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Review Article

The *LONELY GUY* gene family: from mosses to wheat, the key to the formation of active cytokinins in plants

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Summary

LONELY GUY (LOG) was first identified in a screen of rice mutants with defects in meristem maintenance. In plants, *LOG* codes for cytokinin riboside 5′-monophosphate phosphoribohydrolase, which converts inactive cytokinin nucleotides directly to the active free bases. Many enzymes with the PGGxGTxxE motif have been misannotated as lysine decarboxylases; conversely not all enzymes containing this motif are cytokinin-specific LOGs. As LOG mutants clearly impact yield in rice, we investigated the *LOG* gene family in bread wheat. By interrogating the wheat (*Triticum aestivum*) genome database, we show that wheat has multiple *LOG*s. The close alignment of TaLOG1, TaLOG2 and TaLOG6 with the X-ray structures of two functional *Arabidopsis thaliana* LOGs allows us to infer that the wheat LOGs 1-11 are functional LOGs. Using RNA-seq data sets, we assessed *TaLOG* expression across 70 tissue types, their responses to various stressors, the pattern of *cis*-regulatory elements (CREs) and intron/exon patterns. *TaLOG* gene family members are expressed variously across tissue types. When the *TaLOG* CREs are compared with those of the cytokinin dehydrogenases (CKX) and glucosyltransferases (CGT), there is close alignment of CREs between *TaLOGs* and *TaCKXs* reflecting the key role of CKX in maintaining cytokinin homeostasis. However, we suggest that the main homeostatic mechanism controlling cytokinin levels in response to biotic and abiotic challenge resides in the CGTs, rather than LOG or CKX. However, *LOG* transgenics and identified mutants in rice variously impact yield, providing interesting avenues for investigation in wheat.

Introduction

The cytokinins are involved in most aspects of plant growth and development, and there is significant interest in their role in enhancing yield (e.g. Jameson and Song, 2020; Liu *et al*., 2021; Nguyen *et al*., 2021a; Schwartz *et al*., 2020; Werner *et al*., 2021), including wheat (e.g. Beznec *et al*., 2021; Chen *et al*., 2020a, 2021a; Jablonski *et al*., 2020, 2021a, 2021b; Nguyen *et al*., 2020; Ogonowska *et al*., 2019). Historically, cytokinin biosynthesis in plants was considered a two-step process: adenosine phosphate-isopentenyl transferase (IPT) adding an isoprenoid side chain to an adenosine di- or triphosphate (ADP/ATP) to form inactive cytokinin nucleotides (Kakimoto, 2001; Takei *et al*., 2001), which were then predicted to be converted by a nucleotidase to form the ribosides (Chen and Kristopeit, 1981a), and then by a nucleosidase to form the bioactive free bases (Chen and Kristopeit, 1981b; Figure 1). However, while the reverse reactions (free base (nucleobase) to nucleotide via phosphoribosyl transferases; and riboside to nucleotide via adenine kinases) have been shown to occur via the purine salvage pathway (Chen *et al*., 1982; see reviews by Ashihara *et al*., 2018 and Witte and Herde, 2020), the genes/enzymes responsible for the two-step forward reaction have not been identified in *Arabidopsis thaliana* (arabidopsis) or rice (*Oryza sativa*) (Kurakawa *et al*., 2007; Nguyen *et al*., 2021b), although a cytokinin riboside phosphorylase capable of interconverting cytokinin free bases and ribosides was identified in potato (*Solanum tuberosum*) stolons (Bromley *et al*., 2014). In a breakthrough paper, Kurakawa *et al*. (2007) showed that a one-step reaction took place in rice converting the inactive cytokinin nucleotides directly to the active free base forms. This reaction was controlled by LONELY GUY (LOG) (Figure 1). LOG is named after the rice mutant that had only one stamen and no pistil (hence *lonely guy*) and was discovered in a screen for defects in meristem maintenance (Kurakawa *et al*., 2007). LOGs, which exhibit cytokinin riboside 5′-monophosphate phosphoribohydrolase activity, have been misannotated as lysine decarboxylases (Jeon *et al*., 2006; Kurakawa *et al*., 2007; Naseem *et al*., 2018).

In this review, using both sequence analysis and structural biology, we comment on the mega gene family to which the cytokinin nucleotide-specific phosphoribohydrolases belong, consider the wider role of LOG *in planta,* and delve into the wheat genome to assess the potential utility of *LOG* in wheat breeding.

Evolutionary breadth of the *LOG* gene family

LOG gene family in rice

As mentioned above, the *lonely guy* rice mutant was identified in a screen for meristem mutants. *LOG* is expressed in the shoot

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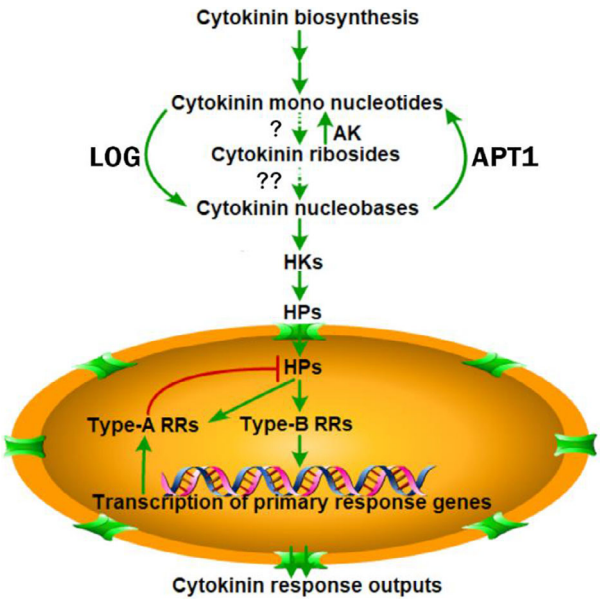

Figure 1 Outline of cytokinin biosynthetic and signal transduction pathway. Cytokinin riboside 5′ monophosphates (cytokinin mononucleotides) are converted in one step by LONELY GUY (LOG) to the active cytokinin free bases (cytokinin nucleobases). In the putative two-step process, '?' refers to the paucity of support for cytokinin-specific nucleotidases to convert the cytokinin nucleotides to ribosides, and '??' refers similarly for cytokinin-specific nucleosidases to convert cytokinin ribosides to nucleobases. Back conversion of nucleobases to nucleotides can occur via adenine phosphoribosyl transferase (APT1; Witte and Herde, 2020) and of ribosides to nucleotides by adenosine kinase (AK; Sakakibara, 2021). Cytokinin signal transduction is mediated by a two-component system (TCS), consisting of histidine protein kinase receptors that sense the input and response regulators (RR) that mediate the output (Wang and Sheen, 2001). Cytokinin free bases are detected by histidine-kinase receptors (HKs) on the ER (Romanov *et al*., 2018) or plasma membrane (Antoniadi *et al*., 2020; Kubiasova et al., 2020). Signal transduction is via a phospho relay with phosphate being transferred from the HKs by intermediary histidine phospho transfer proteins (HPs) leading to phosphorylation of type-B response regulators (type-B RRs) in the nucleus. The type-B RRs are Myb-type transcription factors that regulate transcription of primary response genes, leading to cytokinin responses. Negative regulator type-A RRs are also transcribed (Zubo and Schaller, 2020). It should be noted that, while the essential TCS appears to be the same in monocots and dicots, rice also has a novel serine/threonine kinase receptor known as CHARK (Ito and Kurata, 2006; Halawa *et al*., 2021). Additionally, some rice RRs have acquired novel functions distinct from their roles in dicots (Worthen *et al*., 2019). A detailed figure of cytokinin biosynthesis and metabolism can be viewed in Chen *et al*. (2021a), and a model of the signal transduction pathway in Romanov *et al*. (2018).

apical meristem (SAM), in the panicle meristem at the primary branch initiation stage, and in the floral meristems of rice (Kurakawa *et al*., 2007). The effect of mutations in *OsLOG* resulted in a smaller vegetative meristem, although no abnormalities were apparent during vegetative growth. However, in *log* mutants there was a severe effect during the reproductive phase resulting in a reduction in panicle size, an abnormal branching pattern, a decrease in the number of floral organs and the abnormal floral feature (one stamen, no pistil) after which the gene is named, all of which were attributed to the reduction in proper meristem maintenance (Kurakawa *et al*., 2007; Table 1). Rescue of the *log-2* mutant occurred following the introduction of *OsLOG* cDNA driven by the *LOG* gene promoter. Functional testing showed that recombinant OsLOG converted cytokinin nucleotides isopentenyl riboside monophosphate (iPRMP) and *trans*-zeatin riboside monophosphate (*t*ZRMP) to the free base form with the release of a ribose 5′-monophosphate. Further, LOG reacted only with cytokinin nucleoside 5′-monophosphates, but not with the di- or triphosphates, nor with AMP, cytokinin ribosides and free bases; that is, rice LOG is a cytokinin nucleoside 5′-monophosphate phosphoribohydrolase.

Besides *OsLOG*, 10 other gene family members were recognized (Kurakawa *et al*., 2007), now referred to as *OsLOGL1-10*. Using RT-PCR, the expression of *LOG* gene family members was examined in eight tissues: *OsLOG* was expressed in all tissues, but most strongly in leaf blades; *LOGL3* and *LOGL7* were expressed in all tissues, with *LOGL7* strongly expressed in the shoot apex and immature inflorescence tissues; *LOGL2* and *LOGL4* were not detected, and *LOGL1* was barely detectable and only in the inflorescence meristem; *LOGL5* was barely detectable; *LOGL6* was not detected in stem or tiller buds, but was in other tissues; *LOGL8* was specifically expressed in roots; and *LOGL9* was expressed in root and leaf sheath whereas *LOGL10* was expressed in all tissues except for root and leaf blade (Kurakawa *et al*., 2007).

LOG gene family in *Arabidopsis thaliana*

Nine arabidopsis *LOG* genes (*AtLOG1-9*) were predicted as homologues of rice *LOG* (Kurakawa *et al*., 2007; Kuroha *et al*., 2009). However, the deduced amino acid sequence of AtLOG9 lacked the N-terminal third of the rice LOG sequence, and the sequences of both AtLOG6 and 9 contained predicted premature stop codons (Kuroha *et al*., 2009). The remaining seven AtLOGs were tested for functionality. Purified recombinant proteins were shown to react with iPRMP, but not with the diphosphate or the triphosphate, or with the free base iP or its riboside. The recombinant proteins did not discriminate between the various isoprenoid nucleotide forms (iPRMP, *t*ZRMP, *cis*ZRMP and dihydroZRMP) but were generally less reactive towards the tested aromatic nucleotide (benzyl aminopurine, BARMP). *In planta* analysis, using an inducible promoter to drive overexpression of *AtLOG2*, showed a decrease in iPRPs, a slight increase in iP and iP-9-glucoside and a very strong increase in iP-7-glucoside (Kuroha *et al*., 2009), indicating overexpression of *AtLOG2* resulted in the (subsequent) activation of cytokinin homeostatic mechanisms to deactivate excess cytokinin (Chen *et al*., 2021a).

Analysis of loss-of-function mutants showed that single *Atlog* mutants did not exhibit a visible phenotype. However, the double mutant *Atlog2log7* and the triple mutant *Atlog3log4log7* showed reduced sensitivity to iPR during the lateral root formation assay, but the double combinations of *logs 3, 4* and *7* were not active. Complementation of the triple mutant by *AtLOG7* rescued the reproductive stage, and overexpression of *AtLOGs* showed classic symptoms of excess cytokinin (Kuroha *et al*., 2009; Table 1).

