Review Article

Cytokinin glucosyl transferases, key regulators of cytokinin homeostasis, have potential value for wheat improvement

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Summary
The cytokinins, which are N⁶-substituted adenine derivatives, control key aspects of crop productivity. Cytokinin levels are controlled via biosynthesis by isopentenyl transferase (IPT), destruction by cytokinin oxidase/dehydrogenase (CKX), and inactivation via glucosylation by cytokinin glucosyl transferases (CGTs). While both yield components and tolerance to drought and related abiotic stressors have been positively addressed via manipulation of IPT and/or CKX expression, much less attention has been paid to the CGTs. As naming of the CGTs has been unclear, we suggest COGT, CNGT, CONGT and CNOGT to describe the O-, N- and dual function CGTs. As specific CGT mutants of both rice and arabidopsis showed impacts on yield components, we interrogated the wheat genome database, IWGSC RefSeq v1.0 & v2.0, to investigate wheat CGTs. Besides providing unambiguous names for the 53 wheat CGTs, we show their expression patterns in 70 developmental tissues and their response characteristics to various stress conditions by reviewing more than 1000 RNA-seq data sets. These revealed various patterns of responses and showed expression generally being more limited in reproductive tissues than in vegetative tissues. Multiple cis-regulatory elements are present in the 3 kb upstream of the start codons of the 53 CGTs. Elements associated with abscisic acid, light and methyl jasmonate are particularly over-represented, indicative of the responsiveness of CGTs to the environment. These data sets indicate that CGTs have potential value for wheat improvement and that these could be targeted in TILLING or gene editing wheat breeding programmes.

Introduction
There is increasing interest in the plant hormone group, the cytokinins, because of their potential to improve crop yield (e.g. Chen et al., 2020b; Cucinotta et al., 2018; Jabłoński et al., 2020; Jameson and Song, 2020; Nguyen et al., 2020, 2021; Schwarz et al., 2020; Wang et al., 2020a; Zhang et al., 2020). The cytokinins are considered to have significant but contrasting roles in shoot and root architecture (Werner et al., 2003a) and to control both seed number and seed size (Jameson and Song, 2016). They are also amenable to manipulation to improve yield under stress conditions through either maintenance of leaf function (e.g. Joshi et al., 2019; Rivera et al., 2010) or enhancement of root growth (e.g. Khandal et al., 2020; Nehnevajova et al., 2019; Werner et al., 2010; Zhang et al., 2020; and reviewed in Chen et al., 2020b). While there has been considerable interest in modifying cytokinin levels in plants by targeting either biosynthesis (via isopentenyl transferase, IPT) or cytokinin destruction (via cytokinin oxidase/dehydrogenase, CKX), much less attention has been given to manipulating the genes controlling the formation of the deactivated cytokinin O- and N-glucosides. After introducing the cytokinins more generally, this article focuses on the cytokinin O- and N-glucosyl transferases and their potential roles in modifying yield in bread wheat, under ambient and stress conditions.

Cytokinin biosynthesis and metabolism
Naturally occurring cytokinins, which are adenine derivatives, fall into two groups: those with an isoprenoid side chain and those with an aromatic one. The isoprenoid cytokinins are considered to be the predominant forms (Jaworek et al., 2019; Sakakibara, 2006). The aromatics are not covered in this review as, while not exhaustively looked for in all cereals, they were not detected in either maize leaves (Lacuesta et al., 2018) or in rice (Kudo et al., 2012), although low levels of 6-benzylamino purine were recently reported in wheat spikes (Jabłoński et al., 2020) and low levels of various aromatic metabolites have been detected in germinating seeds of maize and oats (Stirk et al., 2012).
The isoprenoid cytokinins are generated in plant tissues predominantly via two pathways: de novo biosynthesis via ADP/ATP isopentenyl transferases (IPTs) and, indirectly, through the degradation of specific isoprenylated trRNA species (Letham et al., 1983; Miyawaki et al., 2006) (Figure 1). At least one trRNA-IPT likely exists in all organisms including Archea (Wang et al., 2020b), whereas the ADP/ATP-IPTs are found only in higher plants (Nishii et al., 2018).

Nucleotides are the first formed cytokinins and can be converted directly by LONELY GUY (LOG) (Kuroha et al., 2009) to the biologically active free bases trans-zeatin (tZ), isopentenyladenine (iP), cis-zeatin (cZ) and dihydrozeatin (DHZ) (Figure 1). The free bases all exist in riboside form, and these are considered to be the major transport forms (Kudo et al., 2010). The free base, riboside and nucleotide forms can then be further modified by O-glucosylation of the side chain (with the exception of iP), or by 3-, 7- and 9-N-glucosylation of the adenine moiety (Letham and Palni, 1983; see Raspor et al. (2020) for a comprehensive set of molecular structures along with molar masses). More complex modifications have also been identified (Werner et al., 2003b; see additional references in Vyšičlová et al., 2020).

Of all the two dozen or so different isoprenoid forms, it appears likely that the four free base cytokinins are the active forms detected by a variety of histidine kinase (HK) receptors (Lomin et al., 2015). Receptors are located on both the plasma membrane (Antoniadis et al., 2020; Kubiasová et al., 2020) and the ER (Romanov et al., 2018). Following perception of an active cytokinin, a two-component signal transduction pathway is activated leading to the activation of type-B response regulators (RR). These function as transcription factors regulating the expression of cytokinin primary-response genes (reviewed in Zubo and Schaller, 2020), which include the type-A response regulators negatively regulating cytokinin responses.

Cis-versus trans-cytokinins

Side chain modification affects cytokinin stability (Kiba et al., 2013). The isoprenoid side chain of iP is not hydroxylated, but that of Z is (Figure 1). Zeatin can, therefore, occur in both the trans and cis configurations. The hydroxylated side chain may also be unsaturated, providing DHZ, the only one of the four free bases resistant to degradation by CKX.

The trans-forms of cytokinins (tZ-types), biosynthesized via ADP/ATP-IPTs, are ubiquitous in higher plants, with the tZ-types frequently considered the most important forms (Mok and Mok, 2001). The cis-cytokinins (cZ-types) are derived from the turnover of trRNA (Miyawaki et al., 2006). Cis- to trans-isomerization was initially considered as a potential route to tZ, but the identification of a putative cis- to trans-isomerase by Bassil et al. (1993) is now considered likely to have been an artefact (Gajdosová et al., 2011; Hluská et al., 2017; Hošek et al., 2020; Kudo et al., 2012). While there has been considerable debate regarding the cis-isomers because of their lower activity in the classical bioassays (e.g. Schmitz and Skoog, 1972), cis forms are detected by specific HK receptors (e.g. Lomin et al., 2011; Yonekura-Sakakibara et al., 2004).

The general consensus developed that trRNA-IPT genes and the associated cZ-type cytokinins most likely play housekeeping roles, assuring maintenance of minimal cytokinin activity under growth-limiting conditions (Gajdosová et al., 2011; Schäfer et al., 2015; Wang et al., 2020b). However, it soon became clear that a number of plants, notably cereals, accumulated these isomers to very high levels (Schäfer et al., 2015). Kudo et al. (2012) showed cZ to be as physiologically active as tZ in inhibition of seminal root elongation in the rice root growth bioassay and to rapidly activate rice RRs. They consider cZ functions as an active cytokinin in rice.

Gajdosová et al. (2011) showed that cis-cytokinins occur in a wide range of both monocots and dicots and, recently, Schäfer et al. (2015) suggested that the high levels of cis-isomers in many crops (e.g. cereals, chickpeas, potato) may be a result of the plant breeding process itself (Schäfer et al., 2015). However, of significance is the finding by Lomin et al. (2018) that potato receptors show very weak activation by cZ, which contrasts to the strong activation of cereal receptors by cZ. A similar investigation of chickpea is warranted, to determine whether it is only cereal receptors that are strongly activated by cZ.

Cytokinin glucosylation

In addition to degradation by CKX, cytokinin levels are moderated through both O- and N-glucosylation (Figure 1). O-glucosylation of the side chain is carried out by cytokinin O-glucosyl transferases (CGOTs) forming O-glucosides. Glucosylation can also occur at the N3, N7 or N9 positions of the purine moiety by CNGTs, although the 3-N-glucosides are rarely reported (Hou et al., 2004). Differential preference for inactivation of cytokinins by CKX and/or glucosylation occurs both among species and among organs within a plant (Jameson, 1994).

