Is targeted modification of cytokinin regulatory gene activity in Rapid Cycling *Brassica rapa* an appropriate model for forage brassica, *B. napus*?

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Abstract

The cytokinins are rapidly being recognised as having critical and specific roles during seed development. Both seed number and seed size have variously been affected by manipulation of cytokinin levels. We hypothesised that we could identify specific cytokinin regulatory genes as targets for breeders. As both cytokinin biosynthesis and metabolism are controlled by multigene families, our target was to see if specific gene family members expressed in specific tissues at key developmental stages using a qRT-PCR strategy in brassica species. Gene family members for cytokinin biosynthesis and cytokinin degradation were isolated, and showed temporal and organ-specific expression in leaves, flowers and pods of *Brassica napus*. An analysis of seeds separated from pods is yet to be conducted. This information is needed to further refine the tissue-specific expression of selected gene family members as targets for breeding. However, the similarities to date with Rapid Cycling *Brassica rapa* (O'Keefe *et al*., 2011) indicate that targeted modification of cytokinin regulatory gene activity in RCBr is an appropriate model for forage brassica.

Additional keywords: seed, cytokinin synthase, IPT, cytokinin oxidase/dehydrogenase, CKX

Introduction

Consistency of yield and quality of seed are traits not yet optimised by the brassica seed industry in New Zealand. As of 2011, seed producers in Canterbury, New Zealand, exported more than $33 m worth of seed from *Brassica* species (Hampton *et al*., 2012). However, there is a need to increase both seed quantity and/or quality. The plant hormone group, the cytokinins, regulates many stages of plant growth and development, including cell division and enhancement of sink strength, both of which are important processes in seed development and embryonic growth (Riefler *et al*., 2006). In the normal course of seed development the levels of active cytokinins change markedly leading to the suggestion that seed development may be, at least partly, controlled by the balance of cytokinin biosynthetic and metabolism genes (Riefler *et al*., 2006).

Two enzymes have been identified as having key functions in the biosynthesis and metabolism of cytokinin: adenylate isopentenyltransferase (IPT), which is the

rate limiting enzyme in cytokinin biosynthesis and cytokinin oxidase/dehydrogenase (CKX), which deactivates cytokinin by cleaving the N^6 side chain. These enzymes exist as gene families with individual family members expressing in specific tissues and/or at different developmental stages (Song *et al*., 2012 and references therein).

The key objective in this project was to identify which cytokinin gene family members express specifically during pod and seed development of *Brassica* species. O'Keefe *et al*. (2011) have already shown that certain *IPT* and *CKX* gene family members express differentially during development of the model Rapid Cycling *Brassica rapa* (RCBr) The aim of the research reported in this paper was to assess the similarity in cytokinin gene expression between RCBr and forage brassica, *Brassica napus* L., to ascertain whether targeted modification of cytokinin gene activity in RCBr is an appropriate model for forage brassica.

Materials and Methods

Plant material

B. napus was sourced from a commercial property in Tinwald, South Canterbury (45° 55′ S, 171° 43′ E) (December 2010) (Figure 1). Six similar plants were identified and three stages of leaf development (small, very young leaves; expanded leaves; and leaves from near the base of the plant), flowers (young closed buds; buds with petals just emerging; and fully open flowers) and seven stages of pods (emerging pods 1 day post-pollination to full length pods of 8-10 mm with seeds of about 1.5 mm diameter) were collected. Plant tissues were immediately placed into liquid nitrogen and stored at -80°C.

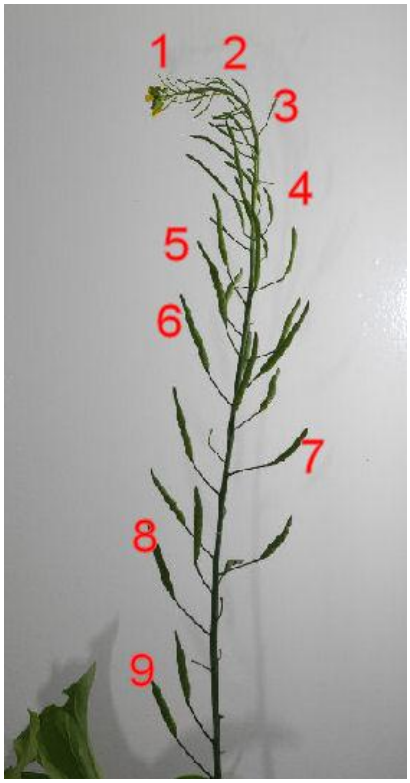

Figure 1: Developmental stages of pods selected for expression analysis along a secondary inflorescence of field-grown *B. napus*.