Using GUS reporter gene constructs, detailed expression patterns of AtLOGs 1-5, 7 and 8 were elegantly shown by Kuroha *et al*. (2009). AtLOGs 1, 4 and 8 were the most widely expressed across tissue types and AtLOG2 the least. The data indicated that AtLOGs were spatially and quantitatively differentiated, albeit with some overlaps between tissues, thus showing that active cytokinins could be synthesized in nearly all parts of the plant body via the direct pathway. Moreover, there are

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Table 1 Impacts of *LOG* mutants or manipulations of *LOG*

Species	Promoter/mutant	Environment	Characteristic	References
Cultivar/ecotype				
Rice				
Taichung65	*log* mutants		Normal vegetative development although smaller vegetative meristem Inflorescence and panicle branch meristems abort Reduction in panicle size and abnormal branching pattern Decrease in floral organs; often one stamen, no pistil Meristematic activity not properly maintained	Kurakawa *et al*. (2007)
	LOG::log2 complementation		Normal phenotype	
O. rufipogon/ *O. sativa*	*LABA1* (*OsLOGL6*)		Long, barbed awns in wild rice	Hua *et al*. (2015)
	laba1 allele		Short, barbless awns; increased seed weight in cultivar	
	RNAi LABA1		Awn length and barbs reduced	
	LABA1::laba1 complementation		Longer awns and barbs	
O. rufipogon/ *O. sativa*	*An-2* (*OsLOG6*)		Long, barbed awns in wild rice	Gu *et al*. (2015)
	An-2 allele in awnless indica cv.		Awns elongated through increased cell division; decreased grain production through fewer grains/ panicle and fewer tillers/plant	
	Complementation		Awned progeny	
	An-2 oe		Awned progeny	
Zhonghua	35S::LOGL5	Hydroponics	Shorter primary root Increased lateral root number	Wang *et al*. (2020)
		Field	Semi-dwarf; narrow leaves Fewer tillers; fewer seeds/panicle; reduced 1000 grain weight	
		Normal and low N	Decreased grain yield	
		Drought conditions	Decreased grain yield	
	OsLOGL5: six knockouts at 3′ end	Hydroponics	Normal roots and shoots	
		Field	Normal vegetative growth	
	Edits A, B & F	Normal and low N	Increased grain yield	
	Edits C, D & E	Low N	Reduced yield	
		Normal N	No effect	
	Edits A-C, E, F	Drought	Increased grain yield	
	Edit D	Drought	Reduced tolerance (ns)	
	Edits B, D, E & F	Drought	Increased seed setting rate, total grain number, full-filled grain numbers/panicle and 1000 grain weight	
Nipponbare	*UNBRANCHED3*	*UB3*-oe	↓tillering; ↓panicle branching; ↓grains/panicle; ↓LOG1	Du *et al*. (2017)
BS208			Abnormal panicle branching pattern; ↓lateral grains on 2^{o} branches	Li *et al*. (2021)
Teqing	*REGULATOR OF GRAIN NUMBER1*	rgn1 mutant	Absence of lateral grains on secondary branches; grain number decreased; grain size and weight increased; yield decreased *LOG* expression downregulated in NIL-rgn1 compared to NIL-RGN1	
		RGN-oe	*LOG* expression upregulated	
		LOG-oe in BS208	Partially rescued the absence of lateral grains in secondary branches	
Taichung 65	OsWOX4	RNAi of WOX4	Severe defect in leaf development; vascular differentiation arrested; ↓*OsLOG3* & *10* in vasculature	Yasui *et al*. (2018)
Arabidopsis thaliana				
Columbia	AtLOG1-9		LOG6 & 9 non-functional	Kuroha *et al*. (2009)
	Single, double and triple mutants of *logs 3, 4* and *7*		Single mutants: no visible phenotype Root growth assay: Single mutants not resistant to iPR; *log2log7* and *log3log4log7* mutants resistant to iPR *log3log4log7* increased adventitious root formation; no change in primary root length; fewer flower buds and flowers	
	LOG7::log3log4log7		Rescued the reproductive stage phenotype	
	35S::LOG2, 4, 5, 7, or 8		Increased cell division in embryos and leaf vascular tissues; reduced apical dominance; delayed leaf senescence; larger seeds; number of emerged lateral roots decreased	

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Table 1 Continued

Species	Promoter/mutant	Environment	Characteristic	References
Columbia	Septuple *log* mutants		Severe retardation of shoot and root growth and development Reduction in size of root and shoot apical meristems Set flowers; Seeds larger than wild type	Tokunaga *et al*. (2012)
	Septuple mutant transformed with *LOG7*		Growth retardation rescued	
Columbia	*Log3,4,7* triple mutant		Abnormal root vasculature: vascular cell number reduced, vascular cell types eliminated (except for protoxylem)	Ohashi-Ito et al. (2014)
	Triple mutant transformed with pTMO5::LOG3	xylem precursor-cell-specific promoter	Rescued triple mutant	
Columbia	*Log1,2,3,4,5,7,8* *Septuple mutant transformed with pTMO5-LOG4*		Reduced vascular cell file number; protoxylem only Cell file number rescued; protoxylem and metaxylem present	De Rybel *et al*. (2014)
	Septuple mutant transformed with pRPS5A-LOG4	Constitutive promoter	Vascular cell file number increased; metaxylem only	
Columbia	*LOG1,2,3,4,5,7,8*	Grafted	Severe vegetative dwarfism due to smaller rosette leaves with reduced epidermal cell number, reduced SAM diameter and a delayed plastochron	Osugi *et al*. (2017)
	LOG1,2,3,4,5,7,8/WT	Grafted	WT root stock rescued leaf size and epidermal cell number, but not SAM size or plastochron number	
	LOG1,2,3,4,5,7,8/abcg14	Grafted onto transporter mutant	Severe dwarfism when root-to-shoot cytokinin translocation impaired Recovered by *t*Z but not *t*ZR application LOG function essential to meristem but not necessarily leaf growth	
Columbia	*LOG1,3,4,7* mutant		Vegetatively normal; smaller inflorescence meristems, fewer organs	Landrein *et al*. (2018)
	LOG4,7/Col-0; *LOG1,3,4,7*/Col-0	Grafted	Wild-type root stock did not rescue LOG mutants	
Columbia	AtLOG4	*AtML1:LOG4* targeted to epidermal cells	Larger rosette and leaves; ↑cell number; early flowering; ↑vegetative meristem size and stem diameter; earlier transition to adult phase; lesser ↑inflorescence meristem size; ↑flower, gynoecia and silique size; ↑seed number/silique; ↑seed yield.	Werner *et al*. (2021)
Tomato	35S::TLOG1		Loss of apical dominance; *de novo* aerial mini-tubers in tomato; extended aerial tuberisation in potato	Eviatar-Ribak *et al*. (2013)
Medicago truncatula	*GUS::MtLOG1* and *2*		Showed expression in the upper dividing cells of the nodule primordia and, in more mature nodules, specifically in the meristem and early differentiating cells; *CRE*-dependent	Mortier *et al*. (2014)
	GUS::MtLOG1		Expressed in the lateral root primordium; CRE-independent	
	MtLOG-oe		Reduced nodule number, with loss of meristem in the nodules that did develop; Root thickening due to vascular tissue expansion and reduced primary root length	
	Silencing of *MtLOG1*		Increased density of lateral roots but decreased the number of nodules formed	
Lotus japonicus	*LjLOG-oe*		Reduced nodule number	Reid *et al*. (2017)
	iPT-Log-Cyp735a-oe		Spontaneous nodule formation occurred in *Lotus japonicus*	
Cotton	*GhLOG3-oe*		↑tolerance of Arabidopsis to NaCl	Wang et al. (2021)
	VIGS of GhLOG3_At		Enhanced sensitivity of cotton to salt stress	
Kiwifruit	Gene edited *SHY GIRL* in male plant		LOG expression increased in 'early flowers'; Enhanced feminisation	Varkonyi-Gasic *et al*. (2021)
Chlorella variabilis	*CvarLOG1-oe*	*In vitro*	"stay-green" phenotype in culture	Nayar (2021)

specific areas of overlap with the spatial expression patterns of the *AtIPT* GFMs shown by Miyawaki *et al*. (2004).

Tokunaga *et al*. (2012) extended the work with *AtLOG* mutants, showing that the sextuple or lower-order mutants showed little change in vegetative phenotype, but the septuple mutants showed severe retardation of both shoot and root growth, and severe impacts on the maintenance of the apical meristems (Table 1). However, some plants did flower and set seed, with those seeds being enlarged. The levels of cytokinin nucleotides were increased and those of the free bases and glucosides decreased in seedlings of the septuple mutant, indicating that the active cytokinins produced by the LOG-

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dependent pathway were critical to maintenance of shoot and root meristems. Considering all the various mutant combinations, Tokunaga *et al*. (2012) concluded that *AtLOG7* was the most important GFM for the maintenance of the SAM and for the provision of a basal level of cytokinin to support normal primary root growth. Moreover, the septuple mutant could be rescued by *AtLOG7* alone (Table 1). Interestingly, in the spatial analyses shown by Kuroha *et al*. (2009), AtLOG7 was expressed in only a limited number of cell types, including strong expression in the elongation zone of roots, but was not shown to be expressed in the SAM. However, *in situ* hybridization by Yadav *et al*. (2009) showed *AtLOG7* expression in the SAM.

Similar work with multiple mutants of *LOG/LOGL* GFMs has not been published for rice or other species. It also appears only OsLOG and OsLOGL6 have been functionally characterized (Hua *et al*., 2015; Kurakawa *et al*., 2007, respectively).

LOG gene family in wheat

In order to identify LOG orthologues in wheat, we used LOG sequences from higher plants to do a genome-wide screening of the wheat proteome sequences using a HMMER model. A total of 45 LOG-like candidate proteins were identified. BLAST and motif analysis showed that five of the 45 LOG-like candidates did not have what is now known as the conserved LOG motif PGGxGTxE (Dzurová *et al*., 2015; Jeon *et al*., 2006) and were excluded. The remaining 40 LOG-like candidates, together with LOG sequences from other species, were then used to perform a phylogeny analysis (Figure S1).

The resulting phylogenetic tree comprised four clades (Figure S1 and Figure 2). Clade I includes those organisms whose LOG proteins are predicted to operate as tetramers (Mayaka *et al*., 2019) and hexamers (Seo and Kim, 2017), whereas LOGs in Clades II, III and IV likely function as dimers (Seo and Kim, 2017). Our initial phylogenetic tree, generated based on amino acid sequences, included archaea, bacteria, cyanobacteria and higher plants in Clade I (Figure S1). Clade II includes a mixture of pathogenic microorganisms, green algae, mosses and higher plants, whereas Clades III and IV contain only higher plants. Members of the wheat LOG gene family were initially found in all Clades and named TaLOG1-12 (Figures S1 and S2). Clades II, III and IV contain functionally characterized AtLOGs. Each of TaLOGs1-11 aligns with a rice LOG GFM. TaLOG11 aligns most closely with OsLOG, functionally characterized by Kurakawa *et al*. (2007); and TaLOG2 with OsLOG2 and OsLOGL6, functionally characterized by Hua *et al*. (2015). Their allocated numbers are detailed in Figure 3 and Table S1.

Motif (Figure 4) combined with MEME analysis (Figure S3) showed that *TaLOG1-11* all coded for the shorter canonical plant LOG motif (PGGYGTxxE), and the extended LOG motifs (MHxRKx(2)Mx(5,20)FlxLxxGGYGTxxEE) (Dzurová *et al*., 2015) and [LR][AS]D[AG]F[VI]x[LM]PGGxGT(L)[DE]E(L)(F)Ex[LW]x(Q)[LT]xx[HVI] (Naseem *et al*., 2018) within motif 1. TaLOG1-11 have the critical amino acids for phosphoribohydrolase activity (Dzurová *et al*., 2015; Seo and Kim, 2017).

The structures of two functionally characterized arabidopsis LOGs, AtLOG3 and AtLOG8, have been determined (Jeon *et al*., 2006). Based on these structures, we have developed homology models for the closest wheat homologues, TaLOG2 and TaLOG6, and TaLOG1, respectively, and compared these with the arabidopsis LOGs. Sequence identities on alignment were 71.2% for TaLOG1 and AtLOG8, 82.9% for AtLOG3 and TaLOG2, and 79.4% for AtLOG3 and TaLOG6 (Table S2). As wheat LOGs 1-11 contain the key amino acids for substrate binding and catalysis, and structural homology to characterized AtLOGs, it is highly likely that the wheat LOGs1-11 are functional cytokinin nucleoside 5′-monophosphate phosphoribohydrolases.

An *AtLOG* has been shown to clade as a hexamer-forming type IIb LOG (Frébortová and Frébort, 2021), and this *AtLOG* sequence was used in our initial phylogenetic analysis. In this analysis, both *TaLOG12* and a *Medicago truncatula LOG* cluster with it (Figure S1). Indeed, the phylogenetic tree constructed by Seo and Kim (2017) shows two *OsLOGs, AtLOGs, MtLOGs,* and *Prunus persica LOG*s cladding as hexamer-forming type IIb LOGs. However, MEME analysis showed *TaLOG12* does *not* have the longer conserved motif 1 sequence within the lysine decarboxylase domain (LDC; Figures 4 and S4). Within the LDC domain of *AtLOG*-type IIa and *MtLOGL3* (Figure S5), motif and MEME analysis showed the *AtLOG* and the *MtLOGL3* genes have a PGGIGTxxE (with an isoleucine replacing a tyrosine) within motif 11 (Figures S6 and S7). The 'I' is indicative of type IIb LOGs as proposed by Seo and Kim (2017). However, there is little consensus with the longer LOG motifs of Dzurová *et al*. (2015) and Naseem *et al*. (2018) within the LDC domain (Figure S7). Additionally, we show that TaLOG12 does not have close structural homology with other hexameric phosphoribohydrolases (Table S2). We consider that it is unlikely that wheat and other higher plants have hexamer-forming cytokinin-specific LOGs, as shown by their being omitted from the amended phylogenetic tree (Figure 2). TaLOG12 was omitted from this and from further analyses. What *TaLOG12* codes for is unknown as the presence of the shorter consensus sequence indicates it is unlikely to code for LDCs (Naseem *et al*., 2018).