O-glucosides

O-glucosides, in which the glucose is attached to the side chain of tZ, cZ, DHZ and their ribosides and even nucleotides (Nishiyama et al., 2011), are widely distributed in plants (Letham and Palni, 1983). The O-glucosides are considered to be storage forms (Parker et al., 1978) able to be reactivated by β-glucosidases (e.g. Brzobohatý et al., 1993; Smith and Staden, 1978; Vyšičlová et al., 2020). While the O-glucosides are not detected by cytokinin receptors (Romanov et al., 2006; Spichal et al., 2004), they do show biological activity in the callus bioassay (e.g. Jameson et al., 1982; Letham et al., 1983; van Staden, 1976), and in the PRRS::GUS-reporter gene assay (Spichal et al., 2004), as a consequence of being readily released by β-glucosidase (Letham and Palni, 1983; van Staden, 1976; Summons et al., 1980). Furthermore, O-glucosides are resistant to cytokinin oxidase (e.g. McGaw and Horgan, 1983; Parker et al., 1978; Zalabák et al., 2013) and, consequently, can accumulate to high levels in plants.

N-glucosides

The N-glucosides are the subject of recent reviews by Hoyerová and Hošek (2020) and Hošek et al. (2020). They suggest these forms accumulate to the highest levels of all cytokinins in planta. This is indeed the case in arabidopsis and tobacco. Even in seedlings of arabidopsis, iP7G accumulated more than any other cytokinin, and tZ7G and tZ9G had also accumulated, and to a much greater extent than O-glucosides. Cis-forms were only evident at low levels (Nishiyama et al., 2011). Under various experimental conditions, Kiba et al. (2019) showed similar patterns in arabidopsis: 7- and 9-N-glucosides of iP and tZ were more prevalent than other cytokinin forms and, on several occasions, iP7G had accumulated to substantial levels; cis-types were present, as were O-glucosides, but at low levels relative to the iP-N-glucosides in particular. N-glucosides are also the predominant cytokinin in potato cultured in vitro (Raspor et al., 2020) and in tobacco (Havlíková et al., 2008). Mytníková et al. (2011) identified the 7- and 9-glucosides of cZ, tZ and iP, with cZ7G clearly the predominant form in tobacco leaves.
Generally, Hoyerová and Hošek (2020) suggest prevalence of CNGs is not proportional to evolutionary age, nor specific to either dicots or monocots. They suggest that N-glucosides gradually accumulate during the life span of both monocots and dicots. Contrary to this assertion, O-glucosides accumulate to very high levels in a number of cereals compared to N-glucosides.
(e.g. maize: Lacuesta et al., 2018; wheat: Nguyen et al., 2020; rice: Takagi et al., 1989; Kojima et al., 2009). After assessing more than 150 representative species of the main land plant groups, Gajdovská et al. (2011) suggest that there is a clear dominance of the O-glucosides of cis- and trans-ZR in the Poaceae versus a strong dominance of 7- and 9-N-glucosides in dicots and, notably, in arabidopsis. In contrast to maize, wheat and rice, barley roots and leaves clearly accumulated Z9G in greater quantities than O-glucosides (Mržová et al., 2013; Pospíšilová et al., 2016), whereas in barley grains cZ/cZROG > iZ/iZROG > iZ9G. The 7-N-glucosides were not detected in barley grains (unpublished data).

Unlike the O-glucosides, 9-N-glucosides can be degraded by CKX: iP9G, iZ9G and cZ9G can be degraded by selected CKXs from arabidopsis, maize and barley, but iZ7G and IP7G are not similarly degraded (Laloue et al., 1977; Galuszka et al., 2007; Mržová et al., 2013; Hošek et al., 2020; summarized in Hoyerová and Hošek, 2020). Although the 7- and 9-N-glucosides have historically been considered inactive forms and of low to negligible biological activity (see references cited in Výlíčilová et al., 2020), iZ9G and DHZ9G have been shown to exhibit low level activity in different bioassays (e.g. Letham et al., 1983; van Staden and Drewes, 1991). Indeed, iZ9G showed similar activity to ZR and ZROG in the radish cotyledon assay, whereas iZ7G showed negligible activity in a variety of bioassays. Interestingly, both iZ9G and iZ7G showed low levels of activity similar to DZ and DZR in the PARS::GUS-reporter gene assay (Spichal et al., 2004). However, as the CNGs cannot interact with cytokinin receptors (Hothorn et al., 2011; Spichal et al., 2004), this implies hydrolysis of the two N-glucosides to form Z. Filipi et al. (2012) showed that maize Zm-p60.1 (β-glucosidase) was able to hydrolyse not only cZOG > iZOG, but also Z9G in vitro, albeit with very low efficiency. In contrast, the hydrolysis of iZ7G was not observed. Most recently, Hošek et al. (2020) presented evidence showing that both iZ7G and iZ9G can be converted to the free base while confirming that the iP-N-glucosides (iPNG) could not be and concluded that they are indeed terminal metabolites.

In contrast to Hošek et al. (2020) and the earlier work referred to above, Hallmark et al. (2020) suggest that activity of iZ7G and iZ9G was not due to hydrolysis to the free base and that iZ7G and iZ9G might be biologically active in their own right. They showed that exogenous application of iZ7G and iZ9G delayed senescence in detached cotyledons and modestly influenced shoot regeneration, while not affecting root elongation in arabidopsis. Moreover, analysis of both the transcriptome and shoot regeneration, while not affecting root elongation in arabidopsis. Moreover, analysis of both the transcriptome and protein levels showed distinct differences following exogenous application of iZ7G and iZ9G compared with Z (Hallmark et al., 2020). However, it is possible that the excess N-glucoside may have prevented endogenous N-glucosylation leading to an increase in Z thereby leading to the observed delay of senescence, as suggested by Hallmark et al. (2020) themselves. There seems no need to invoke unknown receptors/signal transduction pathways. Further, structural analysis (Hothorn et al., 2011) and ligand/receptor analyses (Lomin et al., 2015) would indicate that the intact glucosylated iZ7G and iZ9G are unlikely to be active per se. Irrespective of the mode of action, the potential contribution of these two N-glucosides to the pool of active cytokinins should be borne in mind.

**Lack of attention paid to cytokinin glucosides**

Unfortunately, many researchers choose to ignore the cytokinin glucosides altogether. While it could be the case that relatively few research groups have the knowledge and/or capability to analyse multiple cytokinin forms, these forms should not be ignored (Samsonová et al., 2020). This is because strong homeostatic mechanisms operate (e.g. Gasparis et al., 2019; Schwarz et al., 2020), and the O-glucosides, and potentially also the ZNGs, can be converted to active cytokinins.

Indeed, differential O- and N-glucosylation may impact yield characteristics as exemplified by Rubia et al. (2014) who showed that slow and fast-senescing lines of ‘new plant type rice’ differ in the nature of the glucosylation exhibited in leaves: O-glucosides accumulated in the flag leaves of the fast-senescing line, whereas N-glucosides remained constant in both lines but were at a greater concentration in the slow senescing line.

Currently, there is an unfortunate increase in the use of ELISA detection of cytokinins in crude or partially purified samples not subjected to HPLC separation (e.g. Tsago et al., 2020; Wang et al., 2016, 2019). Banowetz (1994) clearly articulated the absolute requirement for sample purification and HPLC separation prior to immunoassay. While intact O-glucosides will not cross-react with most commercially available antibodies, there is the potential for iZ9G and iP9G, to cross-react with antibodies raised against iZ9 and iP9, respectively (Lewis et al., 1996; Turnbull and Hanke, 1985), providing overestimations of active cytokinin content if HPLC separation has not been undertaken.

**Cytokinin glucosylation genes**

While the existence of O- and N-glucosyl derivatives has been known since the 1970s (Summons et al., 1980 and references therein), and enzymes capable of 7- and 9-N-glucosylation were first identified by Entsch and Letham in 1979, relatively little attention has been paid to the genes coding for the cytokinin glucosyl transferases (CGTs), and their potential application to crop yield.

A gene coding for a trans-zeatin O-glucosyl transferase (iZOGT) was first isolated from Phaseolus lunatus (Martin et al., 1999a) along with a gene coding for a xyosyl transferase (Martin et al., 1999b). Subsequently, a gene coding for a CGT specific to cis-zeatin (cZOGT) was identified and functionally characterized in maize (Martin et al., 2001). According to our phylogenetic analysis, maize has seven CGTs of which five are CGTs (ZmZOG1, 2 and 3; ZmcZOG1, 2 and ZOGT) and two are CNGTs (ZmcZNGT1 and ZmCNGT1) (Figure S1).

