| Rice  | CKX2 [Gn1a]       | cka2 mutant | ↑ panicle branches | Ashikari et al. (2005) |
|       |                   | RNAi       | ↑ grain number | Yeh et al. (2015) |
|       | CKX2              | RNAi       | ↑ tiller number | |
|       | CKX9 [responds only to strigolactone] | CRISPR/Cas9 | ↑ tiller number | Duan et al. (2019) |
|       | CKX2 papers       |            | ↑ flower number (yield not reported) | |
|       | LP                | mutant lp  | ↑ panicle size | Li et al. (2016) |
|       |                   |            | ↑ grain number | |
| Barley| HvCKX1            | RNAi       | ↑ grain number in T4 | Zalewski et al. (2014) |
|       | HvCKX9            | RNAi       | no effect at T4 | |
|       | HvCKX1            | RNAi       | ↑ grain number | Holubová et al. (2018) |
|       |                   | CRISPR/Cas9 | ↑ grain number | Gasparis et al. (2019) |
|       | HvCKX3            | CRISPR/Cas9 | no yield data provided | |
| Wheat | Ta2.2.1-3A        | RNAi       | ↑ grain number/spike | Li et al. (2018) |
|       | GW2               | CRISPR/Cas9 plus TILLING; triple mutant on A, B, D sub-genomes | ↑ grain size | Wang et al. (2018) |
|       | GW2 paper         | Link between GW2 and cytokinin not validated | 1000 grain weight | Geng et al. (2017) |
|       | CKX2.2,1-3D       | Mutation association analysis | ↑ 1000 grain weight | Zhang et al. (2012b) |
|       | CKX2.1-3D         | Mutation association analysis | ↑ grain size | Lu et al. (2015) |
|       | CKX4-3A, 3D       | Variant association analysis | ↑ grain weight | Chang et al. (2015) |

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through metabolism and negative feedback from the type A RRIs, than through perception and the signal transduction pathway. Supporting this contention is the recognition that mutants of OsLOG (LOG converts cytokinin nucleotides to active free bases) have smaller panicles (Kurakawa et al., 2007). However, the obvious necessity for a functional signal transduction pathway was recently shown in rice (Worthen et al., 2019).

As mentioned earlier, cytokinin analyses must be unambiguous. Two papers on CKX2 in rice give cause for concern. Joshi et al. (2018) focused on salt tolerance, and Nayar et al. (2013) focused on MADS29. The techniques used by both Joshi et al. (2018) and Nayar et al. (2013) were inadequate and neither article should be taken as supporting information for a role for CKX and/or cytokinins in elevating yield under salinity stress (Joshi et al., 2013). However, there were differences, as might be expected, between individual cytokinin types and lines, in field and glasshouse-grown plants (Holubová et al., 2018).

Although variable in terms of growth stage and line, decreased root growth was generally seen in both KD and KO plants grown in hydroponics (Holubová et al., 2018). Generally, the cZ types decreased with development, while the tZ types increased. Overall, the KD or KO of HvCKX1 led to an increase in total cytokinin content, although there were differences, as might be expected, between individual cytokinin types and lines, in field and glasshouse-grown plants (Holubová et al., 2018).

Table 3 Impact of manipulating CKX expression in the roots on plant characteristics

