Identification and quantitative expression of cytokinin regulatory genes during seed and leaf development in wheat

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Abstract
Cytokinins are intimately involved in plant growth and development and their concentration is known to change dramatically during early stages of seed development. We propose that the concentration of active cytokinin may be co-ordinately regulated by specific member(s) of the multi-gene families encoding biosynthesis (isopentenyl transferase, *IPT*), catabolism (cytokinin oxidase, *CKX*), and metabolism (zeatin glucosyltransferases (*ZOG*) and β-glucosidase (*GLU*)) genes. Our qRT-PCR data for 22 putative genes showed that the expression patterns of individual members of the *Triticum aestivum* (*Ta*)*IPTs*, *TaCKXs*, *TaZOGs*, and *TaGLUs* multi-gene families were tissue and development specific during seed and flag leaf development, with up to 90-fold changes in mRNA level. Key genes that may be involved in seed yield determination have been identified.
Keywords: Wheat, flag leaf, cytokinin genes, quantitative expression, qRT-PCR

Introduction

Yield in cereals is a function of several components including grain size and grain number. During the last few years attempts have been made to understand the molecular basis of these two important yield-contributing traits. Some of the most significant recent studies include identification of a number of genes and quantitative trait loci (QTLs) for seed size in *Arabidopsis* (Ohto *et al*. 2005) and identification of several genes in rice (Ashikari *et al.* 2005).

Cytokinins (CKs) are important hormones that regulate many developmental and physiological processes in plants. They play a crucial role in regulating the cell cycle, proliferation and differentiation of plant cells, and also the control of various processes in plant growth and development, including delay of senescence (Gan & Amasino 1995), transduction of nutritional signals (Takei *et al*. 2001; Sakakibara 2006), inhibition of root development (Werner *et al.* 2003) and increased seed yield (Ashikari *et al.* 2005). At the molecular level, the bioactive cytokinin concentration may be regulated by several multi-gene families, including isopentenyl transferase (*IPT*) for cytokinin biosynthesis, cytokinin oxidase (*CKX*) for degradation, zeatin *O*-glucosyltransferases (*ZOG*) for reversible inactivation, and β-glucosidase (*GLU*) for reactivation. Most of these multi-gene family members have been elucidated in *Arabidopsis*, with nine *IPT* genes, seven *CKX* genes, three *ZOG* genes and *GLU* genes identified and functionally verified (Bilyeu *et al*. 2001; Kakimoto 2001; Takei *et al.* 2001; Werner *et al.* 2001; Hou *et al.* 2004). Their orthologues have also been annotated and/or functionally verified in rice and a number of other species.

It has been shown that artificial manipulation of the endogenous cytokinin level dramatically affects economically important traits including seed yield. Transgenic tobacco plants over expressing an *IPT* gene driven by the high molecular weight (HMW) glutenin gene promoter from wheat showed increased seed weight, and carbohydrate and protein content by 7-8%, without any morphological abnormalities (Ma *et al*. 2008). In rice, seed number

increased by over 20% in the loss-of-function cytokinin oxidase, *OsCKX2,* mutant. The expression of *OsCKX2* in inflorescence meristems regulates the cytokinin level and controls the number of flowers. Transgenic rice with antisense *OsCKX2* cDNA had reduced expression of endogenous *OsCKX2* and developed more grains (Ashikari *et al.* 2005). Moreover, overexpression of *AtCKX3*, the *Arabidopsis* orthologue of rice *OsCKX2*, in transgenic *Arabidopsis* reduced the number of flowers because of a decreased rate of primordium formation in the floral meristem (Werner *et al*. 2003).

The roles of other families of cytokinin regulatory genes (e.g., the *ZOG* and *GLU* gene families) remain to be elucidated. Additionally, little is known about the effects of any of these cytokinin regulatory genes on seed yield of pasture grass species or cereal crops of economic importance to New Zealand. We hypothesise that cytokinin homeostasis within an organ is co-ordinately regulated by different multi-gene family members to allow the precise control of organ development, and that seed yield can be directly affected by disturbing this regulatory network. Therefore, optimum seed yield could be obtained by fine tuning the endogenous cytokinin concentration at different stages of reproductive growth and seed development.