Origins of *LOG*

Tracking backwards though the evolution of Archaeplastida, it is clear that expansion of the *LOG* gene family took place within this lineage and, more specifically, within the mosses as exemplified in *Physcomitrium* (previously *Physcomitrella*) *patens* (Bryophyta *sensu stricto*; Kuroha *et al*., 2009; Lu *et al*., 2014; Nayar, 2021). Moss appears to have nine *LOG-like* GFMs (Lu *et al*., 2014), whereas the lycophyte *Selagenella moellendorffii* possibly has four, and a red alga possibly two (Lu *et al*., 2014). *PpLOG* XP 001781548 is likely ancestral to all other moss and plant *LOG*s (Figure 2).

Only a single *LOG* gene has been detected in each green alga studied (Lu *et al*., 2014; Nayar, 2021). Recombinant *Chlorella variabilis LOG* showed cytokinin phosphoribohydrolase activity against iPRMP >> *t*ZRMP, leading Nayar (2021) to suggest that the *LOG* enzymes are functionally conserved from unicellular algae to higher plants. This functional conservation aligns with the presence of components of the canonical two-component signalling pathway for cytokinins (Rashotte, 2021; Figure 1), indicative of cytokinin-regulated developmental processes. Critically, the expansion of the cytokinin *LOG* gene family within the mosses (Lu *et al*., 2014; Figure 2) is paralleled by the expansion of gene families associated with each step of the two-component signalling pathway in moss, relative to liverwort and hornwort, and the charophyte green algae, as reviewed by Rashotte (2021).

LOG belongs to a superfamily of genes, and *LOG*-like genes have been shown to be present in all domains of life (Frébortová and Frébort, 2021; Mayaka *et al*., 2019; Naseem *et al*., 2018; Samanovic *et al*., 2015). Burroughs *et al*. (2015) suggest that this superfamily is likely to encompass dual functions, with certain versions serving as (oligo)nucleotide sensors and others as enzymes that operate on nucleotides. Carlsson *et al*. (2018)

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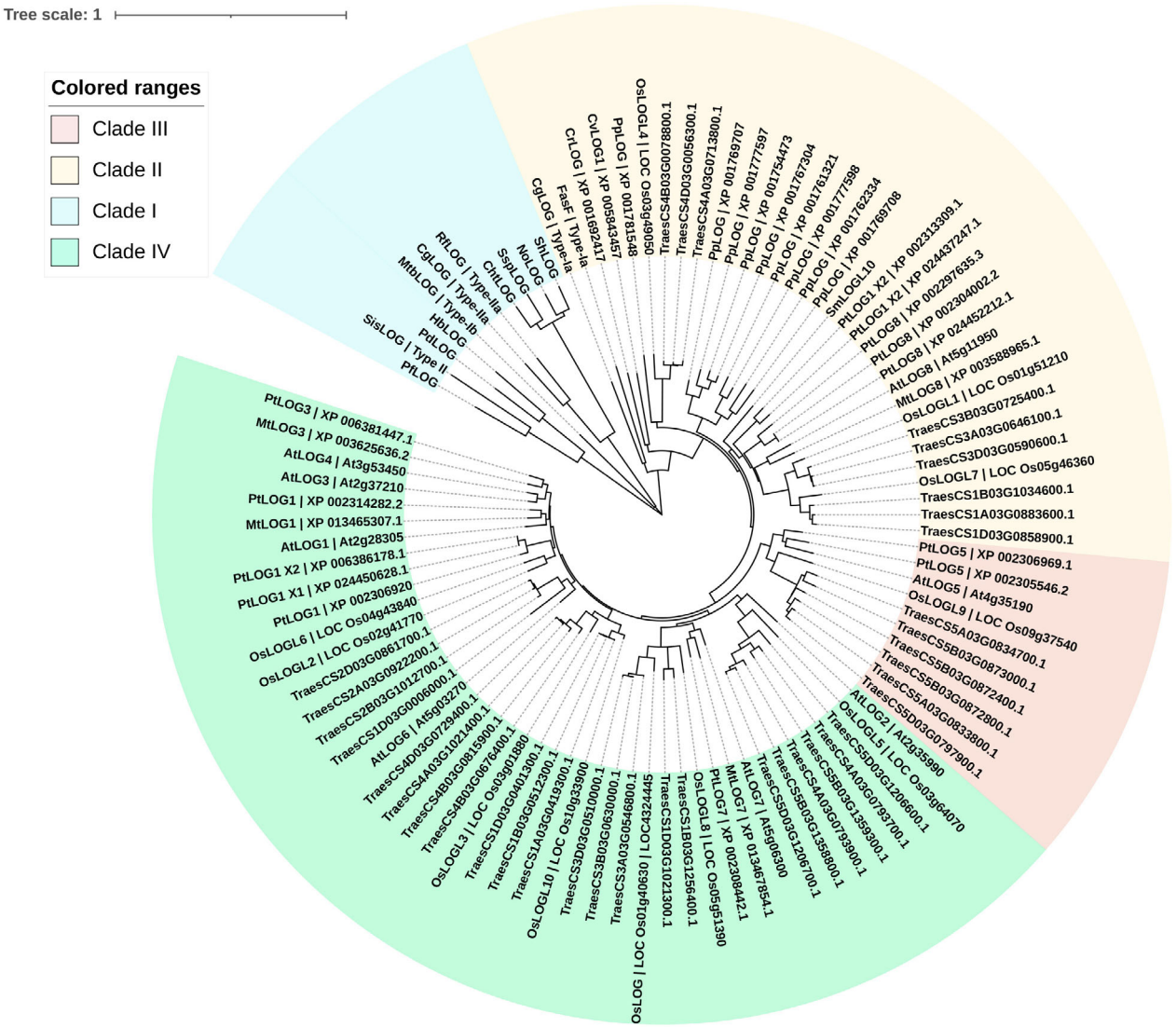

Figure 2 Phylogenetic tree of LOGs from selected species. The wheat LOGs were identified from the latest released version 2.1 of genome-wide wheat peptides (Zhu *et al*., 2021) using the HMMER model and BLAST methods described in Chen *et al*. (2020b; 2021a). The HMMER model was developed based on published LOG data from higher plants (Immanen *et al*., 2013; Kurakawa *et al*., 2007; Kuroha *et al*., 2009; Mortier *et al*., 2014; Pecrix *et al*., 2018; Tokunaga *et al*., 2012), followed by genome-wide screening of the wheat peptide genome (version 2.1). The candidate TaLOG sequences were validated by genome-wide BLAST on the wheat omics database (http://202.194.139.32/blast/blast.html, Ma *et al*., 2021). A total of 45 LOG-like candidate proteins were identified. BLAST and motif analysis showed that five of the 45 LOG-like candidate proteins did not have the shorter conserved LOG motif PGGxGTxE and were excluded. Additionally, MEME analysis showed that the originally named TaLOG12 members do not have the longer conserved motif 1 sequence within the lysine decarboxylase domain (LDC) and were also excluded. The phylogenetic tree was constructed using MEGA X software via the neighbor-joining algorithm (Kumar *et al*., 2018) and then beautified using the iTOL web tool (https://itol.embl.de/).

suggest that the original function of the LOG family of proteins was to degrade non-canonical nucleotides and that their role in cytokinin production was a later development in some organisms. Witte and Herde (2020) suggest that, in addition to recycling nitrogen, purine nucleotide catabolism could dampen stress responses through the production of catabolic intermediates. We suggest 'LOG' should be retained for those enzymes definitively acting as cytokinin riboside 5′-monophosphate phosphoribohydrolases to produce free base cytokinins and which then trigger the canonical two-component signal transduction pathway (Figure 1). We suggest that those enzymes that are not cytokinin riboside 5′-monophosphate-specific phosphoribohydrolases are simply referred to as phosphoribohydrolases (PRH) and not as LOG. We will expand our reasoning in a forthcoming article. Suffice to say that the presence of the PGGxGTxxE motif does not necessarily confer cytokinin riboside 5′-monophosphate-specific phosphoribohydrolase activity, contrary to the inference relating to *E. coli* in Nguyen *et al*. (2021b).

Chromosome location of wheat *LOG*s

There are 11 *TaLOG* GFMs associated with the A subgenome, 14 associated with the B sub-genome and 12 associated with the D sub-genome. There are instances of multiplication and loss of

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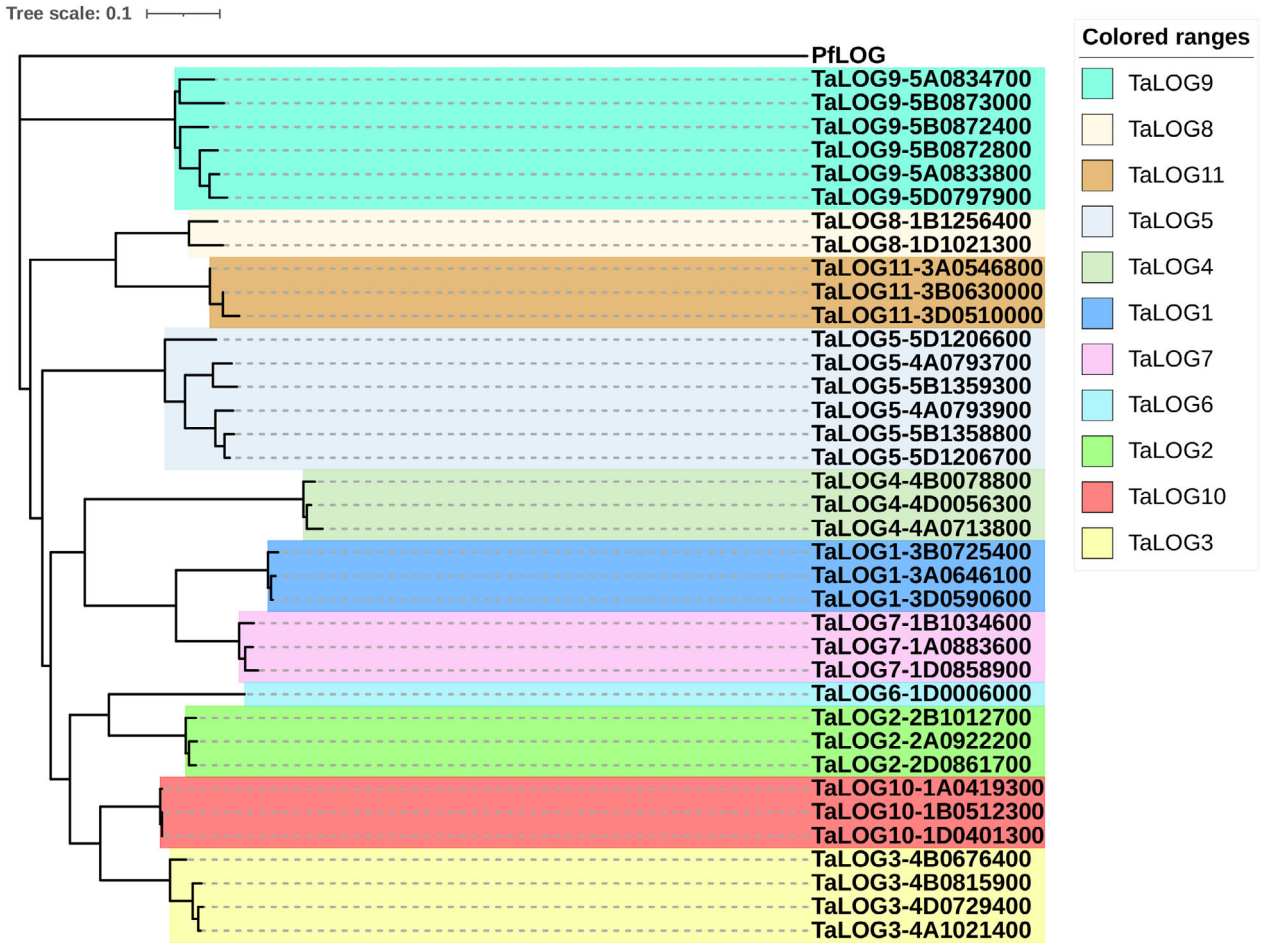

Figure 3 The phylogenetic tree of TaLOGs. The wheat LOG sequences were identified using HMMER model and BLAST methods, combining motif analysis and domain analysis as described for Figure 2. The tree was constructed using MEGA X software via the neighbor-joining algorithm (Kumar *et al*., 2018), and then, beautification was performed using the iTOL web tool (https://itol.embl.de/).