RNA isolation and cDNA synthesis

Total RNA was extracted using a Nucleospin® RNA Plant kit (E&K Scientific Products, Inc., California, USA) according to the manufacturer's instructions. The integrity and quality of isolated RNA was assessed by agarose (1% w/v) gel electrophoresis. The concentration and purity of the total RNA was assessed by Nanodrop™ spectrophotometer. Extracted RNA was converted to cDNA by reverse transcription.

Gene isolation, sequence analysis and qRT-PCR

Primers for PCR (Table 1) were designed using Primer Premier™ 5.00- (PREMIER Biosoft, California, USA) and PCR was run as described in O'Keefe *et al*. (2011). *B.*

napus sequences were sent to Macrogen Inc., Seoul, Korea, for sequencing. Relative gene expression was measured using quantitative reverse transcription polymerase chain reaction (qRT-PCR) as described in O'Keefe *et al*. (2011) and Song *et al*. (2012).

Reference genes were used to normalise each cDNA sample so relative expression could be calculated. However, the manner in which the plates were prepared has meant that relative expression determined for the pods cannot be compared with that for the leaves and flowers.

Table 1: Primers used in this study for *BnIPT*, *BnCKX*, *BnACTIN*, *BnGAPDH* and *BnELF1*.

Gene	Primer name	
BnIPT1	BnIPT1F	5'- GAGATTTTCGTGTAGACGCTTC
	BnIPT1R	5'- GCACAGGTTCCGACACATCTACCC
BnIPT3	BnIPT3F	5'- GTCACTAACAAGATCACGACCGA
	BnIPT3R	5'- TGCTTTCTTGATTCCTCTCGAGTA
BnIPT5	BnIPT5F	5'- GCAAATCCCGTCTCGCCA
	BnIPT5R	5'- CTCTGATTGCTTCCCGCTGA
BnIPT7	BnIPT7F	5'- CACTTGCTCGGGGTATTTGACTCGGA
	BnIPT7R	5'- ACAATGGTCCAGACGAATGGTTCGCA
BnCKX1	BnCKX1F	5'- AGTCCAAGGACTTTGGCAACAGATACCA
	BnCKX1R	5'- CCATAGTTCACCACCTGAGACATC
BnCKX2	BnCKX2F	5'- TCCTAAACGATCCTTCCG
	BnCKX2R	5'- GGGAGGAGACGGGATATCTCA
BnCKX3	BnCKX3F	5'- AAGCGGACAAACGTCTCGGTA
	BnCKX3R	5'- GATCTCCAGTTATCAGGTGGGC
BnCKX5	BnCKX5F	5'- ATCTGGGCTTACGTTAGCTCTC
	BnCKX5R	5'- AGACGGAGGCTAAGTCGGAAG
BnCKX7	BnCKX7F	5'- GGGAAAGACTTCGGTGGCA
	BnCKX7R	5'- CGCCAAGCCATACTCCGA
Actin	BnACTF	5'-TGTGACAATGGAACTGGAATGGT
	BnACTR	5'- ACGGAGGATAGCGTGAGGAAG
GAPDH	BnGAPF	5'- GATCCCTTCATCACCACCGAGTA
	BnGAPR	5'-GGGGAGCAAGGCAGTTAGTG
Elf1	BnELFF	5'-AGGAGGCTGCTGAGATGAACAA
	BnELFR	5'-CCATCTTGTTACAGCAGCAAATCA

Bioinformatics

Sequence data was aligned using ClustalX and ClustalX 2.0 as described in Larkin *et al*. (2007). Sequences were compared to *Arabidopsis* and other known protein and nucleotide sequences using MEGA5 (Tamura *et al*., 2007). The phylogenetic relationship between genes was determined using BLAST to identify homologous genes from the sequence data bases, and phylogenetic trees constructed in MEGA5 (Tamura *et al*., 2007) using Maximum Parsimony methods.