The aim of the present study is to identify the key cytokinin regulatory genes during seed development by elucidating their temporal expression patterns. Such information may be useful in the identification of gene-specific functionally-associated markers for marker-assisted selection and/or for inducing and detecting valuable mutations in the Targeting Induced Local Lesions in Genomes (TILLING) strategy towards the development of high performance commercial varieties.

Bread wheat (*Triticum aestivum* L) is used as our model species, partly because of its economic significance (world total production of 626 million tons in 2005), its well studied physiology and its positive reaction to exogenously applied cytokinins (Gupta *et al*. 2003), but also because of the rapid and dramatic changes in cytokinin concentration that occur during early grain development (Jameson *et al.* 1982; Banowetz *et al*. 1999). Such changes provide fertile ground for the study of the mechanism of hormone homeostasis.

Methods

Putative wheat *IPT*, *CKX, ZOG* and *GLU* homologues were identified using all the annotated family members of these multigene families from *Arabidopsis*, rice, maize and barley as query sequences to BLAST search the publicly available sequence databases, including over one million wheat ESTs. Degenerate primers, with a degree of degeneracy between 4 and 128, were designed to cover as many members of each multi-gene family as possible.

Winter bread wheat, variety Equinox, was grown under prevailing climatic conditions in a nursery plot at the New Zealand Institute for Plant & Food Research, Lincoln, Canterbury. Ovules, developing seeds and flag leaves were harvested from 1 day before anthesis (dba) to 14 days after anthesis (daa) and immediately frozen in liquid nitrogen and stored at –80°C until used. Total RNA was extracted using a modified TRIZOL procedure (Song *et al*. 2008). Two independent tissue samples of each developmental stage were used as biological replicates. About 1 μg of total RNA was used for cDNA synthesis using 50 pmoles of oligo $(dT)_{15}$ primer, 100 pmoles of random primers and 50 Units of Expand Reverse Transcriptase (Roche). Reverse transcription, PCR reaction and PCR product sequencing procedures are described in Song *et al*. (2008).

Temporal expression of putative target genes and selected housekeeping genes was quantified using an Mx3000P® real-time

PCR instrument (Stratagene) and a Faststart SYBR®Green Mix (Roche). At least three replicates, including RNAs from two separate tissue samples, were carried out for samples from each developmental stage.

To overcome the technical difficulties associated with the precise measurement of endogenous cytokinins, particularly in specific tissue types, we attempted to monitor the level of bioactive cytokinins indirectly by quantifying the mRNA levels of the wheat homologues of the *Arabidopsis* response regulators, *ARR5* and *ARR15*. These genes have recently been used as markers to track the *in planta* bioactive cytokinin concentration because their expression levels are reported to be proportionally correlated with the endogenous cytokinin content of the tissue (Kurakawa *et al*. 2007). Multiple housekeeping genes, *GAPDH, β-actin*, *18S rRNA*, and protein phosphatase gene (*PP2A*), were used as internal controls.

Results and Discussion

Identification of cytokinin regulatory genes

After BLAST searching the publicly available sequence databases, including over one million wheat ESTs, using all the annotated members of these multigene families from *Arabidopsis*, rice, maize and barley as query sequences, between 13 and 51 positive hits were identified for each target gene family. Multiple sequence alignment at DNA and amino acid levels and Genbank database BLAST searches, revealed at least four putative *IPT* homologues, 23 *CKX* homologues, 16 *ZOG* homologues, and 11 *GLU* homologues. Specific primers were designed-based on these sequences. Subsequently, representative sequences were selected for further study (Table 1) based on the results of preliminary expression studies using cDNAs from selected seed and leaf samples (data not shown).