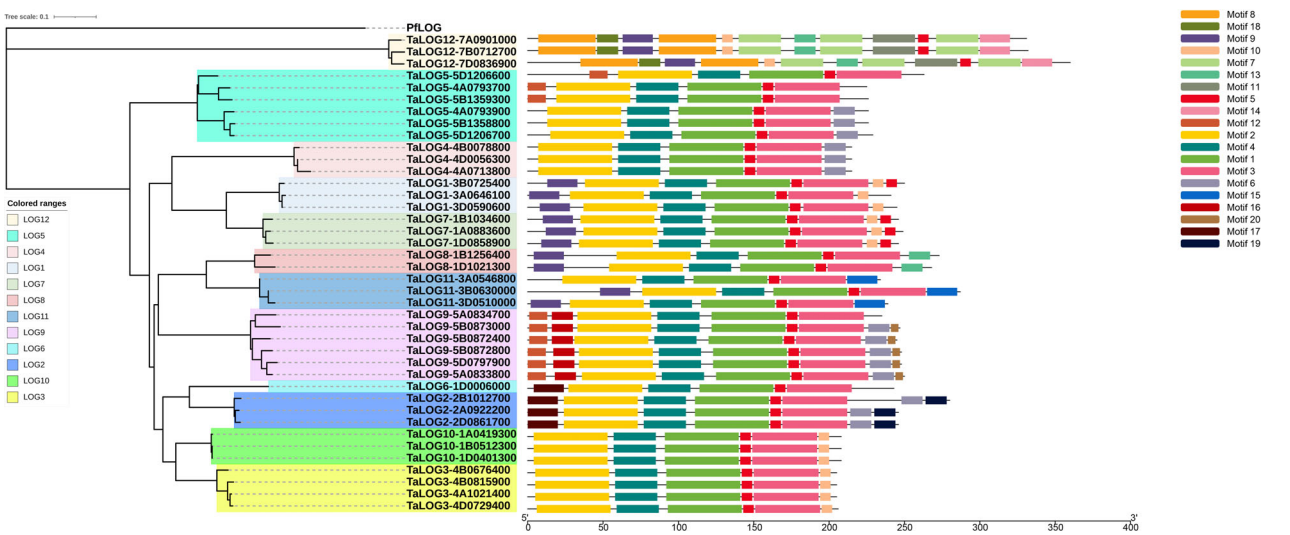

Figure 4 The motif patterns of TaLOGs. The motif patterns were predicted using the MEME database (https://meme-suite.org/meme/tools/meme) and were sorted according to the order in the LOG phylogenetic tree shown in Figure 3. The data were shown using TBtools software (Chen *et al*., 2020b) and merged with the phylogenetic tree in Photoshop software (CS 6).

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specific *TaLOG* GFMs on the sub-genomes. Only the D sub-genome has a copy of at least one of each of the 11 GFMs (Figure 5), whereas the B sub-genome lacks *LOG6*, and the A sub-genome lacks both *LOG6* and *LOG8*. This may relate to the dominance of the D sub-genome in bread wheat (El Baidouri *et al*., 2017).

Multiplication and loss of *LOG* GFMs has been noted in *Populus trichocarpa* and *Prunus persica*, respectively (Immanen *et al*., 2013). *Medicago truncatula* also appears to have lost *LOG* GFMs (Mortier *et al*., 2014). Recently, Wang *et al*. (2021) concluded that there were 28 *LOG* GFMs in *Gossypium hirsutum* (cotton). Cotton is tetraploid and, like wheat, the *LOG* genes are unevenly distributed between the sub-genomes.

Cellular localization of LOGs

It might be anticipated that LOGs will be located close to the production of cytokinin nucleotides by IPT and/or in the vicinity of cytokinin receptors. Kasahara *et al*. (2004) showed, using IPT:GFP fusion proteins, that AtIPT1, AtIPT3, AtIPT5 and AtIPT8 localize in plastids and experiments with stable isotope-labelled substrates indicated that plastids are a major subcellular compartment for the initial step in cytokinin biosynthesis in arabidopsis seedlings. Additionally, AtIPT7 was targeted to mitochondria, and AtIPT4 was localized in the cytosol. The tRNA-IPT fusion protein, AtIPT2-GFP, was also localized in the cytosol (Kasahara *et al*., 2004). Subsequently, Galichet *et al*. (2008) showed that post-

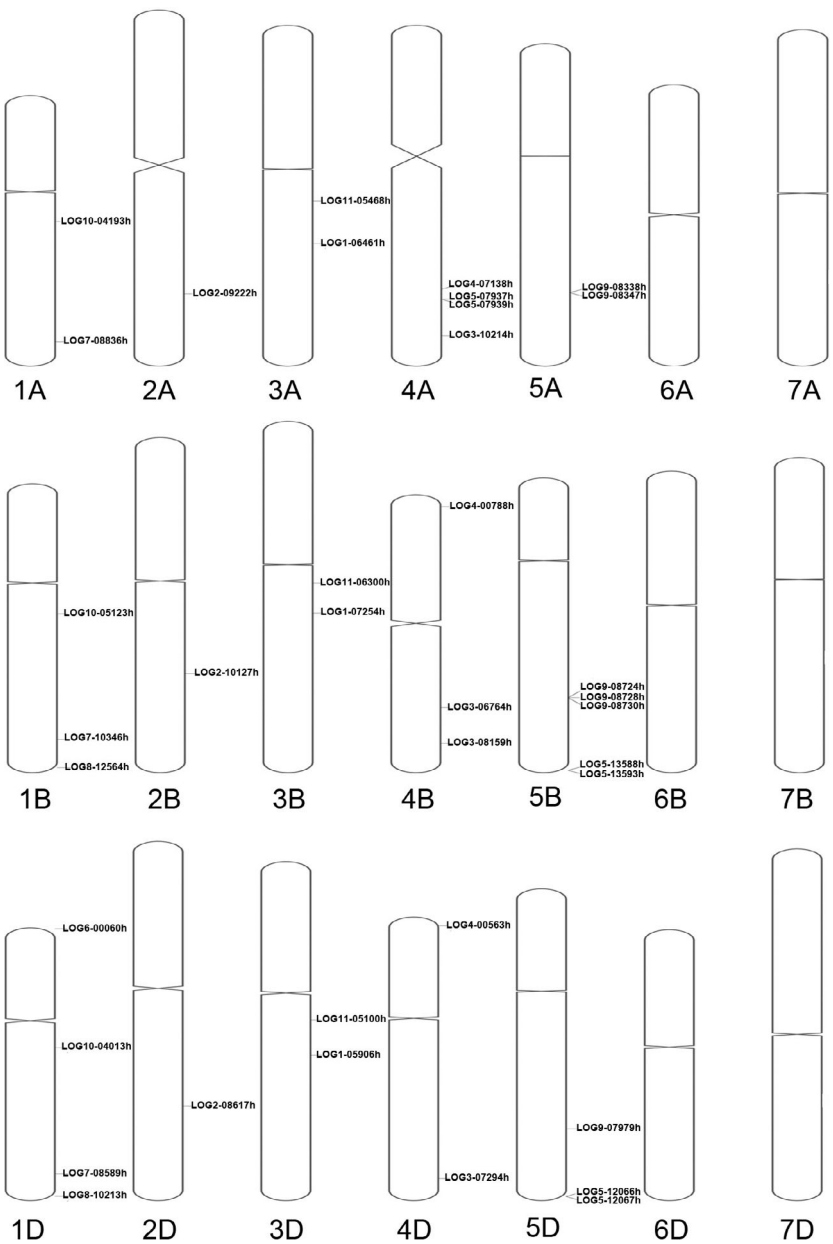

Figure 5 Chromosome location of *TaLOGs*. The position file was extracted via the Linux shell programs from the newly released gff3 file for wheat genome (iwgsc_refseqv2.1_annotation_200916_HC.gff3). The gene positions were then mapped onto the chromosomes using RIdeogram package in R language environment. The images of the ABD sub-genomes were merged and edited in Photoshop CS6 software. In order to shorten the gene names, we omitted the first two letters (Ta) and used 'h' instead of '00' at the end of each name. The corresponding information is listed in Table S2.

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translational farnesylation of AtIPT3 regulated its subcellular localization and directed most of the protein to the nucleus.

Transient and stable expression of rice, arabidopsis and *Chlorella variabilis LOG*-reporter gene fusions showed localization to both cytosol and nuclei (Gu *et al*., 2015; Hua *et al*., 2015; Kurakawa *et al*., 2007; Kuroha *et al*., 2009; Nayar, 2021). In contrast, Wang *et al*. (2021) reported that cotton *LOG3_At* transiently expressed in tobacco leaves 'localized in the cell membrane' – although the authors above have described similarly localized signals as being cytosolic.

Why cytokinin might be produced in the nucleus is an interesting question. Cytokinins themselves are not transcription factors. Cytokinins are recognized by membrane-localized histidine-kinase receptors and are transduced through a His-Asp phosphorelay to activate the type-B response regulators, which are a family of transcription factors in the nucleus. While there are identified differences between arabidopsis and rice (Vaughan-Hirsch *et al*., 2021; Worthen *et al*., 2019), the essential features of signal transduction are conserved (Figure 1). Cytokinin receptors and the initial signal of the signal transduction chain are external to the nucleus (Kieber and Schaller, 2018; Romanov and Schmülling, 2021; Figure 1). As the function of LOG is to release active cytokinins, activity of both an IPT and LOG in the nucleus itself is hard to explain. Cytokinin controls cell division through its control of the cell cycle. As described by Yang *et al*. (2021), the dual regulatory modes of cytokinin in CYCD3 transcription (Riou-Khamlichi *et al*., 1999) and MYB3R4 nucleocytoplasmic shuttling (Yang *et al*., 2021) ensure precise control of cell cycle transitions in response to different levels of cytokinin input. Yang *et al*. (2021) state that the variation in cytokinin levels is most likely perceived by intracellular cytokinin receptors. Indeed, cytokinin receptors have been identified in the membranes close to the perinuclear pore (Romanov and Schmülling, 2021). A role for cytokinin actually within the nucleus remains to be identified. Potentially, an alternative explanation for the location of LOG in organelles where DNA is synthesized could reside in what Carlsson *et al*. (2018) suggest is the original role of LOG: to degrade non-canonical nucleotides thus preventing their incorporation into DNA.

Interestingly, in wheat, our *in silico* analyses showed that none of the TaLOGs were predicted to localize to the nucleus irrespective of the prediction method (Table S3), even with CellPLoc 2.0 and ProtComp producing highly contrasting results. The most popular prediction method, WoLF PSORT (Horton *et al*., 2007; cited 2648 times), showed that more than half of the TaLOGs localize to the cytosol. Additionally, both chloroplast- and mitochondria-localized TaLOGs account for 20%, respectively. Notably, all of the TaLOG3 and TaLOG10 proteins, which are co-located on the phylogenetic tree and appear to be more recently evolved members of the *TaLOG* gene family (Figure 2), are predicted to localize to the mitochondria.

It is likely that TaIPTs and AtIPTs will be similarly located, so it is not unexpected to find TaLOGs located in the chloroplast and mitochondria, in which the free base could be active *in situ* or from where the free base could diffuse into the cytosol. TaLOGs localized in the cytosol could convert cytokinin nucleotides produced in the cytosol, or act on cytokinins arriving in the transpiration stream including cytokinin nucleotides and ribosides (back converted to nucleotides by adenosine kinases (Sakakibara, 2021)).

Clearly, the discrepancy between *in vivo* and *in silico* analyses requires resolving through the careful use of reporter gene constructs, as GFP itself is reported to translocate to the nucleus (Seibel *et al*., 2007). Additionally, different attachment positions can lead to differences in protein subcellular location (Marion *et al*., 2008; Weill *et al*., 2019), although Gu *et al*. (2015) attached GFP to both LOG termini, with no apparent difference in location of LOG.

Role of LOGs *in planta*

Rice LOG was originally detected in meristematic mutants resulting in reduced yield (Kurakawa *et al*., 2007) and, as a consequence, there has been significant focus on meristematic tissues. Cytokinin biosynthetic, catabolic, receptor and signalling mutants have implicated cytokinin in SAM and root apical meristem (RAM) organization in arabidopsis (Bartrina *et al*., 2011; Gordon *et al*., 2009; Landrein *et al*., 2018; Osugi *et al*., 2017), as well as in vascular development (Miyashima *et al*., 2013; Ohashi-Ito *et al*., 2014). Cytokinins are intimately involved in the CLAVATA/WUSCHEL-negative feedback loop of meristem maintenance in eudicots (e.g. Bartrina *et al*., 2011; Chickarmane *et al*., 2012; Gordon *et al*., 2009; Landrein *et al*., 2018; Sablowski, 2009), with *AtLOG4* expression restricted to the L1 (epidermal) layer of the SAM and floral meristem, and *AtLOG7* expressed in the developing primordia (Chickarmane *et al*., 2012; Yadav *et al*., 2009).