Kudo et al. (2012) identified three putative cis-ZOGTs in rice, which were shown to preferentially O-glucosylate cis- and cZ rather than iZ and iZR; additionally, cZOGT1 and 2 showed some activity towards iZ and iZR. Recently, Li et al. (2019) showed a purified Os6 protein N-glucosylated cytokinin in vitro. Heterologous expression in arabidopsis confirmed 7- and 9-NGT activity in planta. Os6 expression was shown in vegetative and reproductive tissues of rice. Our phylogenetic analysis suggests that rice has eight CGTs, of which five are CGTs (OsCOGT1, 2, 7 and 9; OsZOGT1, 2, 7 and OsZOGT1, 2, 5 and 6) and three are CNGTs: Os6–LOC (functionally confirmed by Li et al., 2019), Os01g91000, Os7DG-XM_015780390 and OsUDP773C-XM_015757779 (Figure S1).

Three UGTs (for consistency with the literature, arabidopsis CGTs will be referred to by their UGT prefix) capable of cytokinin glucosylation were originally identified and characterized in arabidopsis by Hou et al. (2004). UGT85S1 O-glucosylated iZ and cZ with similar activity, as well as DHZ, but not iZR. UGT76C1 and 76C2 were shown to be N-glucosylation specific (Hou et al.,...
2004), while Šmehilová et al. (2016) suggest that UGT76C1 may, in fact, be a dual function O/N-glucosyl transferase. However, UGT76C1 does not have the necessary dual O/N GT sequence (312N315Y) as reported by Brazier-Hicks et al. (2007) and, as both UGT76C1 and 76C2 fall into the same subcluster of NGTs (Figure S1), it appears unlikely that UGT76C1 is a dual function O/N-glucosyl transferase. Additionally, we have determined that only the CONGT subcluster aligns well to the structural aspects determined by Brazier-Hicks et al. (2007) for dual function (Figure 2).

Cucinotta et al. (2018) suggested that the UGT85A family consists of six members, A1,2,3,5 and 7, which are clustered next to each other on chromosome 1, and that the UGT73C family has seven GFMs, of which 1 to 6 are clustered in tandem on chromosome 2. While Hou et al. (2004) indicated that both UGT73C5 and UGT73C1 were COGTs that could recognize tZ and DHZ, Jin et al. (2013) suggested that UGT73C5 and 73C1 have only trace GT enzyme activity. Previously, UGT73C5 was characterized as a brassinosteroid-specific glucosyltransferase (Poppenberger et al., 2005) and UGT73C1 was shown to be specific to trinitrotoluene compounds (Gandia-Herrero et al., 2008) with much higher affinity than to cytokinins.

Differential expression is exhibited among CGT gene family members

The tZOGT from P. lunatus exhibited greater expression in very small seeds relative to roots and leaves (Martin et al., 1999a), whereas the maize cZOGT expression levels were highest in roots, but significantly lower in the cob and kernels, and were very low in leaves (Martin et al., 2001). Differential expression of two maize cis-ZOGTs was subsequently shown to occur: both cis-ZOGTs were highly expressed in roots but very weakly in leaves and stems of young plants; however, cisZOG1 expression increased as kernels matured, whereas cisZOG2 expression was low at all stages in kernels (Veatch et al., 2003). Although using one primer pair for both GFMs, Vyrobalová et al. (2009) showed a similar pattern of the cZOGTs with highest expression in the coleoptile + radicle and young roots. The mature leaf had very low expression, while moderate expression was observed in tassels.

Three rice GFMs, cZOGT1, 2 and 3, showed expression in most tissues examined, but with greater expression in leaves (Kudo et al., 2012). Detailed in situ expression analysis by Shang et al. (2016) showed rice OscZOGT1 clearly expressing in root meristem and lateral root primordia, as well as in crown primordia.
OscZOGT1 was also highly expressed in shoot meristematic tissues (both vegetative and floral) and during early seedling growth and tillering. Li et al. (2019) showed Os6 (CNGT) expression in vegetative and reproductive tissues of rice. Gasparis et al. (2019) refer to expression of four putative barley ZOGTs, three of which were differentially expressed in 7 DAP spikes following knockout of barley cxx1 or cxx3. Orthologues of TaZOG2 (TaCOGT2D031000.1) and TaZOG3 (TaCOGT3 A12S500.1) were up-regulated in both lines. A putative HvZOGT, which is an orthologue of TacZOGT1 (TacCOGT7D19 0600.1), was also up-regulated in the C3–21 line.

Jin et al. (2013) refers to the COGT, UGT85A1, as the ‘master’ OGT in arabidopsis. UGT85A1 was mainly expressed in tissues of young seedlings and in maturing embryos. Wang et al. (2011) focussed on N-glucosylation-specific UGT76C2 which was expressed in hypocotyls, cotyledons, young leaves, young lateral roots and immature seeds, but was only weakly expressed in inflorescences, especially inflorescence stems, and other tissues of arabidopsis. Expression of UGT76C1, which had less effect in bioassays relative to UGT76C2, was only apparent in germinating seeds and young seedlings (Wang et al., 2013).

Smehilová et al. (2016) assessed glucosyl transferase activity during leaf development and senescence of arabidopsis. Of the five UGTs monitored, UGT85A1 expression was the most noticeably increased in senescent leaves as was a sharp increase in iZOG and a decline in active forms, cZ and cZR increased, whereas cZOG decreased. However, iPG accumulated to much greater levels in senescent arabidopsis leaves than any other metabolite, although the expression of neither CNGT UGT76C1 nor 76C2 increased in leaves over time. As iPG is still regarded as a terminal metabolite (Hošek et al., 2020), constant gene expression could lead to the increasing levels of this N-glucoside over time (Smehilová et al., 2016).

Knowing that cytokinin levels are supra-optimal in roots (see Chen et al., 2020b), and that they are critically involved in shoot and root architecture (Guo et al., 2020; Kieber and Schaller, 2018), glucosylation is clearly a factor in cytokinin homeostasis in developing tissues and warrants further examination.

### Manipulating cytokinin activity through O-glucosylation

Compared to experiments in which expression of IPT or CXX has been manipulated, those manipulating CGTs are limited and are summarized in Table 1. Over-expression (OE) of COGT has led to some unexpected results: while the levels of O-glucosides were increased, there are several reports where the overall level of cytokinin and/or of free bases also increased, implying that cytokinin biosynthesis had been stimulated (Table 1). Moreover, increased chlorophyll and delayed senescence were reported, along with reduced apical dominance (Kudo et al., 2012; Martin et al., 2001; Pineda Rodó et al., 2008), reflecting increased active cytokinin. On the other hand, shoot growth was reduced while root growth increased reflecting a decrease in active cytokinin (Kudo et al., 2012; Pineda Rodó et al., 2008). Furthermore, in rice, while there were longer seminal roots, there was a reduction in the number of crown roots (Shang et al., 2016) reflecting the specific zones of cytokinin activity in roots (Chen et al., 2020b). Heterologous expression of P. lunatus iZOGT in maize caused a reduction in yield due to the formation of abnormal tassels (Pineda Rodó et al., 2008).

In contrast to over-expression of CGTs, the opposite characteristics were noted when cZOGT1 was targeted by RNA interference (RNAi) in rice (Shang et al., 2016) including, notably, increased yield. The shoot apical meristem (SAM) was enlarged, plant height and tiller number increased, panicles were longer, the grain number per panicle increased as did grain size. However, there was a decrease in lateral roots but an increase in crown root number. Senescence was similar to the WT (Table 1).

Recent experiments with arabidopsis COGTs gave broadly similar results to those with rice (Table 1): UGT85A3-OE led to reduced shoot stature, reduced ovule number and reduced seed number, whereas the ugt85a3 mutant had increased ovule and seed number (Cucinotta et al., 2018). However, earlier experiments with a different GFM showed both UGT85A1-OE lines and mutant ugt85a1-1 plants exhibited normal vegetative phenotypes (Jin et al., 2013; Smehilová et al., 2016). UGT85A1-OE showed increased iZOG, while other cytokinins remained similar to WT levels, again indicating an overall increase in cytokinin biosynthesis (Jin et al., 2013) (Table 1).