Chlorophyll analysis

Chlorophyll was extracted by immersion of leaf material in DMF overnight at 4°C and measured using a Nanodrop™ spectrophotometer as described in Evans *et al*. (2012)

Results

Phylogenetic analysis

Monocot and eudicot *IPT* genes formed two distinct clades (Figure 2). *BnIPT1* grouped together with *BrIPT*, *AtIP1* and a published *BrIPT1* sequence (AB186132.1). These genes were very closely related to *AtIPT4* and *AtIPT8* but appear to be quite diverged from the other *IPT* genes branching from the base of the dicot *IPT* clade. *BnIPT3* grouped together with *BrIPT3*, *AtIPT3*, *GmIPT* and a published *BrIPT3* sequence (AB186133.1). *BnIPT5* grouped together with *BrIPT5*, *AtIPT5* and a published *BrIPT5* sequence (AB186134.1). No other *IPT* dicot genes were closely related. *BnIPT7* was grouped closest to *AtIPT7* and these genes formed a small clade with other dicots. *BnIPT3* and *BrIPT3* are most closely related to the *BnIPT7* clade.

The phylogenetic relationship between the putative *B. napus CKX* genes and *B. rapa*, *Arabidopsis*, *O. sativa*, *Z. mays* and various other *CKX* genes was also determined (Figure 3). *BnCKX1* grouped with *BrCKX1*, *AtCKX1*, *BoCKX1* (AB331918), *BrCKX1* (AB331924), along with other CKX genes from various species. *BnCKX2* grouped together with Br*CKX2*, *AtCKX2* and *AtCKX4* and this group is very closely related to *BnCKX3*, *BrCKX3*, and *AtCKX3*. *BnCKX7* is in the same clade as AtCKX7 and *PsCKX1*. *BnCKX5* is in a clade of its own with *AtCKX5*.

Quantitative gene expression

In flowers, *BnIPT7* expression increased during the second stage of flower development (Flower 2) (Figure 4), whereas there was minimal expression of either *BnIPT1* or *3* at any of the three stages of flower development. In contrast, *BnIPT3* expression increased during leaf development and was most highly expressed in mature leaves (Leaf 3) (Figure 4). There was a gradual increase in expression of *IPT1*, *3* and *7*, during pod development, peaking at Pod4, and then declining to Pod7 (Figure 5). This change in expression levels during pod develoment was most marked for *IPT3* and *7*.