The grafting experiments of Osugi *et al*. (2017) and Landrein *et al*. (2018) using multiple *AtLOG* and other mutants provide convincing evidence that stem cell homeostasis in the SAM is controlled by a systemic signal (*t*ZR) produced external to the meristem, which is converted by LOG to the active free base form (*t*Z) in the SAM, to control the size of the SAM and plastochron number (Table 1).

However, it is the location of receptor and type-B response regulators (RRs) that define the region of cytokinin action below the epidermal layer (Chickarmane *et al*., 2012), with Meng *et al*. (2017) suggesting type-B ARRs bind to the WUS promoter. Recently, Yang *et al*. (2021) confirmed the intimate role played by cytokinin in driving mitosis and cytokinesis (i.e. controlling the cell cycle) in the arabidopsis SAM with cytokinin acting by changing the nuclear concentration of a transcription factor, thereby regulating cell numbers in the shoot stem cell niche.

Although *CLV* and *WUS*-like genes have been identified in wheat, their detailed roles are only beginning to be examined (Li *et al*., 2019, 2020). However, Ohmori *et al*. (2013) suggest that there are two types of meristems in rice – vegetative (not affected by *OsLOG* mutants) and reproductive, which are affected by *OsLOG* mutants through a failure to maintain the floral meristem. As described earlier for rice, *in situ* hybridization shows *OsLOG* GFMs to be clearly located in meristematic regions. Cytokinin-inducible RRs 1 and 5 were also expressed in the floral meristems of wild-type rice, but not in the meristems of a *log* mutant, suggesting that active cytokinin was being produced in the wild-type meristem. In terms of meristem maintenance in rice, OsLOG could control cell-specific activation of cytokinin (Kurakawa *et al*., 2007), but its activity is likely spatially defined by receptor and/or signalling components.

Interesting examples of development implicating LOG include ectopic tuber formation on tomato (Eviatar-Ribak *et al*., 2013), nodule formation (Azarakhsh *et al*., 2020; Mortier *et al*., 2014; Reid *et al*., 2017) and feminization of male kiwifruit plants (Varkonyi-Gasic *et al*., 2021; Table 1).

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Wheat *LOG*s express in multiple organs

Using the RNA-seq data obtained by Ramírez-González *et al*. (2018), we extracted all of the expression data sets for the *TaLOG*s from 70 tissues/organs. The data are displayed in a heatmap (Figure 6) and in detailed graphs (Figure S8) showing expression in roots, shoots, leaves and spikes across development. It is evident that *TaLOG* GFMs are expressed in multiple tissues, both vegetative and reproductive. *TaLOG1* is constitutively and strongly expressed in all tissues and organs analyzed across development, including in the spike. *TaLOG10* and *LOG11* are also constitutively expressed, but much less strongly. There does not seem to be a strong correlation in terms of expression analysis between the *TaLOG*s and the aligned *OsLOGs*. For example, *OsLOGL1* was barely expressed in rice (Kurakawa *et al*., 2007), but *TaLOG1* is constitutively expressed in wheat; *OsLOG10* was widely expressed including the leaf sheath but not leaf blade or roots, whereas *TaLOG10* is constitutively expressed. However, the relative transcript abundances reported by Tsai *et al*. (2012) in rice in shoots and roots [*OsLOG*, *LOGL5*, *LOGL7*, *LOGL9* and *LOGL10* were greater in shoots relative to roots, whereas the transcripts of *LOGL1*, *LOGL6* and *LOGL8* had higher expression in roots] are somewhat replicated in wheat.

RNA-seq data lack the cellular precision of promoter:reporter constructs but, interestingly, *AtLOG8*, which is the most closely associated GFM with *TaLOG1* in Clade II, is the most widely expressed arabidopsis GFM, expressing in vascular tissue and other cells in roots and shoots (Kuroha *et al*., 2009). Clade IV *AtLOG4* is also strongly expressed in vascular tissue, and, between *AtLOG8* and *4*, there is *LOG* expression in both root and shoot meristems. The constitutively expressed *TaLOGs10* and *11* are also in Clade IV. Not surprisingly then, an RNA-seq of extracted whole organs containing vascular tissue revealed expression. As observed by Kuroha *et al*. (2009) for arabidopsis and is also apparent in strawberry (Mi *et al*., 2017) and cotton (Wang *et al*., 2021), cytokinin can be synthesized in most parts of a plant via the direct LOG pathway.

Reproductive Tissues

Clearly there are *TaLOGs* expressing in both vegetative and reproductive shoot apical meristems (Figures 6 and S8). This was expected as Harrop *et al*. (2016), using laser microdissection, showed *OsLOG* to be expressed in the four meristem types (rachis meristem, primary branch meristem, elongating primary branch including axillary meristems and the spikelet meristem), whereas *OsLOGL1* and *LOGL6* (*LABA1*) were expressed more highly in the latter two meristem types (Harrop *et al*., 2016).

More recently, and again using NanoString technology, Yamburenko *et al*. (2017) monitored, among other cytokinin-related genes, expression of all the rice *LOG/LOGL* GFMs, across five stages of panicle development in relation to the shoot apical meristem. Several *LOG*s exhibited greater expression in the SAM and during primary branch initiation (*OsLOGL 2, 3, 4, 5, 7, 8*) than at later stages. Peak expression of *OsLOG* occurred at the time when most branch and spikelet meristems had formed, and expression had decreased at the stage when floret meristems emerge from the spikelets, a stage when *LOGL1, LOGL6, LOGL9* and *LOGL10* were most strongly expressed, although these also expressed earlier in the SAM (Yamburenko *et al*., 2017).

In contrast to the transcript profiling by Feng *et al*. (2017) during wheat inflorescence development, which did not reveal expression of *TaLOG* GFMs, most wheat LOGs, with the exception of *TaLOG6* and *TaLOG2*, expressed during spike and spikelet emergence. The differential expression shown by Yamburenko *et al*. (2017) between rice *LOG* GFMs during panicle development is not apparent in the wheat data during spike emergence due to the limited sampling of early stages of spike development. However, *TaLOG9-5A0834700* [a homologue of *OsLOGL9* that strongly expressed at stage 5 when floret meristems emerge from the spikelets (Yamburenko *et al*., 2017)] showed strong activity in the spikes and spikelets, and in anthers at anthesis, with little expression in other organs. The expression of both *TaLOG9-5A0833800* and *TaLOG9-5B0873000*, although relatively lower, was restricted to the spikes and spikelets. Earlier developmental stages, when meristematic tissues are present, were not included in the RNA-seq samples. This is unfortunate, as elevated levels of *TaIPT8* and the high expression levels of a β-glucosidase GFM (*TaGLU1-1*) and *TaRR9* and *1* (Song *et al*., 2012), all indicate that this is a stage when cytokinin is likely to be critical.

However, wheat spikes are also known to be particularly susceptible to stress at booting, with low temperature stress impacting spikelet, floret and awn development (e.g. Zhang *et al*., 2019), and heat stress causing significant losses to floret fertility (grain set) and hence yield (e.g. Erena *et al*., 2021). All three *TaLOG9* GFMs are, therefore, candidate targets for breeding, as these stresses may decrease cytokinin levels (Cortleven *et al*., 2019a). Moreover, the lack of expression of the *TaLOG9* GFMs in roots and the shoot apical meristem at tillering could be useful in this context, as increased cytokinin levels generally impact root growth negatively (Werner *et al*., 2003). An increased tiller number in wheat is considered undesirable as multiple small tillers redirect resources away from the main tillers and ultimately decrease yield (Hendriks *et al*., 2016; Kebrom *et al*., 2012).

To enhance yield, one would anticipate increasing the cytokinin content of relevant tissues (Jameson and Song, 2016). While it will be challenging to find upregulated mutants of *TaLOG9*, mutations in an upstream negative regulator could be targeted. For example, *UNBRANCHED3*, a member of the *SPL* gene family, binds to the promoter and negatively regulates *OsLOG* and positively regulates *OsCKX*, among other genes (Du *et al*., 2017). Strong overexpression of *UB3* reduced active cytokinin levels and negatively regulated tillering, panicle branching and grains per panicle in rice (Table 1). In the *ub3::mum* maize mutant line, which has additional kernel rows, Du *et al*. (2017) showed that genes associated with cytokinin biosynthesis were upregulated, and those associated with degradation downregulated. Du *et al*. (2017) concluded that *UB3* regulates vegetative and reproductive branching by modulating cytokinin biosynthesis and signalling in both maize and rice. While multiple components interact to control yield in cereals, mutants of *UB3* in wheat would be interesting to investigate.

In contrast to UB3, Li *et al*. (2021) recently identified a positive regulator of *LOG*. A japonica variety of rice, BS208, showed a highly abnormal panicle branching pattern, with reduced numbers of lateral grains on secondary branches. To determine the genetic mechanism underlying the abnormal phenotype, BS208 was crossed with Indica variety Teqing (TQ). Map-based cloning using a segregating population with different grain numbers per panicle led to the identification of an R2R3 MYB transcription factor, *REGULATOR OF GRAIN NUMBER1* (*RGN1*). Near isogenic lines (NIL) were developed: plants with the loss-of-function mutant allele, *rgn1*, displayed highly decreased grain number. *OsLOG* expression was downregulated in *rgn1-1* and NIL-*rgn1* compared with japonica cultivar Nipponbare and NIL-RGN1

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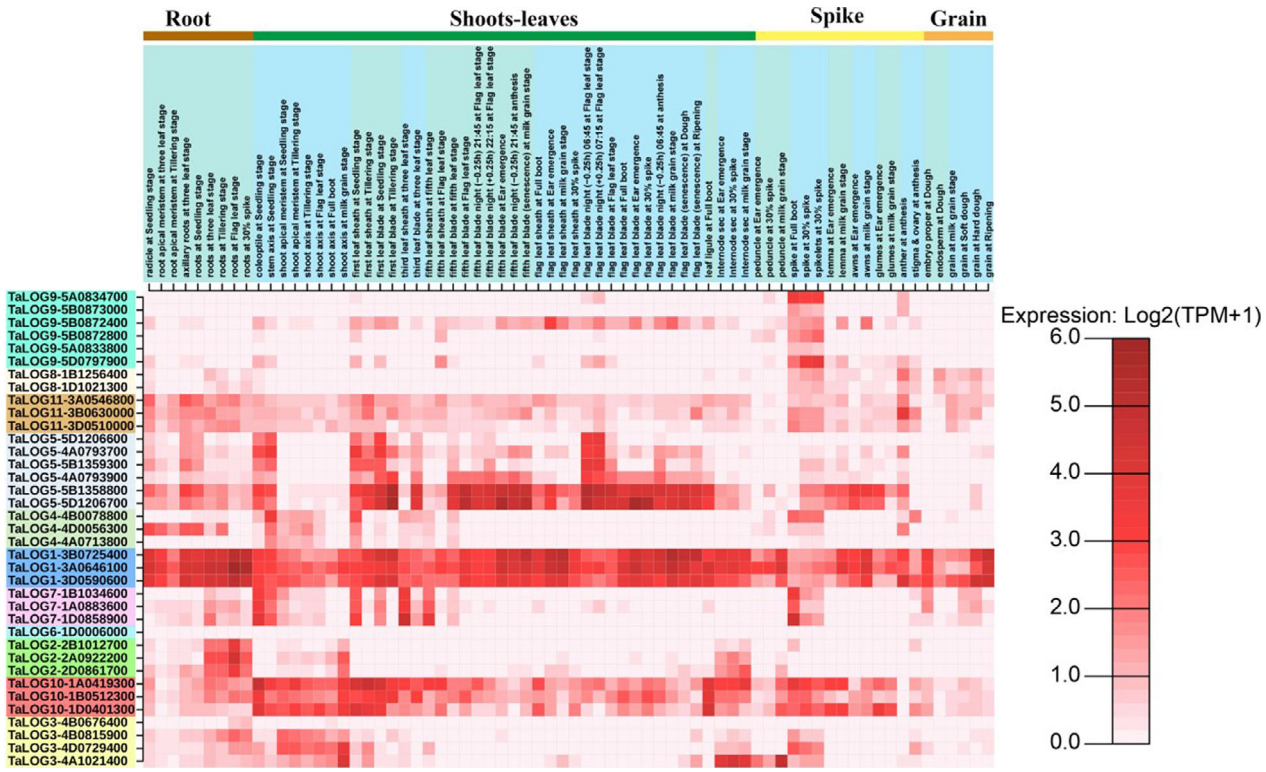

Figure 6 The developmental expression patterns of the *TaLOGs*. The expression data for each *TaLOG* gene family member were extracted from the published data (expVIP: http://www.wheat-expression.com/, Ramırez-Gonzalez et al., 2018), which leveraged 850 RNA-seq samples. The data set was grouped into four tissue types using the R program and is shown on the top of the figure. The order of the genes was sorted using Linux commands and corresponds to the LOG phylogenetic tree shown in Figure 3. The expression data is shown as a heatmap using the image function in the R program. Detailed expression graphs are shown in Figure S8.