### Manipulating cytokinin activity by N-glucosylation

Only a limited number of experiments have been published in which the expression of CNGTs has been manipulated (Table 1). In contrast to the effects of over-expression and RNAi of COGT, similar experiments with arabidopsis CNGT, UGT76C2, showed little effect on vegetative growth, irrespective of whether levels of N-glucosides were increased or decreased (Wang et al., 2011, 2013). Over-expression led to a decrease in sensitivity to cytokinin in root assays but had no effect on seed weight, while the ugt76c2 mutant showed increased sensitivity to cytokinin. However, there was a marked decrease in both N-glucosides and total cytokinin in siliques, which contained seeds with reduced size and weight (Wang et al., 2011).

In summary, there is marked contrast in yield components depending on whether an arabidopsis CNGT or a COGT is mutated: decreased N-glucosylation decreased yield, whereas decreased O-glucosylation increased ovule and seed number in arabidopsis and also increased yield components in rice (Table 1). RNAi of a cereal CNGT would be of interest to compare with the contrasting effects observed in arabidopsis.

### Glucosyl transferases in wheat

#### O- and N-glucosides in wheat

There has been limited investigation of the full complement of O- or N-glucosides in wheat. Early investigators commented on the existence of O-glucosides (Jameson et al., 1982; Lenton and Appleford, 1986), but others have ignored them (e.g. Banowetz et al., 1999; Joshi et al., 2019; Morris et al., 1993). The majority of the isoprenoid cytokinins have now been detected in wheat (Benková et al., 1999; Jablonski et al., 2020; Sayavedra-Soto et al., 1988; Škyrová et al., 2008), but only recently has a comprehensive analysis of cytokinins in developing wheat grains been published (Nguyen et al., 2020). In developing wheat ovaries of five wheat cultivars, monitored from 1 to 2 days after anthesis (DAA) through to 21 DAA, significant amounts of CZOG, cZROG, DZROG and I29G were detected. Among the O- and N-glucosylated forms, only the level of I29G was reducing by 7 DAA which suggests turnover, which agrees with the report in Filipi et al. (2012) relating to the hydrolysis of I29G in maize. However, the levels of glucosides were all markedly less than the peak levels of iZ and cZ that occurred soon after anthesis. cZ nucleotide (NT) was detected at all stages, whereas iZNT had
| Plant     | CGT family member | Alteration†       | Cytokinin changes                                      | Morphological effect                                                                 | Reference                  |
|-----------|-------------------|-------------------|--------------------------------------------------------|---------------------------------------------------------------------------------------|----------------------------|
| Nicotiana tabacum | P. lunatus tZOGT1 | 35S OE            | ↑tZOG; ↑total cytokinin                               | Shorter stature; ↓apical dominance; ↓aerial roots; ↓sensitivity to tZ                  | Martin et al. (2001)       |
| Maize     | P. lunatus tZOGT1 | Ubiquitin OE      | ↑O-glucosides; ↑tZ in leaves                           | Slower development; Shorter stature; Smaller meristems; ↑root mass, length & branching; ↑chlorophyll; Delayed senescence; Feminization of tassels; Smaller seeds; ↓yield | Pineda Rodó et al. (2008) |
| N. tabacum | Maize tZOGT1      | 35S OE            | Dynamic changes in range of cytokinins with both promoters | ‘symptoms of cytokinin elevation in shoots’; ↑root length; ↑root branching; SAG12 faster recovery | Havlová et al. (2008)      |
| Rice      | Rice cisZOGT71 & 2| Actin OE          | ↑cis-O-glucosides                                      | ↓shoot growth; ↑chlorophyll; Delayed senescence; Longer seminal roots; Fewer crown roots | Kudo et al. (2012)         |
| Rice      | Rice cisZOGT71 OE | Actin OE          | ↑↑tZOG; ↑tZ, cis variable among OE and RNAi lines      | ↑lateral roots; ↓crown roots; ↓panicle size and branching; ↓grain number/panicle; ↓grain weight; Accelerated senescence; ↓lateral roots; ↓crown roots; Enlarged SAM; ↑height; ↑tiller number; Longer panicles; ↑branching; ↑grain number/panicle; ↑grain size; Similar senescence to WT | Shang et al. (2016)        |
| Arabidopsis | CNGT: UGT76C2    | 35S OE            | ↑N-glucosides; Free bases comparable to WT            | Normal vegetative growth; Normal seed weight; Decreased sensitivity to CK in root & chlorophyll retention assays | Wang et al. (2011)         |
|           | ugt76c2 mutant    |                   | ↑N-glucosides; ↑O-glucosides; Free bases comparable to WT; Siliques: ↑IP-N-glucosides; ↑Total cytokinin | Normal vegetative growth; Increased sensitivity to CK in root and chlorophyll retention assays; ↓seed size; ↓seed weight |                       |
| Arabidopsis | CNGT: UGT76C1    | 35S OE            | ↑tZNG                                                  | No morphological or sensitivity changes                                                | Wang et al. (2013)         |
|           | ugt76c1 mutant    |                   | ↑N-glucosides; Free bases comparable to WT            | No yield data provided                                                                |                           |
Phylogenetic analyses

Glycosyl transferases are a multigene superfamily in plants. To narrow down the potential CGT GFMs in bread wheat, we initially used 34 available CGT sequences published for other species as a query to perform a whole wheat proteome BLAST search. Considering that wheat is allohexaploid, consisting of the A, B and D sub genomes, the top three BLAST hits were obtained as CGT candidates. In total, 99 genes met the requirements, 42 of which were unique. However, we noticed that none of the 11 TaZOG candidates previously identified (Shoaib et al., 2019; Song et al., 2012) were included in the 42 genes. To determine the appropriate E value for the 11 TaZOG candidates, a HMMER model was developed using the 42 unique genes to identify the TaUDPGT super family genes. A total of 919 UDG sequences were obtained, which containing all the 42 BLAST hit genes and the 11 putative TaZOGs determined by Song et al. (2012) and Shoaib et al. (2019). The lowest E values covering the 42 CGTs and the 11 putative TaZOGs were 2.2e-16 and 1.3e-15, respectively. The results reflect the different effects of BLAST and HMMER model searches. We therefore suggest that 53 genes be accepted as putative TaCGT candidates, and the 1.3e-15 E value be used as the reference threshold in HMMER model identification.

Phylogenetic analysis showed that the UGT sequences grouped into two distinct clades, Clade A and the second clade consisting of sub-clades B to F at different orders (Figure S1; File S1). Clade A consisted of 15 wheat genes in three sub-clades, together with the functionally characterized UGT73C1 and 73C5 in one sub-clade, ZmZOG1 in the second and OsUDPGT73C in the third sub-clade. All the genes in Clade A are suggested to be tZOGTs.

The 13 wheat genes in sub-clade B are suggested to be cis-ZOGTs due to their high sequence similarity to functionally characterized ZmZOG1 and OsZOG1 from other species. Apart from the PIZOG1 in P. turgidum, the all genes in this sub-clade are from monocot species. Interestingly, PIZOG1 has long been accepted as a trans-ZOGT. Martin et al. (1999) stated that cis-zeatin was not a substrate for the recombinant protein of this gene. Multi-sequence alignment of PIZOG1 with cis- and trans-ZOGTs from a variety of species showed that PIZOG1 shared greater sequence similarity with cis-ZOGTs than with trans-ZOGTs (Figure S2). However, heterologous and constitutive over-expression of PIZOG1 in maize led to marked increase in tZOG and a lesser increase in cZOG, although the final level of cZOG in mature leaves of PIZOG1 over-expressing plants was slightly greater than that of tZOG (Pineda Rodó et al., 2008). We therefore suspect that PIZOG1 may be a cis-trans-dual function ZOGT.

The sub-clades C and D superimpose on top of the two parallel sub-clades E and F, and together are parallel to sub-clade B (Figure S1). Nine wheat genes in sub-clades C and D appear to be dual functional OGT/NGTs, as they grouped together with dual function GTs such as UGT72B1 from arabidopsis and Os6 from rice. Seven wheat genes in sub-clade E grouped alongside UGT85A1 from arabidopsis and ZmZOG2 from maize, suggesting that they are most likely OGTs. Sequences of nine putative wheat GT genes in sub-clade F were most similar to those of functionally characterized NGTs such as ATUG776C1 and C2 in arabidopsis, and ZmNGTs in maize. We suggest these genes are N-glucosyl transferases.