Quantitative expression of cytokinin regulatory genes during seed and flag leaf development

In planta, cytokinin activity is considered to be controlled by a balance between synthesis, catabolism and inactivating conjugations (Sakakibara 2006). The spatial and temporal distribution of bioactive cytokinin appears strictly controlled during development, with tissue specific expression of *IPT* and *CKX* genes having been shown in a limited number of plants (Rashotte *et al*. 2003; Gális *et al.* 2005). However, there have been no studies in which the expression of members of these two gene families, let alone members of all four gene families, have been monitored simultaneously. Further, in wheat, there is no direct evidence that the cytokinin required by the developing seed is actually synthesised in the developing seed or whether it is transported from other tissues.

Our qRT-PCR data showed that the expression patterns of individual members of the *TaIPTs*, *TaCKXs*, *TaZOGs*, and *TaGLUs* multi-gene families were tissue and development specific during seed and flag leaf development (Figure 1).

In early seed development, the expression levels of *TaIPTb* were 12- to 15-fold higher at 1 to 4 daa than that at 1 dba, but decreased to 5- to 8-fold the initial level by 14 daa. This strongly suggests that from immediately after anthesis to 2-4 daa, the period of free nuclear division and cellularisation in the endosperm, high levels of bioactive CKs are required. Part of this requirement may be locally synthesised by the products of one or several *IPT* gene family members.

Ta*GLUd* expressed at very high levels at 1 to 4 daa, with moderate expression from 8 to 13 daa. The elevated expression of *TaGLUd* was not completely unexpected as significant levels of *O*-glucosides had been detected prior to, and just after, anthesis in the unfertilised ovule and developmentally

Table 1 Selected putative cytokinin regulatory gene fragments in wheat.

Target gene	Most similar to			Amino acid Similarity (%)
	Gene	Accession No.	Species	
TaIPTa	*ZmIPT6*	EU263129	*Zea mays*	88.4
TaIPTb	*OsIPT3*	AB239799	*Oryza sativa*	76.2
TaIPTc	*ZmIPT4*	ABY78883	*Zea mays*	72.0
TaIPTd	*OsIPT3*	AB239800	*Oryza sativa*	65.4
TaCKXa	*HvCKX1*	AF362472	*Hordeum vulgare*	94.2
TaCKXb	*OsCKX2*	CA705202	*Oryza sativa*	75.6
TaCKXc	*OsCKX4*	BM138354	*Oryza sativa*	89.1
TaCKXd	*ZmCKO1*	NM001112121	*Zea mays*	82.6
TaCKXe	*OsCKX3*	BJ316444	*Oryza sativa*	92.3
TaCKXj	*ZmCKX3*	AJ606944	*Zea mays*	67.5
TaZOGa	*SbZOG*	AAT42161	*Sorghum bicolor*	70.6
TaZOGb	*cisZOG1*	Q93XP7	*Zea mays*	82.4
TaZOGc	*cisZOG2*	Q8RXA5	*Zea mays*	81.2
TaZOGd	*SbZOG*	AAT42163	*Sorghum bicolor*	65.3
TaZOGe	*cisZOG1*	Q93XP7	*Zea mays*	80.1
TaZOGf	*SbZOG*	AAT42161	*Sorghum bicolor*	63.6
TaGLUa	*ScGlu*	AAG00614	*Secale cereale*	91.2
TaGLUb	*OsGlu*	EAZ02122	*Oryza sativa*	87.5
TaGLUc	*ZmGlu1*	NP_001105454	*Zea mays*	75.2
TaGLUd	*OsGlu*	XM_475121	*Oryza sativa*	68.0
TaGLUe	*AsGlu*	AAD02839	*Avena sativa*	65.4
TaGLUf	*MtGlu*	ABW76287	*Medicago truncatula*	61.0
TaRR1	*OsRR1*	AB249661	*Oryza sativa*	78.4
TaRR5	*OsRR5*	AB249654	*Oryza sativa*	95.5

earlier than the peak of either zeatin or zeatin riboside post fertilisation (Jameson *et al*. 1982). Consequently, part of the bioactive cytokinin in the developing seed may be derived from the conversion of storage *O*-glucosides by *GLU* gene member(s).