(Table 1), whereas the expression of *LOG* in RGN1-OE3 plants was upregulated. Moreover, yeast one-hybrid assays showed that RGN1 bound directly to the promoter region of *OsLOG*, and overexpression of *OsLOG* from Nipponbare in BS208 partially rescued the absence of lateral grains in secondary branches (Table 1), leading Li *et al*. (2021) to the conclusion that RGN1 affects lateral grain number and panicle architecture in rice by regulation of *OsLOG* expression.

Investigation of RGN1 sequence variations in wild and cultivated rice revealed two haplotypes, with accessions with the RGN1^{C} allele exhibiting longer panicles and greater expression of *LOG*. They suggested that this elite haplotype RGN1^{C} for panicle length identified in natural germplasm has potential to improve the yield of rice, but had not been selected during domestication, potentially due to linkage drag. This may have been associated with the elimination of undesirable allele(s) controlling awn formation preventing selection of the RGN1^{C} allele during domestication (Li *et al*., 2021).

These data provide evidence of the critical role of OsLOG in controlling determinants of yield. We located three putative wheat *RGN1* sequences in the NCBI database, most likely associated with the A, B and D sub-genomes. This sequence is highly conserved in *Aegilops tauschii* (the progenitor of the D genome of bread wheat; Li *et al*., 2021) and is conserved in the first half of the RGN1 sequence in wheat (Table S4). Additionally, promoter motif scanning analysis showed that all 37 *TaLOG* GFMs have the highly conserved motif, ACCAAA (located at the end of the 18th motif of Figure S9), which was shown by Li *et al*. (2021) to be bound by OsRGN1. This indicates that *TaLOG* may be a conserved downstream target of RGN1. Identification of sequence variants, similar to that found in rice, should be of interest to wheat breeders.

Leaf tissues

TaLOGs, particularly *TaLOG5* and *TaLOG1*, are expressed in leaf tissues at all stages of development, including in senescing flag leaves (Figures 6 and S8). The recent model suggested by Sakakibara (2021) shows *t*ZR (and some *t*Z) moving in the xylem stream, with the *t*ZR being converted initially to *t*ZRMP, by adenosine kinase, prior to activation by LOG either in the SAM or in the leaf. Following activation of the two-component signalling system, the model shows cytokinin positively impacting the leaf emergence rate and leaf maintenance. As several *TaLOGs* are strongly expressed in wheat leaves, and others in the SAM, targeting of the cytokinin produced is likely via positioning of receptors and/or response regulators (Werner *et al*., 2021).

Interestingly, Werner *et al*. (2021) recently showed that elevated *AtLOG4* expression targeted to the epidermis significantly increased the size of the vegetative meristem but less so the inflorescence meristem. Leaf size and rosette size were increased as a result of increased cell number, with the authors suggesting cytokinin was acting on the epidermal cells in a partially layer-autonomous fashion delaying their differentiation. Seed yield was also enhanced due to larger siliques with more seeds per silique, likely as a consequence of increased cytokinin synthesis in the outer layer of reproductive tissues and in the

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placenta, enhancing ovule formation (Table 1). They suggest that the increased seed yield through the expression of *AtML1:LOG4* as a single dominant gene is of potential biotechnological interest (Werner *et al*., 2021).

Leaf development in monocots, with their long, narrow leaves with parallel veins, is quite different from that of the eudicots. However, not surprisingly, LOG is again implicated. In rice, *OsWOX4* plays important roles in early leaf development through its expression in leaf primordia (Yasui *et al*., 2018). OsWOX4 functions upstream of *LOG* as *in situ* analysis showed that knockdown of *OsWOX4* reduced the expression of *OsLOGL3* and *OsLOGL10* in developing vascular bundles. Consistent with this, cytokinin levels were downregulated by *OsWOX4* knockdown and cell cycle progression was reduced (Yasui *et al*., 2018; Table 1). *OsWOX4* is also involved in the maintenance of the vegetative meristem in rice (Ohmori *et al*., 2013; Yasui *et al*., 2018), whereas *AtWOX4* is specifically associated only with the maintenance of vascular stem cells in arabidopsis, indicating diversification of function of the two orthologous genes between rice and arabidopsis.

Root tissues

Multiple *TaLOGs* are expressed in root tissues at various stages of development (Figures 6 and S8). Cytokinins have generally been considered to be negative regulators of root elongation and branching, and reduction in cytokinin levels through overexpression of *CKX* leads to enhanced root growth in multiple species (e.g. Ramireddy *et al*., 2021; and reviewed in Chen *et al*., 2020a). However, interestingly, decreased expression of *CKX* led to greater root mass in *seedling* roots of wheat and barley (Gasparis *et al*., 2019; Jablonski *et al*., 2020, 2021a; Zalewski *et al*., 2010). Cytokinins are critical to cambial activity in roots (Matsumoto-Kitano *et al*., 2008), and the septuple *AtLOG* mutant showed severe retardation of root growth and defects in the RAM (Tokunaga *et al*., 2012; Table 1). Furthermore, Ohashi-Ito *et al*. (2014) showed an arabidopsis bHLH transcription factor complex targets *AtLOG3* and *4* in xylem precursor cells in the RAM, with the produced cytokinin acting as an initial signalling event that induces cell proliferation in adjacent procambial cells (Table 1). De Rybel *et al*. (2014) also showed that *AtLOG4* is a direct target gene of the TMO5/LHW transcription complex and plays a critical role in establishing vascular patterning (Table 1). Moreover, cytokinin, through the inhibitory activity of AtIPT and AtLOG4, is intimately involved in determining the spacing of lateral roots (Bielach *et al*., 2012; Chang *et al*., 2015). Consequently, it is not surprising to find *TaLOG* GFMs expressing in wheat roots.

OsLOG mutants unexpectedly show increased yield

OsLOGL genes have been the target of selection during the domestication of rice (Gu *et al*., 2015; Hua *et al*., 2015). The wild progenitor of rice, *O. rufipogon*, has many long, barbed awns, fewer tillers per plant and fewer grains per panicle in contrast to cultivated rice. *An-1* and *An-2* are two quantitative trait loci (QTLs) associated with awn formation and awn elongation, respectively, and both loci are considered to have been targets of artificial selection during rice domestication (Gu *et al*., 2015; Hua *et al*., 2015; Luo *et al*., 2013). An-1 positively regulates the formation of awn primordia and negatively regulates grain number (Gu *et al*., 2015; Luo *et al*., 2013). Gu *et al*. (2015) fine mapped An-2 to the *LOGL6* gene.

Gu *et al*. (2015) showed that An-2 exhibited a pleiotropic effect during rice development and morphology: it promoted awn elongation by enhancing cytokinin levels and cell division. Additionally, grain production was decreased through fewer grains per panicle and fewer tillers per plant; root length was also decreased (Table 1). *An-2* was mainly expressed in young panicles, branching crowns and roots, and specifically in the epidermal cells and vascular bundles of awns. Complementation and overexpression experiments confirmed that a *LOG* homologous gene was *An*-2. Further, this *LOG* encoded a functional OsLOGL6 in wild rice. The *an-2* allele has a C-nucleotide deletion in exon 1 that resulted in a frameshift and a premature non-functional protein that had lost nearly all the conserved sequences. They concluded that the *An-2* locus was probably a target of artificial selection during rice domestication, for suppression of awn elongation and/or promotion of yield (Gu *et al*., 2015). Additionally, they concluded that the functions of homologous LOG enzymes have differentiated, as the expression pattern of *An-2* in panicles was more dispersed compared with *LOG*. Moreover, the *log* mutant of rice had fewer grains per panicle and abnormal spikelets. Gu *et al*. (2015) suggested this differentiation is controlled by regulatory regions rather than protein functional variations.

In a parallel study, Hua *et al*. (2015) fine mapped the QTL *LONG AND BARBED AWN1 (LABA1)*. Complementation and RNAi interference of the putative gene led Hua *et al*. (2015) to the conclusion that the *LABA1* gene 'conditions' both awn length and the presence of barbs (Table 1). *LABA1* was preferentially expressed in developing panicles, 1–2 cm in length, notably in young epidermal cells of awn primordia, with lesser expression in vegetative tissue and the awn itself after heading. The *laba1* mutation (a frameshift deletion) was associated with short, barbless awns, with increased unit weight and was selected during rice domestication (Hua *et al*., 2015). Functional analyses showed *LABA1* encodes cytokinin nucleoside 5′-monophosphate phosphoribohydrolase activity, with sequencing indicating it to be a rice *LOG* homologue. Hua *et al*. (2015) concluded that *LABA1* encodes the cytokinin-activating enzyme *OsLOGL6* that increases cytokinin content and cell division in epidermal cells of the awn primordia in wild rice.

Wang *et al*. (2020) selected a drought-tolerant rice activation tagged line with 100% amino acid match to *OsLOGL5*. Focusing on the fact that there are diverse N-terminal and C-terminal ends among the *LOG* GFMs, they developed a series of gene edits targeting the final 25 amino acids of the C-terminal end of the protein. Tagged lines overexpressing *OsLOGL5* were semi-dwarfed and showed reductions in primary root growth, fertile panicles, seeds per panicle and seed weight, with yield significantly reduced under drought conditions. Gene-edited lines with mutations at the 3′-end of the coding sequence resulted in normal vegetative growth, and with increased yield in the field under normal conditions as well as under drought and low N (Table 1). Under drought, this was a consequence of increases in grain setting rate, total grain number and 1000 grain weight. Of the six gene-edited lines, two showed an increase in tiller number, while two others had fewer tillers. Wang *et al*. (2020) concluded that *OsLOGL5* was likely to be involved in both seed development and grain filling and that the C-terminal end of the protein was important in the response of *OsLOGL5* to abiotic stress conditions.

In the above cases, it is apparent that reduced expression of *OsLOGL6* and *OsLOGL5* leads to increased yield (Table 1).

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Response to biotic and abiotic stressors

Cytokinins are generally considered to be negative regulators of stress (e.g. Nishiyama *et al*., 2011; Pavlů *et al*., 2018; Tran *et al*., 2010). Wang *et al*. (2021) showed that expression levels of *GhLOG* were modulated by drought, high salt and hormone stress. They suggested that overexpression of *GhLOG3*_At improved tolerance of cotton to NaCl potentially because root growth was less inhibited by salt relative to control roots, whereas reduced expression enhanced sensitivity (Wang *et al*., 2021), a result that appears contrary to cytokinins as negative regulators of stress.

In wheat, those *LOG* GFMs constitutively expressed in all organs (*TaLOG1, TaLOG10* and *TaLOG11*; Figure 6) showed little response to biotic or abiotic stressors (Figure 7). *TaLOG6* showed no response. *TaLOG5* GFMs, two of which were strongly expressed in leaves (Figures 6 and S8), showed only a low level of expression in response to the biotic and abiotic stressors. Overall, there were no consistent patterns of up- or down-regulation in response to various stressors; for example, in terms of drought, both upregulated and downregulated GFMs can be seen. In a similar fashion, the analyses conducted by Pavlů *et al*. (2018) showed little overlap in the expression patterns between *AtLOG* GFMs and a wide range of stress-associated genes, in contrast to expression of *IPT* and signal transduction components.

LOGs are intron-rich

Jeffares *et al*. (2008) suggest that there has been selection against introns in genes that require rapid expression changes. They showed that genes whose expression changed rapidly in response to stress contained significantly lower intron densities. Specifically relating to plants, Liu *et al*. (2021) speculate that loss of introns may be a factor in early land plant evolution allowing fast response to stress conditions. They also suggested loss of introns may have facilitated faster developmental adaptations to life on land.