Based on the topology of the phylogenetic tree rooted with a UGT sequence from a gymnosperm species (Figure S1), we predict that, from an evolutionary point of view, the original cytokinin conjugation enzymes are likely to have been the O-glucosyltransferases. The cis-ZOGTs may have evolved early and been maintained in the monocots due to the prolific production of cis-isomers. This production may be related to loss of introns in class I tRNA-IPTs in the Poales clade shown by Nishii et al. (2018), who suggest this loss may affect the regulation of cis-type cytokinin production in these plants.

Hoyerová and Hošek (2020) stated that N-glycosylation seems to be an evolutionarily recent mechanism to inactivate biologically active cytokinins. In terms of NGTs, we suggest that partial N-glycosylation function was gained first in some family members of O-glucosyltransferases to generate the dual functional O/N-glycosyltransferases. This scenario is supported by data from Hou et al. (2004) who reported that some arabidopsis CGTs did not discriminate between cis- and trans-isomers and were able to

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Table 1  Continued

| Plant | CGT family member | Alteration | Cytokinin changes | Morphological effect | Reference |
|-------|-------------------|------------|-------------------|---------------------|-----------|
| Arabidopsis | COGT: UGT85A1 | 35S OE | ↑35S OE; others comparable to WT | Normal phenotype; ↑chlorophyll in the detached leaf bioassay | Jin et al. (2013) |
| Arabidopsis | COGT: UGT85A3 | 35S OE | No cytokinin analyses | | Cucinotta et al. (2018) |
| Arabidopsis | COGT: UGT73C1 | mutant | | | |
| Arabidopsis | ugt85a3 | mutant | | | |
| Arabidopsis | COGT: UGT73C1 | 35S OE | | | |

1Alteration: includes (i) use of promoters (e.g. Cauliflower mosaic virus 35S; tetracycline-inducible; ubiquitin; SAG12; actin) to drive over-expression (OE) of the named CGT gene in the named plant; (ii) RNA interference (RNAi); or (iii) named mutant.
glucosylate both Z isomers efficiently. Subsequently, we suggest some of the dual functional O/A-glucosyltransferases evolved into N-glucosyltransferases and others mutated back to O-glucosyltransferases. The side chain of ip-types is unavailable for O-glucosylation and, with the differentiation of the tz-types and ip-types into xylem- and phloem-specific signalling molecules (Sakakibara, 2006; Wheeldon and Bennett, 2020), N-glucosylation may have provided extra metabolic control.

Based on the phylogenetic relationship between wheat CGTs and identified CGTs and combining this with their chromosome position (Figure S3), we suggest unambiguous symbols for the TaCGTs (Tables 2 and S1; Figures 2 and S1) making five potential functional groupings of wheat CGTs: the COGTs, c/o-COGTs (cCOGT), CNGTs, CONGTs and CNOGTs. In terms of potential functionality, the COGT, cCOGT and CNGT clusters group with functionally characterized UGT members with high bootstrap values. These include OsZOG1, AtUGT85A1 (COGT), ATUGT76C1 and C2 (both CNGTs). However, the wheat CGTs remain to be functionally characterized. File S2 provides the protein sequences of the 53 CGT GFMs in Figure 2.

**Protein motifs**

Protein motif analysis showed that most of the motifs have a consistent pattern corresponding to the phylogenetic clustering: for example motifs 15 and 25 are specific to the potential bifunctional CGTs, TaCONGT and TaCNOGT, respectively; motif 9 is specific to cCOGTs; and motif 18 is specific to CNOGTs (Figure S4). As mentioned earlier, the CONGT subcluster aligns well to the structural aspects determined by Brazier-Hicks et al. (2007) for dual function (Figure 2).

**CGTs lack introns**

The early reports on CGTs highlighted the fact that neither *P. lunatus* t2ZOGT nor maize cZOGT contained introns (Martin et al., 1999b; 2001). More recently, of the seven wheat CGTs reported by Shoaib et al. (2019), TaZOG2 had one intron, while the remainder had no introns. By gene transfer format analysis, we show that 19 of the 53 TaCGTs have one obvious intron within the coding region, one has two introns in the coding region and a further five have introns in the UTR or have uncertain predictions, while the remaining 28 have no introns. The 48 genes with reliable structures show consistent phylogenetic patterns (Figure S5). However, in all cases the CGTs could be described as intron-less or intron poor (Liu et al., 2021). In contrast, many of the putative TaUGTs, with low E value, are intron-rich (Figure S6). Liu et al. (2021) speculate that loss of introns may be a factor in early land plant evolution allowing fast response to stress conditions and also may have facilitated faster developmental adaptations to life on land. Indeed, Vyroubalová et al. (2009) commented that O-glucosylation ‘is a mechanism by which the plant cell could react very fast to the disturbed homeostasis induced by physiological stimuli’. Moreover, several CKX and CGT gene family members are regarded as primary-response genes in arabidopsis (e.g. Brenner et al., 2012; Zubo and Schaller, 2020).

**Subcellular location of CGTs**

Over 90% and 85% of arabidopsis and barley leaf cytokinins, respectively, were determined to be located to the extracellular space (Jiskrová et al., 2016). Moreover, the majority were TPNG and iTPNG (arabidopsis) or Z9G and cZOG (barley). The location of the CGTs is, therefore, of interest. It has generally been considered that plant UGTs for secondary products are cytoplasmic enzymes (Ross et al., 2001). However, GFP-tagged UGT76C2 (Šmehlíková et al., 2016), COGT UGT85A1 (Jin et al., 2013) and OsZOG1-GFP (Shang et al., 2016) show localization to both cytosol and nucleus.

Shoaib et al. (2019) reported that of their seven wheat ZOGTs, three of the four TaZOGT proteins were predicted to be localized to the plasma membrane. In contrast, all three cZOGTs were predicted to be ‘secretory in nature’. We used four online databases, Cell-Plloc 2.0, pLoc_Deep-MPlant, WoLF PSORT and ProtComp (Table S2), to help predict the locations of the TaCGTs. Clearly, the information from these databases differs (as also shown by Šmehlíková et al., 2016) with the most consistent allocation across the databases being to chloroplasts, but with no pattern among the five groups of CGTs. Based on data from WoLF PSORT (which is currently the most cited database for predicting protein locations), there is strong support for CGTs additionally being located to the cytoplasm, and weaker support for mitochondria, vacuole, ER, peroxisome, nucleus and extracellular.

With cytokinin biosynthesis occurring in plastids (Kasahara et al., 2004), it is perhaps not surprising that enzymes involved in homeostasis are located likewise. The full spectrum of cytokinins (*Z*, *DHZ*- and ip-free bases, ribosides and nucleotides) as well as Z9G, DZ9G and iP were detected in chloroplasts of tobacco and wheat; and Z7G, DZ7G and ZOG and DZOG in tobacco chloroplasts. Clearly, the potential for N- and O-glucosylation exists in chloroplasts, along with CKX activity (Benková et al., 1999). Predicted location to the cytoplasm is as expected (Šmehlíková et al., 2016), although this is not strongly indicated for the cCOGTs. However, resolution of the location of the wheat CGTs requires reporter gene tagging of the different CGT GFMs, as clearly database predictions and reporter gene tagging results can differ (Šmehlíková et al., 2016).

**Expression of CGTs in wheat**

Recently, Nguyen et al. (2020) analysed the expression of the three cCOGTs in wheat 1-2, 4 and 7 DAA. Generally, the expression of cZOGT1, cZOGT2-1 and cZOGT2-2 was elevated across the three stages examined with cZOGT2-2 being the most strongly expressed and particularly elevated at 1-2 DAA. Importantly, Nguyen et al. (2020) note that the levels were variable among their five wheat cultivars, with the higher yielding cultivars having lesser expression of the cZOGTs.