| Plant | Gene targeted | Alteration (↑ OsCKX4) | Morphological response | References |
|-------|---------------|------------------------|------------------------|------------|
| Rice  | OsCKX4        | Enhancer line (↑ OsCKX4) | ↑ crown root growth (number and length) | Gao et al. (2014, 2019) |
|       | Root-specific promoter | ↑ shoot growth | | |
|       | RNAi          | ↑ graviotropic response | | |
|       | Overexpression | ↑ Zn in seeds | | |
|       | RNAi and CRISPR/Cas9 | ↑ yield in field | | |
|       | cis-OsNAC2 [transcription factor that expresses in roots] | ↑ cytokinin | | |
| Barley | ZmCKX1 | Barley phosphate transporter promoter | ↑ root growth | Mržová et al. (2013) |
|       | AtCKX1 | Maize root-specific promoter | ↑ root growth | Vopšílová et al. (2016) |
|       | AtCKX2 | Root-specific promoter | ↑ root length, total root surface area, root DW | Ramreddy et al. (2018a, 2018b) |
|       | AtCKX2 | Root-specific promoter | ↑ Zn, Fe in seeds in field trial | | |
|       | RNAi and CRISPR/Cas9 | ↑ root growth | | |
|       | CRISPR/Cas9 | ↑ CKX4 | | |
|       | CRISPR/Cas9 | ↓ crown root number | | |
|       | CRISPR/Cas9 | ↓ Zn | | |
|       | CRISPR/Cas9 | ↓ yield in field | | |
|       | CRISPR/Cas9 | ↓ cytokinin | | |

CKX and yield in barley

Following RNAi targeting of HvCKX1 and HvCKX9 by Zalewski et al. (2010, 2012), selected because they both express in developing kernels (Zalewski et al., 2014), Holubová et al. (2018) used both hairpin RNAi targeted to ‘knockdown’ (KD) HvCKX1 and a CRISPR/Cas9 construct targeted to the first exon of HvCKX1 to ‘knockout’ (KO) CKX1. Reduction in HvCKX1 expression and CKX activity was shown in selected RNAi KD lines. No effect was shown on the expression of other HvCKX or HviPT genes indicating that the targeting was specific to CKX1. CKX activity was significantly decreased in the KO lines at all stages measured.

The cytokinin content of the KD and KO lines was carefully assessed: increases in cis- and trans-zearin (Z)-type cytokinins and in isopentenyl (IP)-type cytokinins were detected (Holubová et al., 2018). Generally, the cZ types decreased with development, while the tZ types increased. Overall, the KD or KO of HvCKX1 led to an increase in total cytokinin content, although there were differences, as might be expected, between individual cytokinin types and lines, in field and glasshouse-grown plants (Holubová et al., 2018).

Although variable in terms of growth stage and line, decreased root growth was generally seen in both KD and KO plants grown in hydroponics (Holubová et al., 2018). In terms of yield, of the KD lines grown in the glasshouse, two showed increased spike numbers and one line substantially increased grain number leading to an overall increase in yield even though the TGW was reduced. Similarly, for field-grown KD lines: spike numbers were increased, TGW was decreased, and an increase in yield was shown for one line over two seasons. No yield data were provided for the KO lines. These data from Holubová et al. (2018) are in agreement with the T4 generation of RNAi-CKX1 silenced barley plants, where increased yield was influenced by increased grain...
number, to a lesser extent by increased spike number, even though TGW was reduced (Zalewski et al., 2014).

In the most recent barley paper, Gasparis et al. (2019) generated gene-edited mutants of *HvCKX1* and *HvCKX3*, both of which are reported to be expressed in developing spikes of WT 0–14 days after pollination (DAP), and in seedling roots. In the Hvckx1 lines, a significant decrease in CKX activity was measured in 7 DAP spikes and in the seedling roots. In contrast, in the Hvckx3 lines, CKX activity in the spike was little changed relative to control, but a significant increase in CKX activity was observed in the roots, as was a significant decrease in *HvIPT10* expression.

There was no consistency between lines in terms of yield components: notably, there was limited effect for ckx1 mutants; and apart from one ckx3 line showing an increased number of spikes, other lines showed decreased numbers of grains and grain weight. Based on gene expression combined with RNA-seq data, the authors explain the limited effect on yield by the activation of strong homeostatic mechanisms via reduced *IPT* expression and enhanced inactivation via 0-glucosylation. They suggest yield increases noted in RNAi-CKX plants (Zalewski et al., 2010) may have occurred because the lesser reduction in CKX may not have invoked the strong up-regulation of homeostatic mechanisms as

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**Figure 5** Wheat ideotype based on selected *TaCKX* mutants exhibiting increased/decreased expression in specific parts of the wheat plant.