However, the elevated expression of *TaCKXb* post-anthesis is less easy to explain, although there are a number of examples in the literature that suggest high levels of cytokinin oxidase activity are found in tissues of high endogenous cytokinin content (Gális *et al.* 2005). We suggest that accumulation of a large amount of bioactive cytokinin may result in a negative feedback reaction leading to activation of the *CKX* genes for degradation, and *TaZOGb* and *d* genes for inactivation by conjugation.

Marked changes in the expression of members of all four gene families occurred during leaf expansion and senescence. During expansion of the flag leaf there was a significant increase in expression of *TaIPTd* and *b* and *TaZOGf*, suggesting a balance between synthesis and storage and the maintenance of cytokinin homeostasis for the normal functioning of leaves including photosynthesis. However, at anthesis, when the flag leaf is providing significant resources to the developing ear, these genes showed decreased levels, which contrasts with the 97-fold increase in expression of *TaGLUd.*

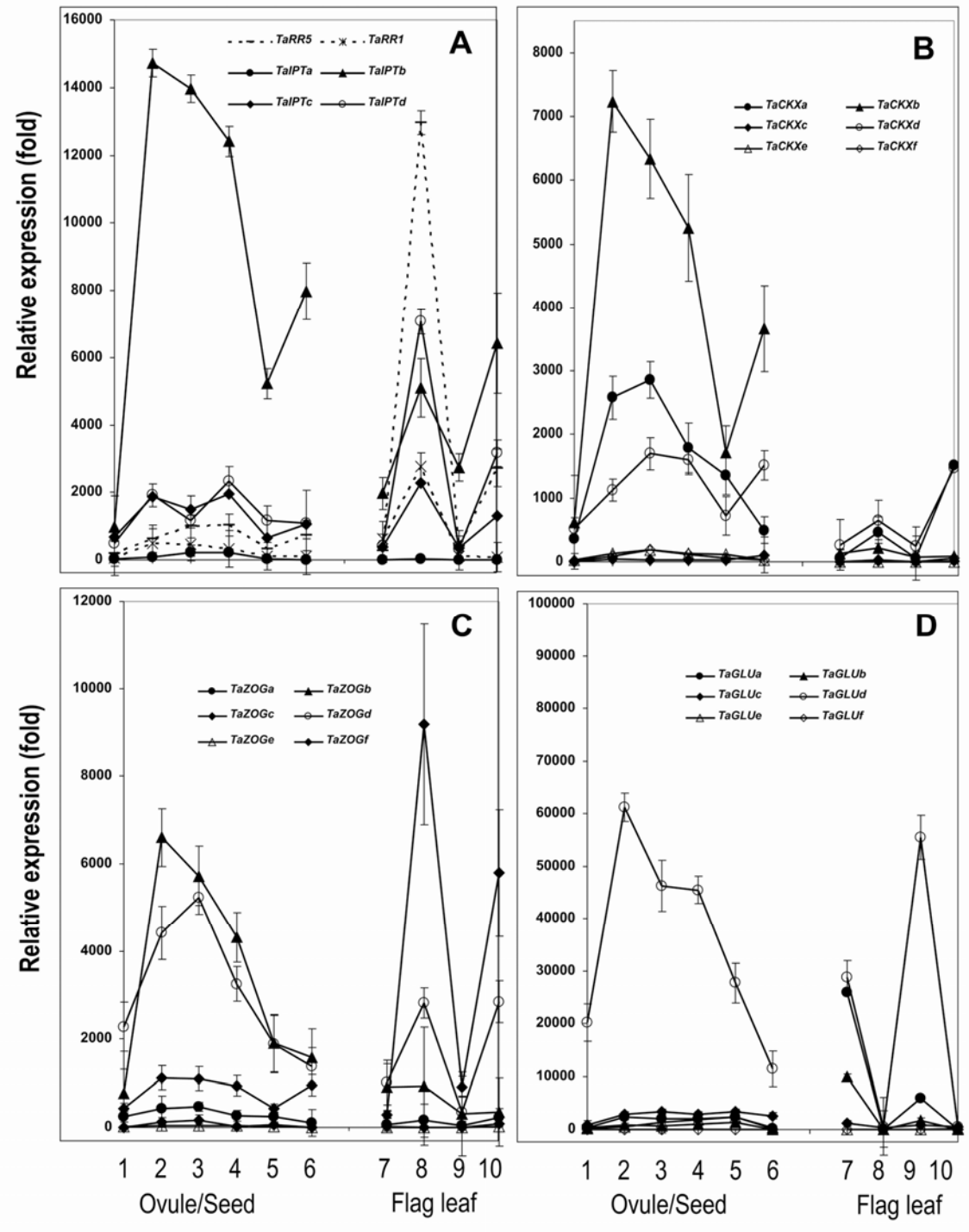

Figure 1 Quantitative expression of putative cytokinin regulatory genes during seed and flag leaf development in wheat. A. *ARR* and *IPT* genes; B. *CKX* genes; C. *ZOG* genes; D. *GLU* genes. 1. 1 day before anthesis (dba); 2. 1 day after anthesis (daa); 3. 2 daa; 4. 4 daa; 5. 8 daa; 6. 14 daa; 7. young leaf 2-3 cm in length; 8. fully expended leaf; 9. leaf at anthesis; 10. leaf showing senescence at 14 daa. Values were means of relative mRNA levels in fold changes with 3-4 replicates including two biological replicates.

β-glucosidase activity could lead to the release of cytokinin from conjugation in the leaf and provide a source of cytokinin for the developing ovules/seeds.

Leaf senescence was occurring about 14 daa. Expression of *TaCKXa* and *d* increased, as did that of *TaZOGf*. Cytokinins have well known anti-senescent properties so a reduction in active levels is not surprising either by side chain removal (CKX) or conjugation (ZOG). High levels of *O*-glucosides have been detected in senescing leaves (Jameson 1994).