The three cZOGs referred to above were originally isolated by Song et al. (2012). We have now identified these as the A, B and D alleles of the same locus (Figures 2 and S3; Tables 2 and S1): TaZOGT1 (TraesCS2D02G407200) is on chromosome 2D; TaZOGT2-1 (TraesCS2A02G410000) is on chromosome 2A; and TaZOGT2-2 (TraesCS2B02G428300) is on chromosome 2B. Song et al. (2012) showed differential expression among these three GFMs: TaZOGT1 (2D) peaked in 10 cm spikes and was again highly expressed in ovules just before and after pollination. TaZOGT2-2 (2B) was similarly highly expressed between 1 and 7 DAA, whereas expression of TaZOGT2-1 (2A) was much less.
There was little expression of \( tZOGTs \) in spikes, ovules/seeds or flag leaves. In the flag leaf, \( cZOGT1 \) (2D) and \( TacZOGT2-1 \) (2A) increased in expression post-anthesis, with \( cZOGT1 \) (2D) increasing markedly as the flag leaf senesced, whereas \( TacZOG2-2 \) (2B) showed little expression in the flag leaf (Song et al., 2012).

### Table 2: Correspondence of TaCGTs gene ID, gene symbol and former name

| Gene ID | Gene symbol | Song et al. (2012) | Shoaib et al. (2019) |
|---------|-------------|--------------------|----------------------|
| TraesCS3B02G143000.1 | TaCOGT3B143000.1 | TaZOGG3 | TaZOG3AA |
| TraesCS3B02G144800.1 | TaCOGT3B144800.1 | TaZOG1 | TaZOG4DD |
| TraesCS3A02G125200.1 | TaCOGT3A125200.1 | TaZOG3 | TaZOG3BB |
| TraesCS3D02G126300.1 | TaCOGT3D126300.1 | TaZOG4AA |
| TraesCS3A02G125500.1 | TaCOGT3A125500.1 | TaZOG4BB |
| TraesCS5A02G144600.1 | TaCOGT5A144600.1 | TaZOG1BB |
| TraesCS7A02G189500.1 | TaCOGT7A189500.1 | TaZOG1DD |
| TraesCS7B02G094400.1 | TaCOGT7B094400.1 | TaZOG1 |
| TraesCS7D02G190600.1 | TaCOGT7D190600.1 | TaZOG1 |
| TraesCS3A02G119000.1 | TaCOGT1D119000.1 | TaZOG1 |
| TraesCS3B02G369200.1 | TaCOGT3B369200.1 | TaZOG1 |
| TraesCS3B02G467800.1 | TaCOGT3B467800.1 | TaZOG1 |
| TraesCS5A02G527900.1 | TaCOGT5A527900.1 | TaZOG1 |
| TraesCS5A02G383900.1 | TaCOGT5A383900.1 | TaZOG1 |
| TraesCS5B02G388700.1 | TaCOGT5B388700.1 | TaZOG1 |
| TraesCS5D02G393300.1 | TaCOGT5D393300.1 | TaZOG1 |
| TraesCS3B02G034800.1 | TaCONGT3B034800.1 | TaZOG1 |
| TraesCS3D02G001400.1 | TaCONGT3D001400.1 | TaZOG1 |
| TraesCS2A02G010300.1 | TaCONGT2A010300.1 | TaZOG1 |
| TraesCS2B02G428800.1 | TaCONGT2B428800.1 | TaZOG1 |
| TraesCS2D02G407500.1 | TaCONGT2D407500.1 | TaZOG1 |
| TraesCS7A02G369200.1 | TaCOGT7B369200.1 | TaZOG1 |
| TraesCS7A02G467800.1 | TaCOGT7A467800.1 | TaZOG1 |
| TraesCS5D02G031300.1 | TaCOGT5D031300.1 | TaZOG1 |
| TraesCS2B02G428300.1 | TaCOGT2B428300.1 | TaZOG1 |
| TraesCS2D02G407200.1 | TaCOGT2D407200.1 | TaZOG1 |
| TraesCS2B02G428500.2 | TaCOGT2B428500.2 | TaZOG1 |
| TraesCS2D02G407300.1 | TaCOGT2D407300.1 | TaZOG1 |
| TraesCS2A02G041000.1 | TaCOGT2A041000.1 | TaZOG1 |
| TraesCS3A02G010300.1 | TaCOGT3A010300.1 | TaZOG1 |
| TraesCS2A02G0474700.1 | TaCOGT2A0474700.1 | TaZOG1 |
| TraesCS2B02G498300.1 | TaCOGT2B498300.1 | TaZOG1 |
| TraesCS2D02G031000.1 | TaCOGT2D031000.1 | TaZOG1 |
| TraesCS2B02G031000.1 | TaCOGT2B031000.1 | TaZOG1 |
| TraesCS3B02G533100.1 | TaCOGT3B533100.1 | TaZOG1 |
| TraesCS4B02G388700.1 | TaCOGT4B388700.1 | TaZOG1 |
| TraesCS5A02G553100.1 | TaCOGT5A553100.1 | TaZOG1 |
| TraesCS4B02G070700.1 | TaCOGT4B070700.1 | TaZOG1 |
| TraesCS5A02G553100.1 | TaCOGT5A553100.1 | TaZOG1 |
| TraesCS5B02G500700.1 | TaCOGT5B500700.1 | TaZOG1 |
| TraesCS5D02G501000.1 | TaCOGT5D501000.1 | TaZOG1 |
| TraesCS6B02G079600.1 | TaCOGT6B079600.1 | TaZOG1 |
| TraesCS6A02G059300.1 | TaCOGT6A059300.1 | TaZOG1 |
| TraesCSU02G121700.1 | TaCNGTU121700.1 | TaZOG1 |

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TacZOG2-2 (2B) showed strong expression across a range of cultivars immediately post-anthesis (Nguyen et al., 2020; Song et al., 2012). As this wheat GFM is closely related to rice cZOG1, which had increased yield when expression was decreased by RNAi, it may be a useful GFM to target via TILLING or gene editing. Moreover, as TacZOG2-2 (2B) showed little expression in the flag leaf (Song et al., 2012), decreased expression may not lead to delayed senescence in the flag leaf and establishing it as a cultivar immediately post-anthesis (Nguyen et al., 2020). Increased expression of RNAi, it may be a useful GFM to target via TILLING or gene editing in the stay green wheat mutant, TacZOG1, with the increasing expression of cZOG1 (2D) being reduced.

Recently, Wang et al. (2019) showed reduced expression of TacZOG1 in the stay green wheat mutant, tasg1. This aligns well with the increasing expression of TacZOG1 (2D) demonstrated in senescing leaves (Song et al., 2012). Knockout mutants of the ‘cisZOGT1-B locus’ in a Durum wheat mutant line (i.e. the B genome) showed delayed senescence (Wang et al., 2019), which is in contrast to little expression of the equivalent GFM (TacZOG2-2 (2B)) in the flag leaf of bread wheat cv. Equinox (Song et al., 2012) – but see below.

A total of 850 RNA-seq data sets obtained from expVIP (http://www.wheat-expression.com/, Ramirez-González et al., 2018) provide extensive expression analysis of CGTs both within organs and over time in bread wheat (Figures 3 and S7). However, there is a notable lack of tissues analysed between the critical developmental stages of anthesis and maturity stage.

Clearly, there are a large number of cis- and trans-COGTs, CNGTs and putative dual COGT GFMds in bread wheat. As a general overview, several CGTs are constitutively expressed (e.g. COGT3A125500 and CONGTA010300 (except endosperm at dough)), while others have no (e.g. CONGTD001400) or little (e.g. COGT1D119000; COGT5A444500; COGT5A383900; COGT5B388700; COGT5D393300; COGT5A553100 CONGT68079600; CONGTU121700) expression in the wheat tissues analysed.

At any stage of development, there is expression of a CGT, with the most limited expression being in grain at soft dough. As a group, the cCOGTs show little expression in the developing grain which is in general agreement with the relatively low expression detected in the RT-qPCR analyses from both Song et al. (2012) and Nguyen et al. (2020). However, expression of CONGTA3, B and D, CONGTA010300, and several CNGTs is evident in the developing grain. There is a general increase in expression of CGTs in grain at ripening.

In-depth investigation of the heat maps reveals various patterns in the roots and leaves: several GFMds of the various types of CGTs are not expressed in root tissues, whereas COGT2D031300 expression is limited to root tissues. COGT7B094400 is one of the more highly expressed GFMds in the 5th leaf blade and flag leaf, whereas COGT3D126300 and 3B144500 were the more highly expressed GFMds in the 1st and 3rd leaf blades. While several cis-COGTs and COGTs expressed in both developing and senescing 5th and flag leaves, expression of COGT2B428300, CNGTs and CNGTs increased as these leaves aged. This increasing expression of COGT2B428300 (i.e. TacZOG2-2) in cv. Chinese Spring, while not detected in flag leaves of Equinox, may explain the delayed senescence in the mutant Durum wheat line reported by Wang et al. (2019). Expression of TacZOGT1 (TacCOTG2D407200) showed a more specific increase in senescing flag leaves – in agreement with Song et al. (2012).