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seen with the knockout gene-edited plants (Gasparis et al., 2019). This observation may be pertinent to the gene-edited Oskck2 mentioned earlier, where the morphology was similar to lines overexpressing CKX9. Duan et al. (2019) offered no explanation for their apparently contradictory results. Interestingly, the root phenotypes of T2 plants showed opposite effects for the ctk1 and ctk3 mutants: ctk1 mutants generally showed enhanced root parameters, whereas ctk3 mutants showed inhibited root growth relative to control. This was potentially due to enhanced CKX9 activity and up-regulation of type A RRs in the ctk1 mutant. The unexpected, negative effect of the ctk3 mutant is explained by perturbations in cytokinin signalling via up-regulation of the AHP4 component of the signal transduction pathway (Gasparis et al., 2019). Evidently, the homeostatic control mechanisms operate just as strongly in roots as they do in the shoot.

**CKX and yield in wheat**

**TaCKX alleles associated with yield in wheat**

Based on the hypothesis that, as in rice, wheat CKX genes might regulate agronomic traits and be useful to improve productivity, Zhang et al. (2012b) adopted a candidate gene approach. They focused on finding the orthologue of OsCKX2. They showed TaCKX6 (renamed TaCKX2.2.1-3D) to be the wheat orthologue of rice OsCKX2 and to be located on chromosome D. To determine the function of TaCKX2.2.1-3D in wheat, they undertook linkage analysis, association analysis and gene expression. Cultivar Yanzhan, which has an 18-bp deletion in intron 2 of CKX2.2.1-3D, was shown to be associated with a greater TGW than Neixiang 188. They confirmed this by testing for association between the 18-bp deletion and TGW across 115 wheat accessions and concluded that TaCKX2.2.1-3D may underlie the greater or lesser TGW of individual cultivars.

Expression analysis showed transcripts of TaCKX2.2.1-3D reaching a maximum level in grains 8 DAP (Zhang et al., 2012b), in a similar pattern to that shown by Song et al. (2012) for TaCKX2 (renamed 2.2-1) in winter bread wheat variety Equinox, and in the RNA-seq data set (Fig S2C,F). A comparison of TaCKX expression between haplotypes carrying the deletion (haplotype a) and those without the deletion (haplotype b) showed significantly reduced CKX expression in those with haplotype a. The deletion was associated with increased TGW (Zhang et al., 2012b).

Increased grain size is associated with domestication of cereals (Purugganan and Fuller, 2009). Zhang et al. (2012b) further showed that the karyotypes carrying the deletion in TaCKX2.2.1-3D are essentially restricted to landraces and modern cultivars indicating that the deletion associated with increased TGW is likely to have been derived relatively recently. Because of the domestication bottleneck, the TaCKX6-D1 locus has little remaining genetic variation in either Chinese landraces or cultivated varieties (Zhang et al., 2012b).

Zhang et al. (2012b) also suggest that functional divergence has occurred in CKX2, as OsCKX2 is associated with grain number (Ashikari et al., 2005), and TaCKX2.2.1-3D is associated with grain weight. Expression analyses across various organs confirm this suggestion, with TaCKX2.2.1-3D expressing mainly in kernels and relatively less in inflorescences (Zhang et al., 2012b), whereas OsCKX2 is mainly expressed in inflorescences and flowers (Ashikari et al., 2005).