Interestingly, most of the highly expressed genes in the seeds had low expression in the leaves and *vice versa*, further suggesting that expression of individual family members of the cytokinin regulatory genes are tissue specific.

The expression levels of *RESPONSE REGULATOR* genes (*ARRs*) are reported to be proportionally correlated with the endogenous cytokinin content of the tissue (Kurakawa *et al*. 2007). On this basis, we might have expected the levels of *ARRs* to be higher in developing seeds and at lesser expression levels in the leaves. While we did detect *TaRR* expression in the developing seed as early as 1 daa, and higher concentration at 2 to 4 daa, being 5- to 8-fold higher than that at 1 dba (Figure 1a), the expression pattern does not reflect the substantial increase in bioactive cytokinin detected previously (e.g., Jameson *et al*. 1982). In fully-expanded flag leaves, the expression levels of *TaRR1* and *TaRR5* were 10- and 50-fold higher than those in young expanding leaves, respectively, and dropped rapidly to the base level at anthesis. While the expression pattern of *TaRR5* mirrors that of the *TaIPTd* in the leaves, it remains possible that we have not isolated the appropriate ARR from the seed.

In conclusion, the large number of wheat ESTs in public databases enabled us to isolate at least 10 gene fragments for each cytokinin regulatory gene family. The specific expression patterns we have identified for particular gene fragment(s) will provide useful information for testing their roles in seed development, and to develop molecular markers for valuable mutations or QTL detection in plant breeding programmes. Of significance from this current study is the readiness with which this information can be adapted to pasture grass species (for which sequence availability is extremely limited), thanks to the functional conservation and high sequence similarity between homologous genes in these species. Such species may include ryegrass which is of considerable economic importance to New Zealand.

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