Responsiveness of CGTs to abiotic and biotic stressors

Abiotic stress

There is consistent evidence that cytokinin acts as a negative regulator of, among others, drought/osmotic /saline stress resistance (reviewed in Cortleven et al., 2019; Pavlú et al., 2018). The negative regulation has been associated with a decline in active cytokinin in above ground parts, a differential response between lower leaves and upper leaves (establishing a gradient of active cytokinin in favour of the upper leaves) and an increase in active cytokinin in roots (e.g. Havlová et al., 2008 in tobacco). The negative regulation was further confirmed through functional analysis in cytokinin-deficient abaridopsis plants (Nishiyama et al., 2011).

In response to osmotic stress in 7-d-old maize seedlings, cZOGT expression decreased in shoots but increased in roots (Vyrubalová et al., 2009), which could be in response to the gradient of active cytokinins referred to above. Vyrubalová et al. (2009) comment that O-glucosylation ‘is a mechanism by which the plant cell could react very fast to the disturbed homeostasis induced by physiological stimuli.’

Over-expression or reduced expression of CGTs and assessment of stress responsiveness is relatively limited. Havlová et al. (2008) used 6-week-old tobacco expressing a maize tZOGT under control of either the constitutive CaMV 35S promoter or the drought-inducible SAG12 promoter as their model to investigate response to drought/rewatering. In the SAG12::tZOG plants, expression increased in the older leaves relative to control and reduced rapidly after rewatering. Increases in total cytokinin in the lower leaves were mostly O- and N-glucosides. This increase was associated with a delay in senescence of the stressed older leaves, and a generally faster recovery, presumably from cytokinin released by β-glucosidase that had been protected from degradation by CKX during the drought.

Manipulation of CGTs and response to stress was investigated more recently by Li et al. (2015), focusing on AtUGT76C2 (an arabidopsis CNGT). Expression of 76C2 was down-regulated when the arabidopsis plants were exposed to drought, osmotic stress and abscisic acid (ABA) and UGT76C2pro::GUS seedlings showed decreased GUS over time after stress exposure. As expected, mutant and over expresser lines showed different effects: mutant ugt76C2 lines germinated faster on mannitol, and seedlings were more tolerant of mannitol and ABA compared with 76C2-OE lines. However, in contrast, the more mature over-expressing plants were more tolerant of stress and wilted less, lost less water, had reduced stomatal aperture and had greater survival than the mutants or WT. Stress-related genes were more up-regulated in the 76C2-OE lines, while cytokinin signalling was down-regulated, providing supporting evidence of cytokinin as a negative regulator of stress.

We conducted an analysis of the cis-regulatory elements (CRE) of the S3 TaCGT genes, analysing the 3 kb putative promoter sequences. The data set was submitted to the plantCARE database (Chou and Shen, 2007; 2008; 2010) for CRE analysis. A total of 12,016 CREs were detected (Table S3), in which there are 1979 annotated biotic-, abiotic- and hormone-responsive elements. These regulatory elements are shown in bar graphs (Figure S8). Multiple elements were detected, and these are highly variable in nature and position. To determine whether the CREs are over- or under-represented within the 3 kb putative promoter sequences of the CGTs, we compared them with those on 100 randomly selected wheat gene promoters (Table S4; File S3). These data are presented as heat maps (Figure 4).

Not unexpectedly, ABA-responsive elements are over-represented on many CGT promoters (Figure 4), indicative of the significant and frequently antagonistic crosstalk between ABA and cytokinins (Zubo and Schaller, 2020). There is less consistency with
auxin- and gibberellin-responsive elements, being both over- and under-represented on the CGTs, and with no obvious phylogenetic pattern. However, no cytokinin- or ethylene-responsive elements were located in the 3 kb region of the CGT promoters. It is well known that increases in cytokinin are frequently paralleled by increases in CKX (Jameson and Song, 2016), but as certain CGTs have been identified as primary-response genes to cytokinin (Brenner et al., 2012), this lack of cytokinin-responsive elements is somewhat surprising. However, such elements may be located beyond the 3 kb region analysed (Liu et al., 2019).

Light-responsive elements appear over-represented among the CGTs (Figure 4). Cytokinins play a key role in the response of the plant to the light environment (reviewed in Cortleven et al., 2019; Pavlu et al., 2018). As the identification of CREs does not reveal whether they interact with, for example, promotive or repressive transcription factors, only further investigation can reveal in what way CGTs are regulated by light.

As a general overview, the several CGTs which were shown to be constitutively expressed (e.g. COGT3A125500) had an over-representation of both ABA- and light-responsive elements but other CREs were generally under-represented, particularly with respect to biotic response elements (Figure 4). CONGT3A010300 (constitutive except in endosperm at dough) also had relatively few over-represented CREs in addition to ABA and light. Other CGTs which had no or little expression during normal development exhibited a range of elements: for example both ABA- and light-responsive elements are over-represented on COGT1D119000 and COGT5B388700 has an over-representation of drought- and ABA-responsive elements, as well as to methyl jasmonate and salicylic acid. Clearly, the CGTs are a critical component of the response of the plant to its environment.

In a similar manner to the heat map showing arabidopsis cytokinin metabolism gene responses to environmental cues, and which included two NGTs (Ramireddy et al., 2014), we show a heat map of bread wheat CGTs in response to various abiotic and biotic stresses (Figure 5). To compile the heat map, the raw data were downloaded from the expVIP database (http://www.wheat-expression.com/, Table S5). While Ramireddy et al. (2014) were able to restrict their analysis predominantly to Col-0 accessions, caution needs to be exercised with Figure 5, as here the compilation includes different cultivars of bread wheat.

As a general overview, Cluster A (COGTs), Cluster B (cis-COGTs), Cluster C1 (CNOGTs) and Cluster E (CNGTs) were variably responsive to the applied stressors, whereas Cluster C2 and particularly Cluster F COGTs (which we suggest are more recently evolved) were noticeably less responsive. The wheat CGTs that were constitutively expressed (Figure 3) showed modest responses to the stressors (e.g. COGT3A125500; CONGT3A010300 (Figure 5)), while CONGT3D001400, whose expression was not detectable during development, showed a general up-regulation to stressors, as might be predicted by its range of over-represented CREs (Figure 4).

Those GFMs showing low level responses during development generally showed minimal responses to abiotic and biotic stress although this did not necessarily correlate strongly with the representation of CREs (Figures 4 and 5). For example, COGT1D119000 showed minimal response to stress but several CREs are over-represented, especially ABA-RE; COGT5B388700 showed minimal response to stress but ABA-, drought- and SA-responsive elements are over-represented; CNGT6B079600 showed only a slight up-regulation to biotic stress but has over-representation of several CREs.
Analysis of the series of drought/heat/water/osmotic stress experiments on bread wheat shows considerable variation in the expression of CGT GFMs (Figure 5). Under the combined drought and heat stress, by 6 h almost all responding GFMs showed down-regulation, whereas after 6 h of drought or heat stress alone there was a more general up-regulation of responding CGTs. However, responses within the different CGTs were variable to the individual stressors and varied between cultivars. For example, in terms of osmotic stress induced by PEG6000, the COGTs that were up-regulated in one cultivar were down-regulated in the other. Generally, the CNGTs were more likely to be down-regulated in response to the abiotic stressors. Similarly, Ramireddy et al. (2014) showed a general down-regulation of UGT76C2 in arabidopsis leaves subjected to drought or heat but a more variable response by UGT76C1 (Ramireddy et al., 2014).

Cortleven et al. (2019) suggest that a function for cytokinin in response to cold stress has not been well demonstrated. However, there was clear up-regulation of 11 CGT GFMs in shoots exposed to two weeks of cold and also down-regulation of three GFMs, although the more strongly expressed GFMs did not necessarily have an over-representation of low-temperature-REs (Figures 4 and 5). The strong up-regulation of both cis- but especially trans-COGTs may be indicative of a reduction in active cytokinin, as occurs in response to many stressors. As CONGT3B034800 and CONGT3D461200 were down-regulated, and O-glucosylolation up-regulated, this may conserve cytokinin for reactivation. Interestingly, Ramireddy et al. (2014) showed down-regulation of NGT UGT76C2 in response to cold.