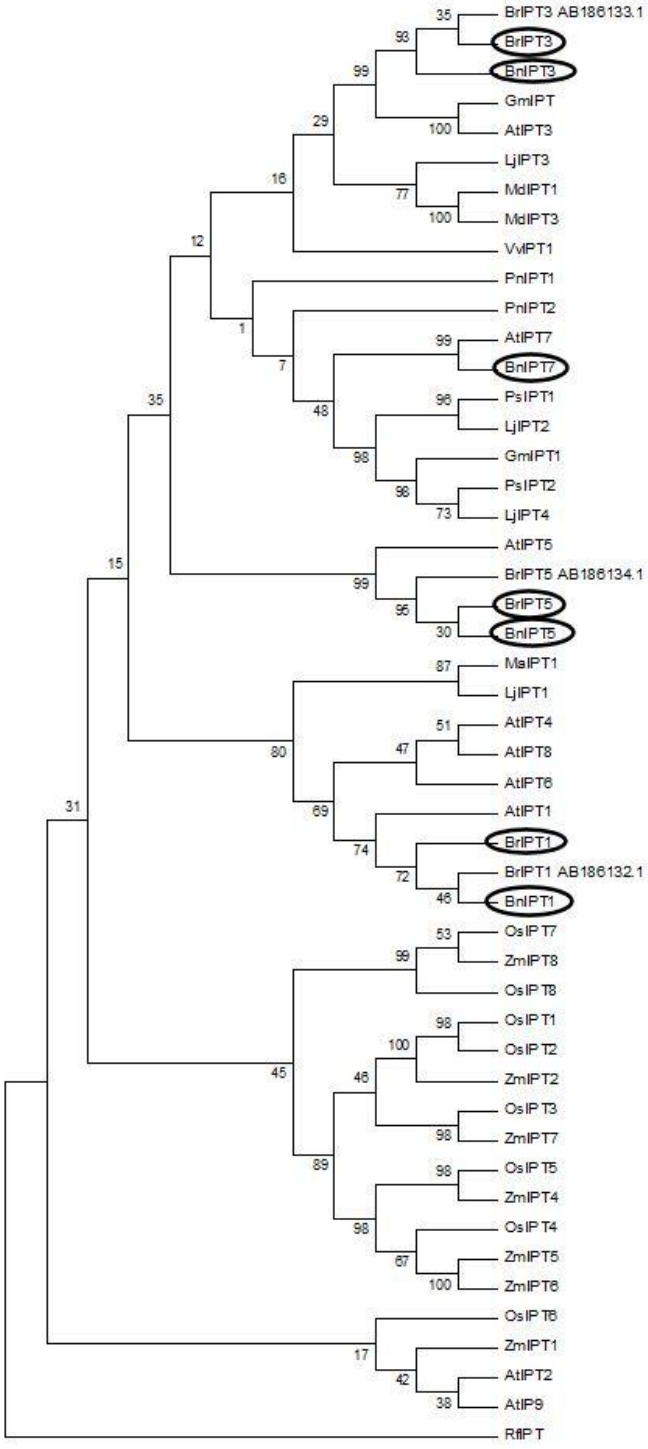

Figure 2: Phylogenetic tree showing the relationship between *Brassica rapa* and *Brassica napus IPT* genes and *Arabidopsis thaliana, Oryza sativa, Zea mays* and various other *IPT* genes. Maximum parsimony tree rooted with *Rhodococcus fascians IPT genes*. Bootstrap tree branch support values shown (10000 bootstrap replications). Gene family members identified in this study and in O'Keefe *et al*. (2011) circled in black.