Lu et al. (2015) also isolated a novel allele of CKX2. TaCKX6a02 has been allocated to the TaCKX2.1 gene family (Ogonovska et al., 2019) and renamed TaCKX2.1-3D (Table 1). Lu et al. (2015) showed this gene was located to chromosome 3D. They set out to identify allelic variations of CKX genes for grain filling, grain size and grain weight using 169 recombinant inbred lines (RILs) developed by a cross between a high yielding winter wheat variety and a low yielding Chinese landrace differing in grain size, grain weight and grain filling, along with 102 other wheat varieties. Of the six TaCKX gene families assessed, only TaCKX2.1-3D had a significant correlation with grain size, weight and grain filling rate. Two alleles were identified, with one, TaCKX2.1-3Da, having significantly higher values for these characteristics than varieties with allele ‘b’. Following an association analysis amongst the 102 cultivars, 68% had the ‘a’ allele, while the rest had the ‘b’ allele. They concluded through the analysis of different genetic backgrounds, RILs and natural populations that the ‘a’ allele was positively correlated with improved yield characteristics. A 29-bp insertion in the 3’ UTR region after the stop codon was detected in this allele compared with allele ‘b’. A full-length sequence of this gene still needs to be obtained, and an expression analysis of TaCKX2.1-3D is still to be carried out, as is a comparative analysis of CKX expression between lines carrying the two different alleles. However, the RNA-seq database analysis indicates that peak expression of TaCKX2.1-3D is in grains at the milk stage (Fig S2B), a stage relevant to grain filling and ultimate grain weight.

Chang et al. (2015), using the same RILs and varieties as described above for Lu et al. (2015), searched for alleles significantly associated with chlorophyll content and grain weight. Allelic variation between the two parental lines showed genotype A to contain two alleles, TaCKX4-1 and TaCKX4-2 (corresponding to TaCKX4-2A and TaCKX4-2D in Fig S1) that were absent in genotype B. Only genotype A was positively associated with chlorophyll content and grain yield. However, no expression analysis has been carried out, nor full-length sequences obtained. While the RNA-seq data set (IWGSC, 2018) indicates TaCKX4-3D is strongly expressed in the lemma at the milk stage and is also expressed in the shoot axis (Fig. 4; Fig. S2H), more information is needed concerning the nature of the CKX variations in the RILs.

**Down-regulated CKX genes and yield in wheat**

Grain number per spike in wheat was enhanced when a conserved fragment of TaCKX2.2.1-3A (originally TaCKX2.4) was targeted by RNA interference (Li et al., 2018). Li et al. (2018) showed a strong correlation between grain number and reduced expression of TaCKX2.2.1-3A in T3 plants. Moreover, increased grain number was due to an increased grain number per spike. As there was no increase in spikelet number, this implies more filled florets. In the light of the results for rice where OsCKX2 was associated with grain number (Ashikari et al., 2005), and wheat where TaCKX2.2.1-3D was associated with grain weight (Zhang et al., 2012b), the results by Li et al. (2018) suggest a difference in function between the TaCKX2.2.2.1 genes on chromosomes 3A and 3D. This leads to the potential of stacking mutations in TaCKX2.2.1-3D and TaCKX2.2.1-3A to address both seed number and seed size in bread wheat, even though the RNA-seq data show similar profiles for both gene family members (Fig S2C).

GW2 has been associated with grain weight in rice and bread wheat, particularly wider grains and greater TGW (Geng et al., 2019).
expression of levels were increased in the overexpression lines, leading to the et al. expression of OsNAC2 transcription factors on root development in rice. Using both without reducing shoot growth. achieved enhanced crown root growth (more and longer roots) and Schm optimal: overexpression of CKX is associated with root growth and mineral Werner et al. cytokinin has been shown to be required for maintenance of both the shoot apical meristem and root apical meristem (Werner et al., 2003; Wybouw and De Rybel, 2019). Additionally, cytokinin is involved in regulating root system architecture in arabidopsis: it acts as a positional cue through its interaction with auxin, regulating lateral root spacing through the action of IPT and LOG4 genes (Chang et al., 2015), and in the gravitropic response of lateral roots (Waidmann et al., 2019).

The root system of cereals differs markedly from that of arabidopsis. Both the crown roots and lateral roots of cereals play key but different roles in maintaining plant yield. Root depth is a critical feature for productivity under non-irrigated conditions (in which most of the world’s wheat is grown), a feature associated with crown root growth—both length and angle of the roots (Julkowska, 2018). Lateral roots form the major bulk of the root system, for both water and nutrient acquisition, and spread laterally covering a wide surface area. Recent publications are showing the critical role that the cytokinins, CKX4 in particular, play in cereal root growth, drought tolerance and the acquisition of micronutrients. Critical in this research has been the recognition of the inhibitory role that the cytokinins play in roots in contrast to the promoting role they have in shoots (Table 3).