Biotic stress

There is an extensive literature on the involvement of cytokinins in plant-microbe interactions (see recent reviews by Akhtar et al., 2020; Albrecht and Argueso, 2017; Cortleven et al., 2019; Spallek et al., 2018), with the most well-known cytokinin-producing pathogens being the gall-forming bacteria (Jameson, 2000), where the cytokinin (and auxin) produced affect organogenesis and nutrient acquisition (e.g. Dhandapani et al., 2017; Spallek et al., 2018). A general picture suggests that supplementation of cytokinin can enhance or reduce resistance to pathogens, the ‘cytokinin-induced immunity’ vs ‘cytokinin-induced susceptibility’ as described by Albrecht and Argueso (2017). Cytokinins have also been shown to have plant-protective effects: for example, cytokinin production by Pseudomonas fluorescens G20-18 determines its biocontrol activity against Pseudomonas syringae in arabidopsis (Großkinsky et al., 2016). Spallek et al. (2018) suggest that ‘the prevalence of pathogenic strategies to produce or modify host plant cytokinin homeostasis across different kingdoms of plant pathogens speaks to its biological relevance’.

Defence against biotrophic pathogens involves crosstalk between cytokinins and salicylic acid (SA) (summarized in Zubo and Schaller, 2020). It is notable that there is an over-representation of SA-responsive elements scattered among the CREs of the CGTs (Figure 4). While clear links have been established between the cytokinin and SA interaction, such links have not been well-established for cytokinin–jasmonate interactions (see Cortleven et al., 2019) although Zubo and Schaller (2020) suggest any interactions are generally viewed as antagonistic. Methyl-JA responsive elements are over-represented in the CRE heat map (Figure 4). Wounding through insect chewing is well known to up-regulate JA signalling (Schuman et al., 2018), but as wound-responsive elements are under-represented (Figure 4), it would appear that changes in cytokinin responses are downstream of the wound event itself. However, it now appears that certain biotrophic fungal species can also trigger the activation of JA-mediated responses, suggesting a role for JA signalling in resistance against biotrophs, such as powdery and downy mildews (see Guerreiro et al., 2016).
GFMs responsive to abiotic stress were generally responsive to biotic stressors, but the opposite was not necessarily the case. For example, COGT3B144800, COGT3A125200, and COGT3D126300 were much more responsive to biotic stress than to abiotic stress and were strongly up-regulated in response to *Z. tritici*, stripe rust, and powdery mildew particularly. A consistent pattern of up-regulation of CGTs occurred in response to *Z. tritici*, a filamentous, ascomycete fungus that causes the important foliar disease of wheat, septoria tritici blotch (STB), and to stem rust and powdery mildew. Responses to *Fusarium* were less consistent across the different wheat cultivars and CGTs, but cluster A COGTs were generally down-regulated.

These variable responses are not surprising considering the ‘cytokinin-induced immunity’ vs ‘cytokinin-induced susceptibility’ as described by Albrecht and Argueso (2017), but also highlight the risk of moderating cytokinin levels without considering susceptibility to biotic challenges. As cytokinins are involved in the growth-defence trade-offs (Cortleven et al., 2019), it is highly likely that cytokinin glucosylation will play a role in the reduction of growth, through reduction of active cytokinin levels, in favour of defence.

**Future perspectives**

Cytokinin glucosyl transferases warrant in-depth investigation as they are clearly implicated in responses of the plant to both their

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**Figure 5** Expression patterns of TaCGTs under different stress conditions. The raw data were downloaded from expVIP (Table S5). The expression values are shown in fold change (FC): \( FC = \frac{\text{Control/Treat}}{} \). To avoid a meaningless calculation when the denominator was zero, 0.001 was added to both the numerator and denominator. The FC data were normalized using log2 transformation. The heat map was generated using the image function of the R program and merged using Photoshop CS6. *Magnaporthe* OS: *Magnaporthe oryzae* symptomatic; *Spikelets_PLR*: only using palea, lemma and rachis; PAMP: Pathogen-associated molecular pattern; *Fusarium* Gra.: *Fusarium graminearum*; *Fusarium* Pse: *Fusarium pseudograminearum*. The raw data and experimental details are in Table S5. The phylogenetic tree is consistent with Figure 2.

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internal and external environments. At a fundamental level, their sub-cellular location remains an open question while, from an evolutionary perspective, whether the NGTs derived originally from OGTS requires further investigation, as does the relative timing of the evolution of control through degradation (CKX) versus glucosylation (CGTs).

From an applied perspective, progress has been made with agronomic species by manipulating internal cytokinin levels through controlling expression of IPT or CKX. Following work with rice and arabidopsis, both Shang et al. (2016) and Cucinotta et al. (2018) concluded that CGTs are also good candidates for improving components of seed yield in agronomically important plants. However, as summarized in Table 1, while the COGT mutant, ugt76c2, showed decreased seed size and seed weight (Wang et al., 2011). Importantly, in rice, both grain number/panicle and grain size were increased when zOGT1 expression was decreased by RNAi (Shang et al., 2016), so yield components have been increased in both a monocot and a dicot on reduction of, specifically, COGT activity. Moreover, Nguyen et al. (2020) noted that the levels of expression of the COGTs were variable among their five wheat cultivars, with the higher yielding cultivars having lower expression of the zCOGTs.

Wang et al. (2011) showed that the expression of several genes changed and suggested that the decreased IPT5 and increased CKX3 expression in the ugt76c2 mutant reflected a homeostatic response to the reduced N-glucosylation, leading to the decrease in seed yield, whereas enhanced cytokinin biosynthesis has been reported following reduced O-glucosyla-
tion (Table 1). We suggest that targeting COGTs may be a ‘softer’ approach as O-glucosides are not terminal metabolites, whereas inhibiting N-glucosylation (especially of the CKX-resistant 7-N-Gs) could lead to an over-abundance of active cytokinin, and a strong feedback response to both degrade cytokinin and reduce biosynthesis.

Given that loss-of-function mutations of COGTs showed increased seed yield and stress tolerance, identification of specific CGT genes could be of importance for genetic improvement of bread wheat. Based on our wheat mutant gene bank covering 99% of the high-confidence genes in the IWGSC RefSeq v2.0, developed through whole-exome capture of a TILLING population of cultivar Jima22 (Chen et al., 2020b), we have identified multiple mutations for all of the 53 TaCGT genes listed in this work, with 21.8 missense mutations and 1.0 stop-gained mutation per gene on average. Jima22 was the most widely grown wheat cultivar in China over the last decade. Serial backcrossing and stacking of triple mutants for ABD sub genomes is currently being undertaken prior to phenotype evaluations and incorporation into wheat breeding programmes.

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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 relevant figures. J.S. interrogated the phylogenetic data and prepared the relevant figures. J.Z. accessed and interrogated the databases for sub-cellular location and constructed Figure 1. P.E.J wrote the first draft of the manuscript 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 tree for 93 CGTs in bread wheat and selected monocot and dicot species.

Figure S2. Multi-sequence alignment of PlZOG1 with cis- and trans-ZOGTs from other species.

Figure S3. The chromosome locations of TaCGTs.

Figure S4. Protein motif patterns of TaCGTs.

Figure S5. Gene structures of TaCGTs.

Figure S6. Intron locations of TaUGTs.

Figure S7. Expression patterns of TaCGTs at different developmental stages.

Figure S8. Distribution patterns of hormone-, biotic- and abiotic-responsive cis-elements in the 3 kb putative promoter regions of TaCGTs.

Table S1. Correspondence of TaCGT gene ID, symbol, and homolog.

Table S2. Database predictions of sub-cellular locations of TaCGTs.

Table S3. Data sets of cis-regulatory elements in the 3 kb putative promoter regions of TaCGTs.

Table S4. Cis-regulatory element information obtained from 100 random wheat genes.

Table S5. Data sets of TaCGT expression patterns under different stressors.

File S1. Protein sequences of the 93 CGT GFMs in Figure S1 phylogenetic tree.

File S2. Protein sequences of the 53 CGT GFMs in Figure 2 phylogenetic tree.

File S3. Promoter sequences of 100 randomly sampled wheat genes used to develop Figure 4 and Table S4.

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