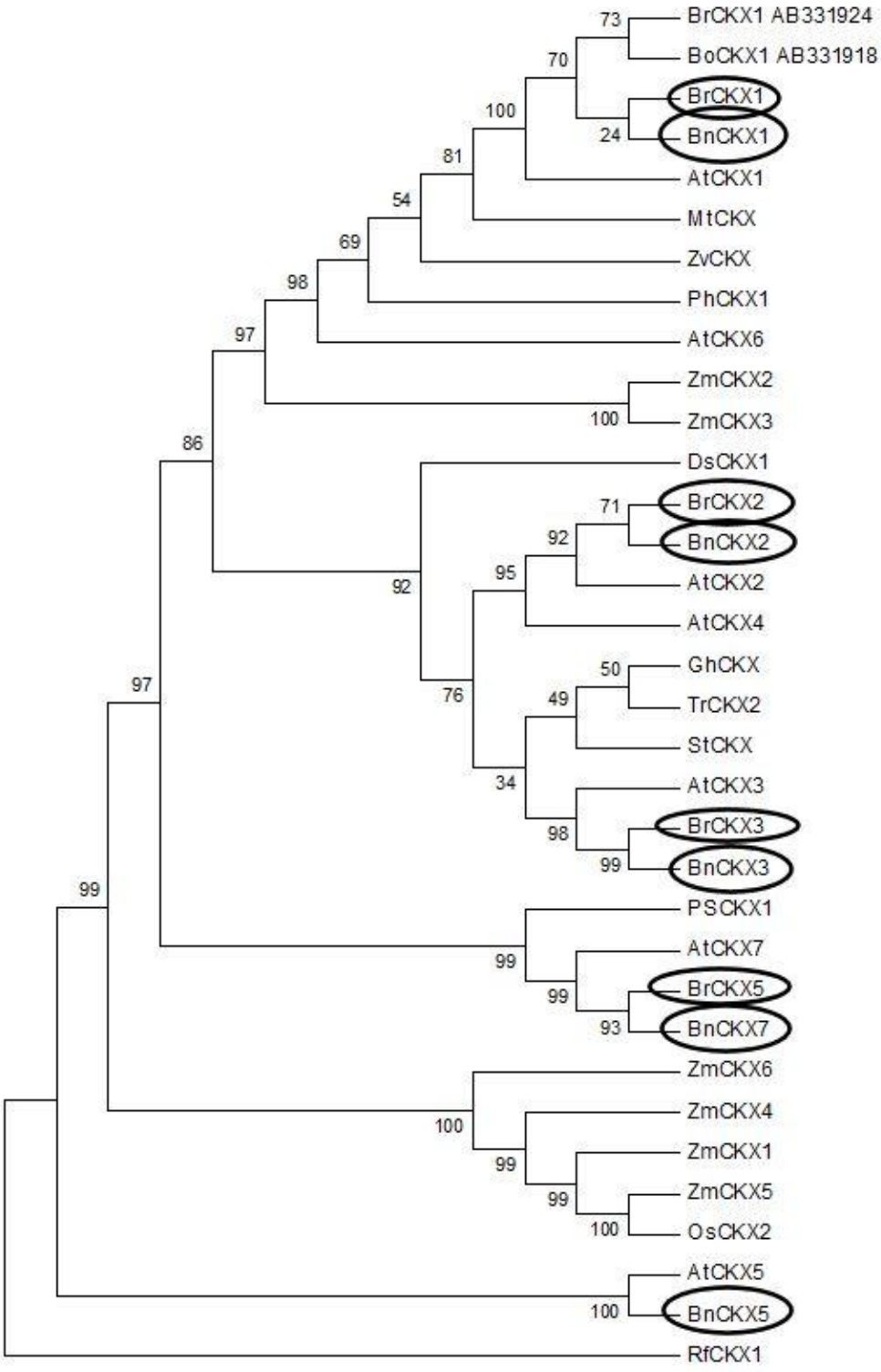

Figure 3: Phylogenetic tree showing the relationship between *Trifolium repens CKX* genes and *Arabidopsis thaliana*, *Zea mays* and legume *CKX* genes. Maximum parsimony tree rooted with *Rhodococcus fascians CKX* gene. Bootstrap tree branch support values shown (10000 bootstrap replications). Gene family members identified in this study and in O'Keefe *et al.* (2011) circled in black.