Gao et al. (2014) showed that OsCKX4 has a key role in the initiation of crown roots in rice. Using an enhancer line, a mutant was isolated that exhibited greater root growth and more crown roots, a stronger root gravitropic response, but reduced plant height. Molecular analysis showed that the enhancer line had enhanced expression of OsCKX4. Overexpression and RNAi of OsCKX4 led to greater and lesser crown root growth, respectively. The Gao et al. (2014) model shows OsCKX4 mediating the interaction between cytokinin and auxin biosynthesis. As the enhanced OsCKX4 expression also reduced shoot growth, Gao et al. (2014) then linked OsCKX4 to a root-specific promoter and achieved enhanced crown root growth (more and longer roots) without reducing shoot growth.

Most recently, Mao et al. (2019) investigated the role of NAC transcription factors on root development in rice. Using both down-regulated (RNAi and CRISPR/Cas9 lines) and overexpression of OsNAC2, they showed that OsNAC2 is a negative regulator of root growth, reducing both crown root number and root length. Expression of OsIPT3 and OsIPT5, and OsLOG4.3 was increased, expression of OsCKX4 and OsCKX5 was decreased, and cytokinin levels were increased in the overexpression lines, leading to the conclusion that OsNAC2 stimulates cytokinin accumulation by repressing CKX expression and stimulating cytokinin biosynthesis. More specifically, this occurred through the binding of OsNAC2 to the promoter of OsCKX4 (in addition to binding to promoters of several auxin-related genes). The authors concluded that OsNAC2 functions as an upstream integrator of the auxin and cytokinin signals regulating the root meristem and root growth, and that its specific cytokinin target is OsCKX4.

Undoubtedly, an investigation of TaCKX gene family members is warranted in wheat, as enhanced crown root growth—especially longer and lesser-angled roots—would be of benefit in wheat grown in water-limited environments. TaCKX4 clusters with OsCKX4 and is in a different sub-clade to the TaCKX2 cluster associated with grain yield (Fig. S1). According to the RNA-seq database, TaCKX4 expresses weakly in roots (Fig. S2H) including in axillary roots and in the root apical meristem (RAM) at the three-leaf stage. However, the two TaCKX gene family members that express specifically in roots (TaCKX7 and TaCKX10) are expressed at very low levels relative to other gene family members (Fig. S2I,M; Ogono et al., 2019).

No comparative analysis of TaCKXs in crown root primordia versus lateral root primordia has been undertaken, nor has any analysis in cereals relating to the control of lateral root spacing. However, artificial constructs have been used to modify CKX expression and to investigate the impact on the plant of modified root growth. Transgenic barley overexpressing an arabidopsis CKX1 gene under the control of a maize root-specific promoter showed enhanced tolerance to drought, an effect ascribed to altered root morphology (Pospíšilová et al., 2016). More recently, barley plants, transgenic for arabidopsis CKX1 or CKX2 under one of three root-specific promoters (pEPP, pPER and pRET), were constructed and exhibited enhanced root systems (Ramireddy et al., 2018a). Ramireddy et al. (2018a) selected AtCKX1 and AtCKX2 because they represented two lines of CKXs that diverged before the divergence of the monocots from the dicots, and because they have different subcellular localization, biochemical characteristics and substrate preferences (Schmulling et al., 2003).

CKX expression was elevated in the roots but not the shoots, with 2-type cytokinin conjugation reduced in the roots. Total root length, total root surface area and root dry weight were all increased in the transgenics, with no reduction in shoot biomass, while seed yield was maintained. The concentrations of several macro- and micronutrients were increased, especially for the CKX2 transgenics, and this enhancement was maintained under drought conditions. In the CKX2 transgenic lines then analysed, Ca, Cu and Zn were shown to be enhanced in the seeds.