Generally, the reported *LOG* GFMs appear to be relatively intron-rich (having ≥4 introns; Liu *et al*., 2021). The *LOG* gene of rice has seven exons and six introns (Kurakawa *et al*., 2007), Strawberry (Mi *et al*., 2017) and *Medicago truncatula LOG*s have six to seven introns (Azarakhsh *et al*., 2020). While 10 of the 11 wheat *LOGs* are relatively intron-rich (with between five and six introns), *TaLOG5* on all sub-genomes is intron-poor (Figure 8). Notably, *OsLOGL5* is also reported to have no introns (Wang *et al*., 2020), in contrast to *OsLOG*. The single *TaLOG6* has six introns, with one being extremely long. The intron pattern is in general agreement with Wang *et al*. (2021) where most cotton *LOGs* had six introns, although one GFM had an extremely long intron.

The generally intron-rich character of LOGs is in contrast to the intron-poor character of the cytokinin glucosyl transferase (CGT) gene family (Chen *et al*., 2021a), which is considered to respond fast to stress (Vyroubalová *et al*., 2009) and to decrease active cytokinin levels (Chen *et al*., 2021a). Intron numbers for *TaCKX* are somewhat in between, ranging from 0 to 4, with the majority of GFMs having either 2 or 4 introns (Figure S10). Indeed, 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). However, *TaLOG5* GFMs (0-1 intron) showed little response to biotic or abiotic stress (Figure 7) and did not have more over-represented stress-associated *cis*-regulatory elements (CREs) than other *TaLOGs* (Figure 9), and so potentially do not follow the general rule relating to intron-poor genes responding rapidly to stress.

Long introns have been considered to be costly in terms of transcription (Seoighe *et al*., 2005). The very long intron in *TaLOG6D*, and the loss of *TaLOG6A* and *B* (Figure 8), possibly reflects selection against this gene. *TaLOG6D* was not expressed during development nor responsive to stress (Figures 6 and 7). We subsequently discovered that *TaLOG6* is likely truncated on the A and B genomes of Durum wheat and, as such, was not captured in our analysis of bread wheat. As TaLOG6D protein has high sequence similarity and possibly similar function to OsLOGL6/LABA1 in rice, the long intron might be the result of unconscious selection events towards the reduction and/or elimination of its negative effects on grain number as shown for OsLOGL6 in rice, leading to loss-of-function of the gene. Selection might have induced the complete elimination of the non-functional sequence fragments of this gene loci on chromosomes A and B. We noted that TaLOG6D protein aligns closely to AtLOG6 which was shown to be non-functional. Interestingly, no MtLOGs or PtLOGs aligned with AtLOG6, and *FvLOG3* (homologue of *AtLOG6*) was not expressed in strawberry (Mi *et al*., 2017), so it is possible that this LOG is non-functional across the angiosperms.

Cis-regulatory element analysis

We conducted an analysis of the *cis*-regulatory elements (CRE) on 3 kb of the putative promoters of the *TaLOG* GFMs, as this has been used as an indicator of responsiveness of genes to various signals. Here, we grouped the CREs according to responsiveness to abiotic, biotic, hormone and 'internal control'. The heatmap shows whether the various elements are over- or under-represented relative to 100 randomly selected wheat genes (Figure 9).

There are several consistent patterns among the CREs on the promoters of *TaLOG* GFMs: light-responsive elements and anaerobic-induction response elements were over-represented, whereas *CMA3*, cell cycle and binding site of ATBP.1 were under-represented (Figure 9).

As cytokinin is intimately involved in the response of the plant to the light environment (reviewed in Cortleven *et al*., 2019a; Ikeda *et al*., 2021; Pavlü *et al*., 2018), it is not surprising that light-responsive elements are over-represented. *CMA3* (conserved DNA module array) is a binding site for 3AF3, which, along with other proteins, activates light transcription of the small subunit of RUBISCO (Sarokin and Chua, 1992). As this *cis* element is under-represented, it implies expression of *TaLOGs*/cytokinin is not required for this particular process.

ATBP-1 (AT-rich DNA binding protein-1), first identified by Tjaden and Coruzzi (1994), binds to AT-rich promoter elements. These elements are considered to play a role in transcription of plant genes. As they are under-represented on the *TaLOG* promoters, we infer that the expression of the *TaLOG* genes is under the regulation of specific regulators, rather than a common regulator.

An anaerobic environment is one totally lacking in free or bound oxygen. Anaerobic-induction response elements are over-represented on the *TaLOG* promoters. Interestingly, during a genome-wide association study (GWAS), *OsLOGL8* was highly associated with anaerobic germination in rice (Hsu and Tung, 2015). Maintenance of the RAM under such conditions is essential.

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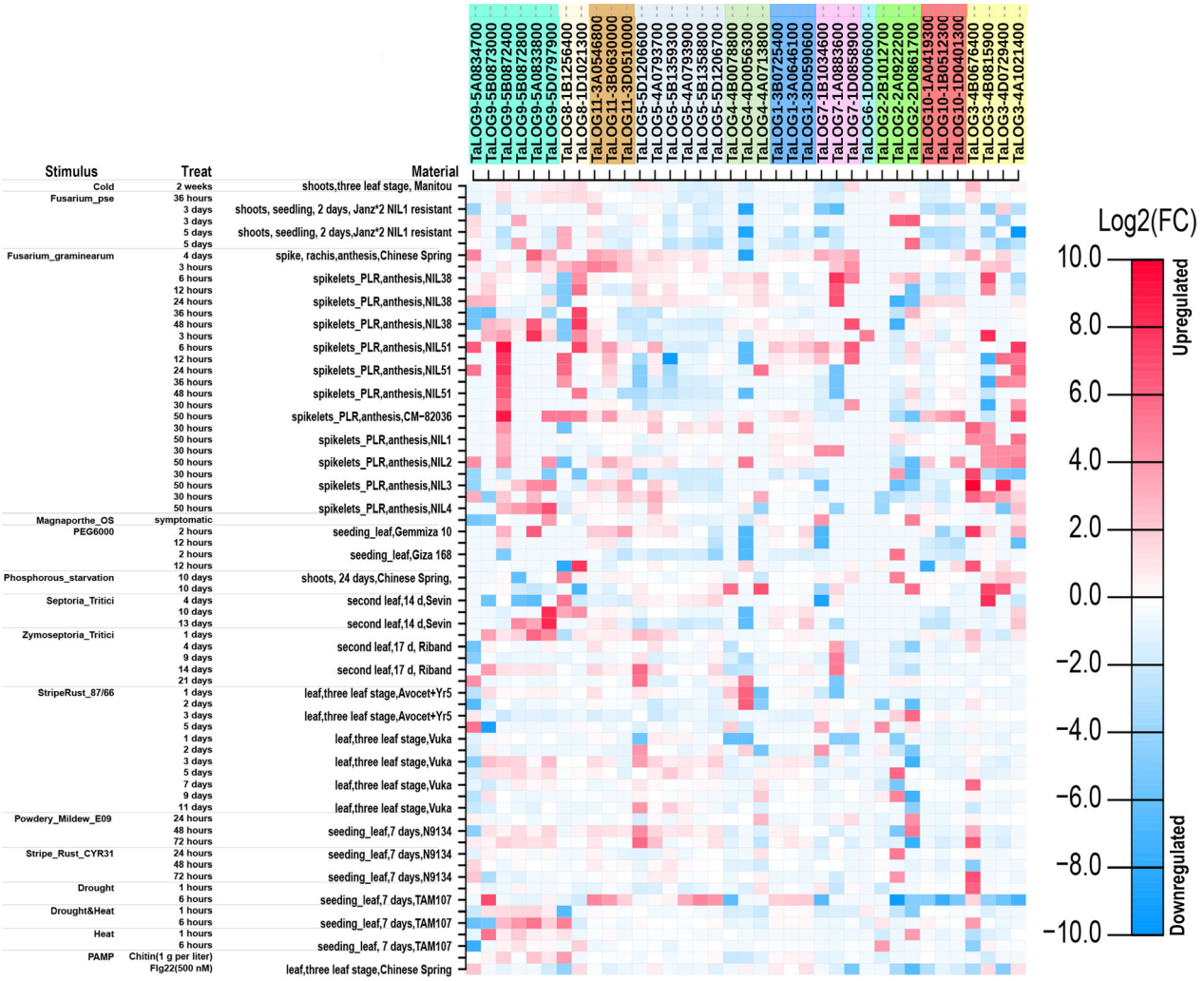

Figure 7 Expression response pattern of the *TaLOGs* under biotic and abiotic stressors. The expression data were extracted from the expVIP database (http://www.wheat-expression.com/) and shown as fold change. The expression values are shown in fold change (FC). FC is defined as: FC = Control/Treat. In order 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 gene order corresponds to the LOG phylogenetic tree (Figure 3). The expression data are shown as a heatmap using the image function in R program.

The MYBHv1-binding site is generally underrepresented. MYBHv1 is a positive regulator of drought tolerance and is associated with mediating the action of abscisic acid (ABA; Alexander *et al*., 2019). Lee *et al*. (2007) showed the wheat orthologue (TaMYB1) to be upregulated by abiotic stress in wheat roots, and overexpression of the wheat paralogue TaMYB1D in tobacco enhanced drought and oxidative stress resistance (Wei *et al*., 2017). However, the MYB drought response element is generally over-represented on the *TaLOG* promoters, although there is evidence to suggest that some of these elements may be negative regulators of stress responses (e.g. Baldoni *et al*., 2015; Gao *et al*., 2016). As cytokinin levels and signal transduction are generally reduced during drought (e.g. Nishiyama *et al*., 2011; Nguyen *et al*., 2016; reviewed in Cortleven *et al*., 2019a), negative regulation would appear likely.

Elements responsive to ABA, gibberellin and auxin are generally averagely to under-represented on the TaLOG promoters (Figure 9). As this is in marked contrast to the over-representation of these CREs on the CGTs (Chen *et al*., 2021a), we also analyzed the CREs on the *TaCKX* promoters (Figure S11). Interestingly, the patterns of CREs on the *TaCKX* promoters matched those on the *TaLOG* promoters, which reinforces suggestions in the literature that CKX plays a strong role in maintaining cytokinin homeostasis (refer Chen *et al*., 2020a). However, the well-known antagonism between cytokinin and ABA (Zubo and Schaller, 2020) would appear to be better reflected in the over-representation of ABA-REs on the promoters of the *TaCGT*s (Chen *et al*., 2021a) but not on *TaLOG*s or *TaCKX*s. This may reflect the conservation (rather than destruction) of cytokinin during stressful conditions, and its availability for subsequent release.

Jasmonates (JA) are involved in many aspects of plant development and are viewed as part of the growth-defence trade-off (Howe *et al*., 2018). They are key factors in the plant's response to wounding by chewing insects including in rice (Fu *et al*., 2021), resistance to necrotrophs (e.g. Chen *et al*., 2021b) and certain biotrophs (Figueiredo *et al*., 2017). Interactions between cytokinin and jasmonates are generally viewed as antagonistic (Pavlů *et al*., 2018; Zubo and Schaller, 2020), and

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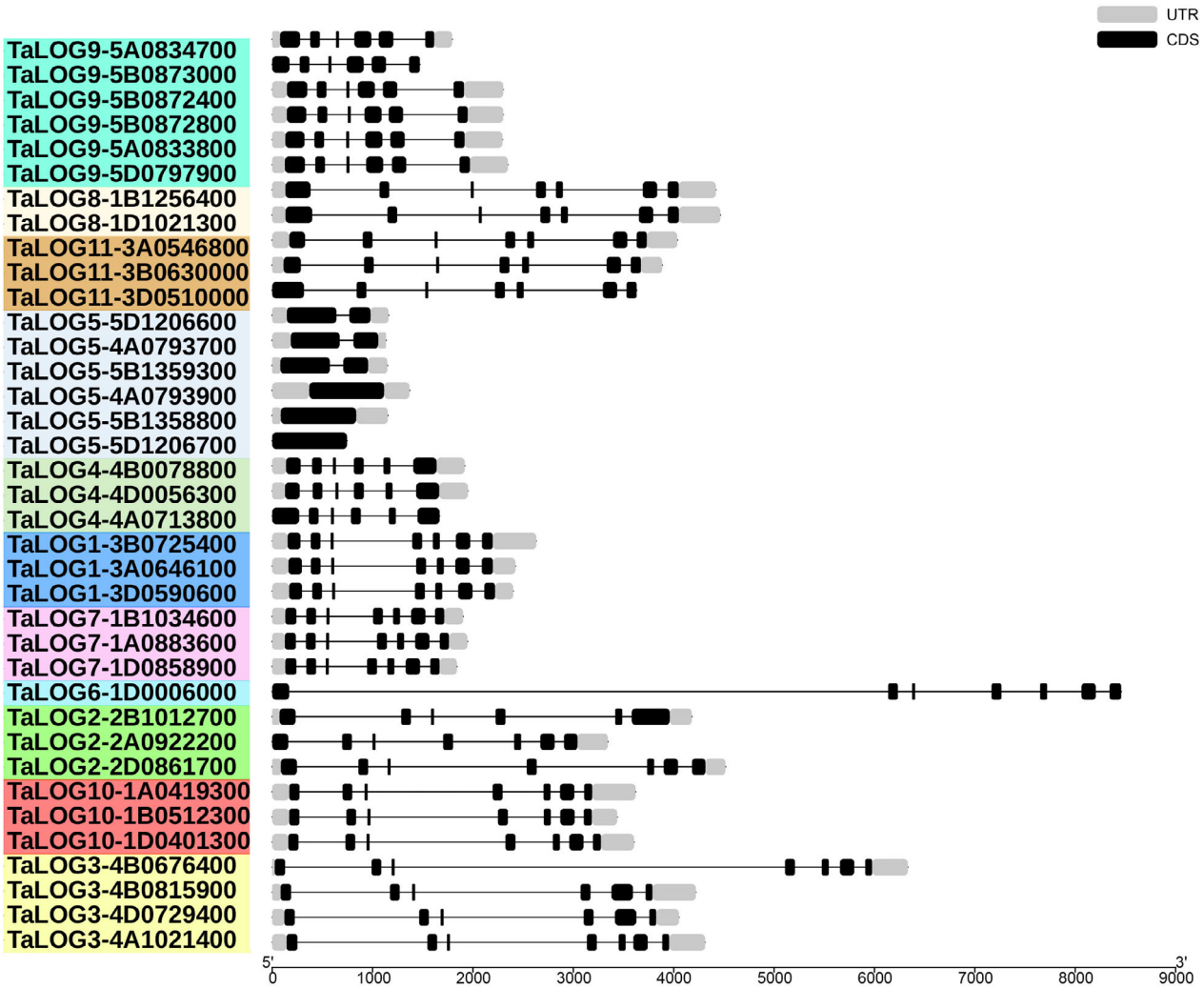