Furthermore, the pEPP::CKX2 transgenic lines, which showed the strongest root growth also withstood prolonged drought better (Ramireddy et al., 2018a). The increased concentration of Zn is particularly significant as it is an essential element in the human diet and can be in limiting quantities when cereals are a staple food (Ramireddy et al., 2018b). Field trials of pEPP::CKX1 and CKX2 plants showed not only enhanced Zn but also Fe in the seeds, along with similar grain yield and TGW indicating, significantly, that the enhanced root growth did not confer a yield penalty (Ramireddy et al., 2018b). Enhanced Fe uptake is a significant feature, as previously it has been shown that cytokinin suppressed genes for Fe uptake (Séguelé et al., 2008).

Andersen et al. (2018) show how cytokinin might influence mineral nutrient uptake in arabidopsis through investigating the
formation of passage cells. These cells disrupt the suberized endodermis and facilitate water and mineral uptake. Mineral nutrient transporters have been demonstrated to be associated with passage cells (Andersen et al., 2018). They suggest that endodermal cells acquire passage cell features when cytokinin is repressed. Mineral nutrient stress leads to reduced cytokinin, which may facilitate passage cell production. Likewise, enhanced CKX activity in roots should lead to enhanced passage cell production and facilitate the uptake of mineral nutrients, as was shown in the barley transgenes overexpressing CKX (Ramireddy et al., 2018a, b).

Most recently, using the overexpressing CKX4 ‘root-enhancer’ mutant of rice, Gao et al. (2019) showed that reduced cytokinin elevated Zn uptake in roots and shoots under both normal and Zn-limiting conditions. Application of cytokinin reduced Zn uptake via a reduction in the expression of the metal transporters OsZIP1 and OsZIP5 in root cells. They further showed that cytokinin levels were reduced under Zn deficiency. Not only was cytokinin interacting through type B RR2 to directly reduce the expression of OsZIP1 and OsZIP5 in the roots, but the cytokinins were shown also to be negative regulators of chelators necessary for the internal transport of Zn (Gao et al., 2019). Seeds of OsCKX4 overexpressing plants and mutants of the side chain-hydroxylating enzyme that catalyses the formation of the active t2-type cytokinins both showed elevated Zn while those of the Osckx2 mutant had reduced Zn concentration. Moreover, seeds from transgenic plants exhibiting root-specific expression of OsCKX4 had increased seed Zn (but not Fe) and, furthermore, increased overall yield when grown in the field.

This multiple action of cytokinin on Zn uptake and transport is similar to the multiple effects the cytokinins have on N uptake and assimilation, where under C limitation/excess N, cytokinin reduces N uptake while enhancing assimilation (Guo et al., 2017).

The ramification of lateral roots through soil is a key aspect of the uptake of less mobile mineral nutrients. The orientation of lateral roots has generally been associated with auxin (e.g. Friml et al., 2003). Lateral roots establish a distinct gravitropic set-point angle (GSA) (Digby and Firn, 1995) at an angle to gravity that differs from the primary roots and that suggests a suppressive response to gravity (Waidmann et al., 2019). Based on a genome-wide association study (GWAS) of arabidopsis lines, Waidmann et al. (2019) identified a prominent peak associated with a single nucleotide polymorphism located in the AtCKX2 gene that was associated with increased GSA values, reflecting more perpendiclar LR growth to gravity. As root angle could be increased or decreased by enhancing or reducing AtCKX2 activity, respectively, Waidmann et al. (2019) concluded that cytokinin signalling defines directional lateral root growth by reducing lateral root bending after emergence, through the activity of AtCKX2.

Various transgenic manipulations show that the cytokinins are involved in multiple different aspects of root growth and their physiology in rice and barley, but there is no information about the impact of CKX mutants on root growth in wheat. There is limited information about the expression of TaCKX gene family members in roots (Fig 4), and no information about spatial expression. As Julkowska (2018) suggests, bulk root mass may be disadvantageous when resources are limited, whereas root distribution is critical: wheat plants with lesser root angle would be ideal. As most of the world’s wheat is grown in non-irrigated areas (Ud Dowlia et al., 2018), information on the control of root growth is critical.

Future perspective

The wheat ideotype has a limited number of strong tillers—all of which are productive—more fertile florets per spikelet and more large grains per floret, a root profile that has longer, lesser-angled crown roots, and a flag leaf that is resilient to abiotic and biotic stressors and yet senesces at an optimum time for seed fill (Fig. 5). There is a clear divergence in TaCKX gene family members between those that contribute to grain yield and those that are expressed in vegetative tissues. Moreover, the contrasting effects of cytokinins on roots and shoots must be accommodated. Root growth is currently manipulated by CKX overexpression driven via root-specific promoters. Reducing CKX to promote seed number by increasing spikelet number while not providing a cytokinin environment that might promote increased tillering or indeed reduced root growth is likely to be challenging. Moreover, any manipulation of the cytokinins via gene technology interventions will be further challenged by the strong homeostatic mechanisms controlling cytokinin levels in plants (Mrčová et al., 2013; Ogonovska et al., 2019).

While orthologues of CKX1 and CKX2 express in the reproductive structures of rice, barley and wheat and can be related to yield in all three cereals, other CKX gene family members appear to have diverged. For example, OsCKX11 is the most strongly expressing CKX gene family member in the rice panicle (Yamburenko et al., 2017) and yet in wheat no expression was detected in reproductive structures, and its greatest expression was in the flag leaf including the senescing blade (Fig. 4). Consequently, as previously suggested (Ninan et al., 2017), it is critical to identify targets for breeding to the gene family member level in the specific species and, potentially, cultivar of interest and which, in wheat, may necessitate the identification of mutations on all three genomes. TILLING followed by whole-exome sequencing is a mechanism by which mutations on the A, B and D genomes can be identified and subsequently stacked (Krasileva et al., 2017). We suggest the most useful mutations in terms of yield enhancement are likely to be those in TaCKX1 and the CKX2.1 and 2.2 gene family members. In contrast, mutations in TaCKX4, TaCKX7 and TaCKX10 could enhance cytokinin levels in the roots but which would, in fact, be undesirable. In such cases, when gain of function of the target genes is required, mutations of the upstream regulators of the target gene could be used. For instance, Maa et al. (2019) were able to enhance the OsCKX4 function in rice by down-regulating its inactivator, NAC29.

As stated by Borrill et al. (2015), genomics is the key to unlocking the polyploid potential of wheat. Unlike arabidopsis, there have been very few mutations available in wheat. We have been using speed breeding (Ghosh et al., 2018; Watson et al., 2018) and whole-exome capture of a TILLING population of Jimai22 to overcome this limitation. Jimai22 is the most popular cultivar of wheat currently grown across the major wheat-producing regions in China. Our aim is to sequence over 1000 mutant lines to develop a hexaploid wheat mutant gene bank containing over one million missense and stop codon mutation sites covering 99% of the high-confidence genes in the IWGSC RefSeq v2.0. We plan to identify and functionally characterize beneficial gene alleles, focusing initially on cytokinin regulatory genes including TaCKX gene family members. Our initial database, containing over 770,000 EST-type mutants in 94,900 high-confidence genes, is now available online (http://www.yjebc.com). So far, we have identified multiple missense/stop
codon mutant alleles in each of the A, B and D sub-genomes for all the TaCKX gene family members. Functional analysis of these mutants is currently underway.

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Conflicts of interest

The authors declare they have no conflicts of interest.

Author contributions

L.C. interrogated the wheat genome databases and prepared the gene position and expression figures; J.S. and J.Z. interrogated the phylogenetic data and prepared the relevant figures; and P.E.J. wrote the first draft of the review with input subsequently from all authors.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Phylogenetic cladogram showing the basis of the TaCKX naming.

**Figure S2** RNA-seq graphs for all TaCKX gene family members.

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