Figure 8 The intron–exon gene structure pattern of *TaLOG*s. The gene intron–exon structure information was extracted from the gff3 file of wheat genome (Version 2.1, Zhu *et al*., 2021) using Linux shell commands. The intron–exon gene structure pattern was drawn in TBtools (Chen *et al*., 2020b).

a second obvious contrast in the CREs relates to the JA-REs. These are over-represented on the *TaCGT* promoters and under-represented on both the *TaLOG* and *TaCKX* promoters, again indicating control of cytokinin levels during stress responses through glucosylation rather than through transcription of *TaLOG* or destruction of cytokinin by CKX. This conclusion is supported by the overall low level and inconsistent responses of the *TaLOG*s to biotic stressors (Figure 7).

In contrast to the above, the light-REs were over-represented on the promoters of the *TaLOG*s, *TaCKX*s and *TaCGT*s, potentially reflecting the critical involvement of cytokinin in light-regulated responses (e.g. Cortleven *et al*., 2019a, 2019b; Pavlů *et al*., 2018).

Two steps or just the one?

In a recent paper, Nguyen *et al*. (2021b) endeavor to reactivate the two-step pathway as well as suggesting that the cytokinin ribosides may be active compounds and have significant roles beyond being transport forms. Interestingly, they do not refer to Lomin *et al*. (2015) who used a plant-based system to confirm that only the free bases and not the ribosides interacted with the cytokinin receptors, in contrast to previous receptor work where bacterial or yeast-based systems were used (see references in Nguyen *et al*., 2021b). Indeed, they cite a yeast complementation assay that showed binding to receptors not only of free bases and ribosides but also of nucleotides and *O*-glucosides (Daudu *et al*., 2017) and infer that *Brassica napus* receptors (Kuderová *et al*., 2015) also bind a wide range of cytokinin forms (see Nguyen *et al*., 2021b). This inference by Nguyen *et al*. (2021b) is in contrast to that by Hluska *et al*. (2021) who state that *O*-glucosides or ribosides exert cytokinin activity in various bioassays, but *O*-glucosides do not bind to receptors at all and ribosides very weakly. They are active because they can be metabolized to the active forms. Moreover, structural biology assessments of the ligand-binding sensor modules of the cytokinin receptors indicate free bases are a 'good fit' but not so for ribosides (Hothorn *et al*., 2011; Lomin *et al*., 2015); binding of nucleotides and *O*-glucosides to cytokinin receptors is, therefore, highly improbable.

More worryingly, Nguyen *et al*. (2021b) cite several papers in which cytokinin ribosides were applied to plants and suggest that the impacts on growth and/or development were due to the riboside itself. This ignores the metabolism papers from the Letham and Wareing labs that show that exogenously supplied

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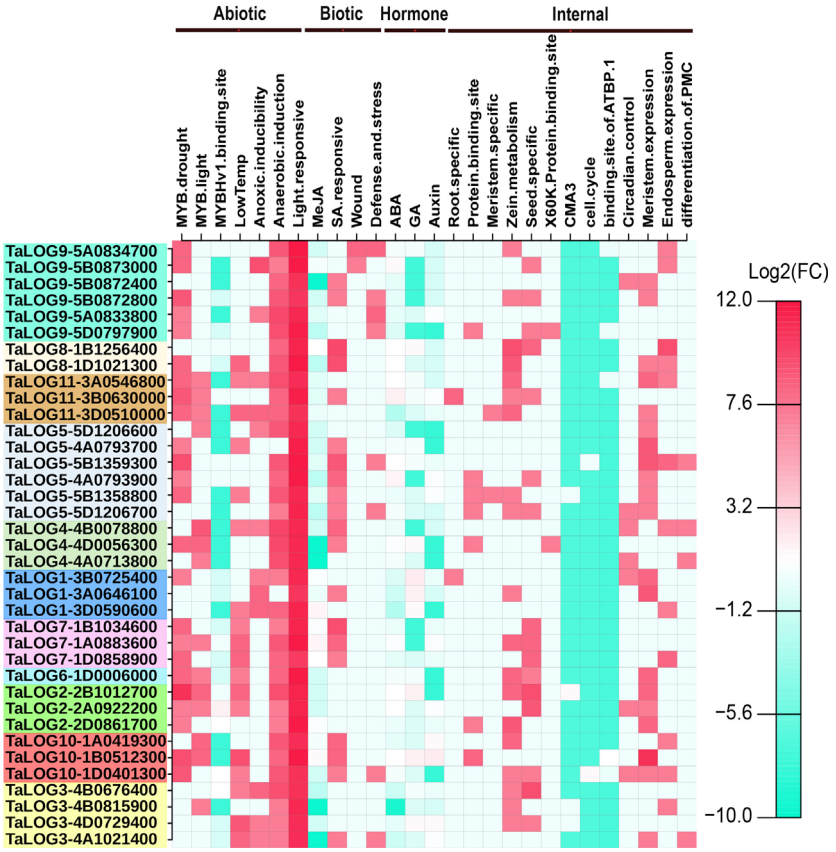

Figure 9 *Cis*-regulatory element distribution patterns on 3 kb potential *TaLOG* promoter. The 3 kb potential promoter sequences were extracted from the wheat genome sequence (Zhu *et al.*, 2021) via Linux shell commands. The *cis*-regulatory elements (CRE) were predicted in plantCARE database (Chou and Shen, 2007; 2008; 2010). The heatmap was made based on a comparative analysis of CREs on the *TaLOG*s with a representative promoter CRE data set of 100 genes chosen randomly from the wheat genome as described in Chen *et al*. (2021a). We calculated a value of FC (Fold Change, TaLOG CRE number/average CRE number) to confirm over- (FC > 1) or under-represented (FC < 1) CREs. The FC values were normalized by log2 calculation and developed into the heatmap using the R program.

cytokinins are rapidly converted to the nucleotides in various plants: for example ^{3}H-ZR/^{3}H-DZR/^{3}H-Z supplied to *Lupinus angustifolius* (e.g. Jameson *et al*., 1987; Parker *et al*., 1978; Zhang *et al*., 2002) and *L. luteus* (Knypl *et al*., 1985); ^{3}H-Z/^{3}H-ZR supplied to soybean (e.g. Singh *et al*., 1988); ^{3}H-Z supplied to *Zea mays* (e.g. Parker and Letham, 1974) and radish (Gordon *et al*., 1974). Early work from Wareing *et al*. (1977) indicated that 8-^{14}C zeatin supplied to roots and buds of *Phaseolus vulgaris* was converted to zeatin nucleotide rather than to glucosides, and ^{14}C-dihydrozeatin supplied to stem segments of *P. vulgaris* was rapidly metabolized to dihydrozeatin nucleotide (Palmer *et al*., 1981). Indeed, in the seminal paper testing functionality of arabidopsis LOGs, iPR was supplied to arabidopsis on the *expectation* of conversion to nucleotide and subsequent direct conversion by LOG to active free bases (Kuroha *et al*., 2009). While endeavoring to reactivate the two-step pathway, Nguyen *et al*. (2021b) do admit that strong and specific activity towards cytokinins of the enzymes of this pathway has yet to be confirmed (see references cited in Nguyen *et al*., 2021b; Figure 1). The most recent model presented by Sakakibara (2021) shows xylem-translocated *t*ZR converted by adenosine kinase to *t*ZRMP and thence by LOG to *t*Z to control both leaf emergence rate and leaf expansion. The recent commentary by Romanov and Schmülling (2022) is a critique of Nguyen *et al*. (2021b) and concludes that the free bases and not the ribosides are the active forms of cytokinins.

Conclusion

Structural biology comparison of wheat LOGs with arabidopsis LOGs indicates that wheat LOGs 1-11 likely form dimers. Sequence and expression analyses indicate that TaLOGs 1-5 and 7-11 are functional cytokinin riboside 5′-monophosphate phosphoribohydrolases. We suggest that the main homeostatic mechanism controlling cytokinin levels resides with CKX but, in response to biotic and abiotic challenges, the activities of the CGTs, rather than CKX or LOG, become more important. The *TaLOGs* variously expressed in all tissues analyzed and those tissues constitutively expressing LOGs may provide the plant with basal levels of cytokinin (Hluska *et al*., 2021). Even though cytokinins are generally regarded as negatively impacting root growth, a basal level of cytokinin is required for maintenance of the RAM (Tokunaga *et al*., 2012), as well as cytokinin being implicated in lateral root spacing (Chang *et al*., 2015). Even senescing leaves need to maintain basic mitochondrial functionality and energy production well into senescence (Kim *et al*., 2018). The more subtle roles for cytokinin, such as implicated in the SAM, are more dependent on the precise positioning and activity of RRs and other components of the signal transduction pathway, than on active cytokinin forms *per se*.

However, results such as those from the overexpression of *AtLOG4* in epidermal cells leading to enhanced yield components (Werner *et al*., 2021) provide avenues for future investigation. Interestingly, selection during domestication of a mutation in rice *LOGL6* is implicated in increased yield (Gu *et al*., 2015; Hua *et al*., 2015), and overexpression of *OsLOGL5* led not only to a reduction in primary root growth, as might be expected, but also to a reduction in yield. Further, gene editing the C-terminal of *OsLOG5* conferred enhanced stress tolerance and enhanced yield (Wang *et al*., 2020). Consequently, targeted mutations in wheat *LOGs* may provide useful material for breeders.

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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. G.B.J. provided the structural biology analyses. Y.G. collected the raw seq data of LOG and the RNA-seq data, and did some manual pre-processing of the raw data sets. J.S. interrogated some phylogenetic data and prepared the relevant figures. 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 The cluster pattern of the unrooted LOG phylogenetic tree clades.
Figure S2 The rectangular pattern of the unrooted LOG phylogenetic tree.
Figure S3 Detailed information of the TaLOG motif patterns.
Figure S4 Conserved domain analysis of TaLOGs.
Figure S5 Conserved domain analysis of TaLOGs, TaLOG12, AtLOG Type IIb and MtLOGL3.
Figure S6 Motif patterns of MtLOGs, AtLOGs, OsLOGs and MtbLOG.
Figure S7 The detailed information for the motif patterns of MtLOGs, AtLOGs, OsLOGs and MtbLOG.
Figure S8 RNA-seq graphs for each of the *TaLOG* gene family members.
Figure S9 Detailed information of the *TaLOG* promoter motif patterns.
Figure S10 The intron-exon gene structure pattern of *TaCKX*.
Figure S11 *Cis*-regulatory element distribution patterns on 3 kb potential *TaCKX* promoter.

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Table S1 *TaLOG* gene family members: gene ID, positions, symbols, and homologues.
Table S2 Percent identity of TaLOG2, 6, 1 and 12 with AtLOG3 and 8, and other sequences.
Table S3 Cellular localization prediction of TaLOGs.
Table S4 Rice, *Aegilopes tauchii*, and bread wheat RGN1 amino acid sequence alignment.

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