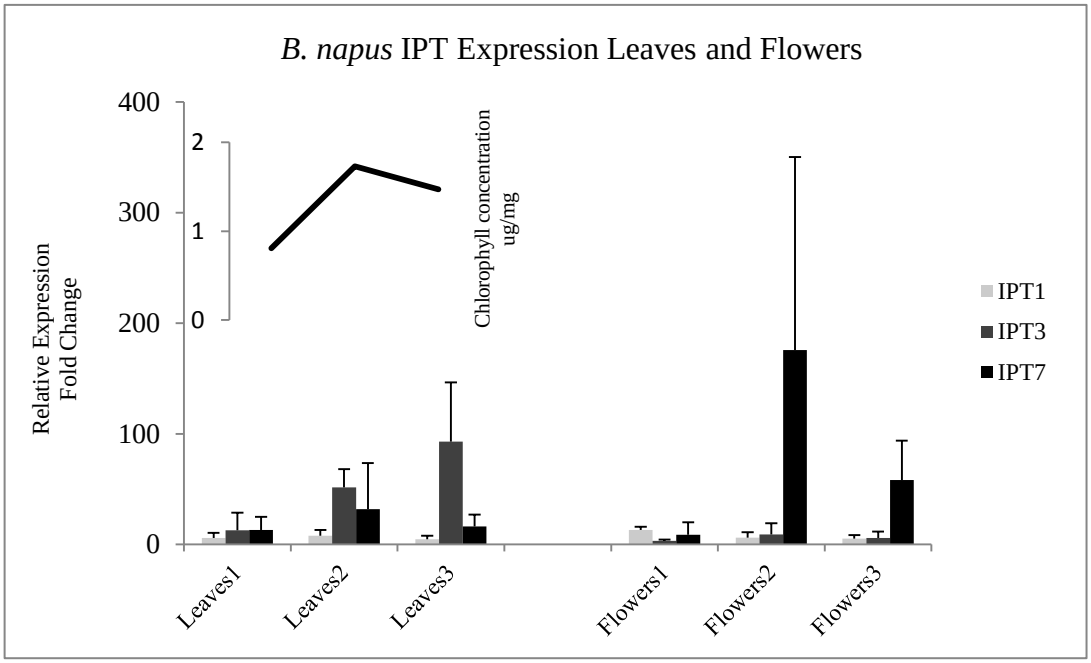

Figure 4: Expression of *BnIPT* gene family members during leaf and flower development determined by qRT-PCR. Leaves (leaves 1, 2, and 3, depicting youngest to oldest) and flowers (flowers 1, 2, and 3, depicting bud to fully open flower). Inset is chlorophyll concentration in leaves 1, 2 and 3. Bars show SD of the mean of two biological and three technical replicates based on corrected CT number.

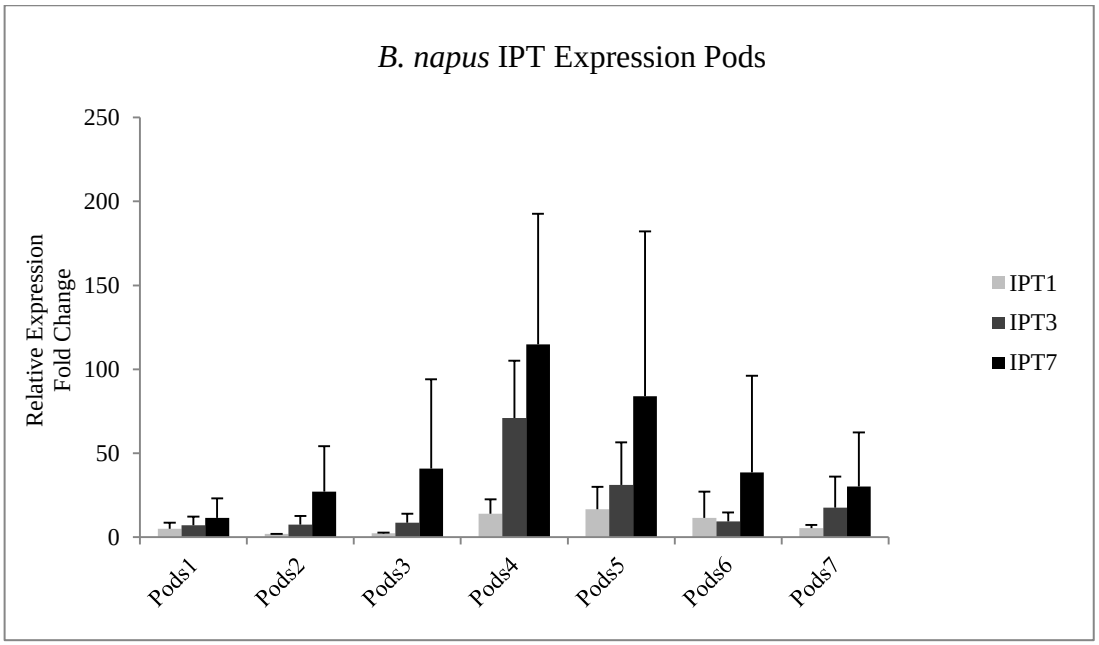

Figure 5: Expression of *BnIPT* gene family members during pod development determined by qRT-PCR. *Pods* 1, 2, 3, 4, 5, 6 and 7 depicting earliest fertilised pods through to fully expanded pods. Bars show SD of the mean of two biological and three technical replicates based on corrected CT number.

In *B. napus* plants, *BnCKX2* showed a marked increase in expression during flower development, and a peak in expression in developing leaves (Figure 6). In contrast, expression of *BnCKX1*, *5* and *7* in leaves and flowers was relatively very low (Figure 6). As pods developed, expression of *BnCKX1* and *2* increased to maximum at Pod 5 (Figure 7). *BnCKX5* and *7* showed only a slight increase in expression as pods developed (Figure 7).

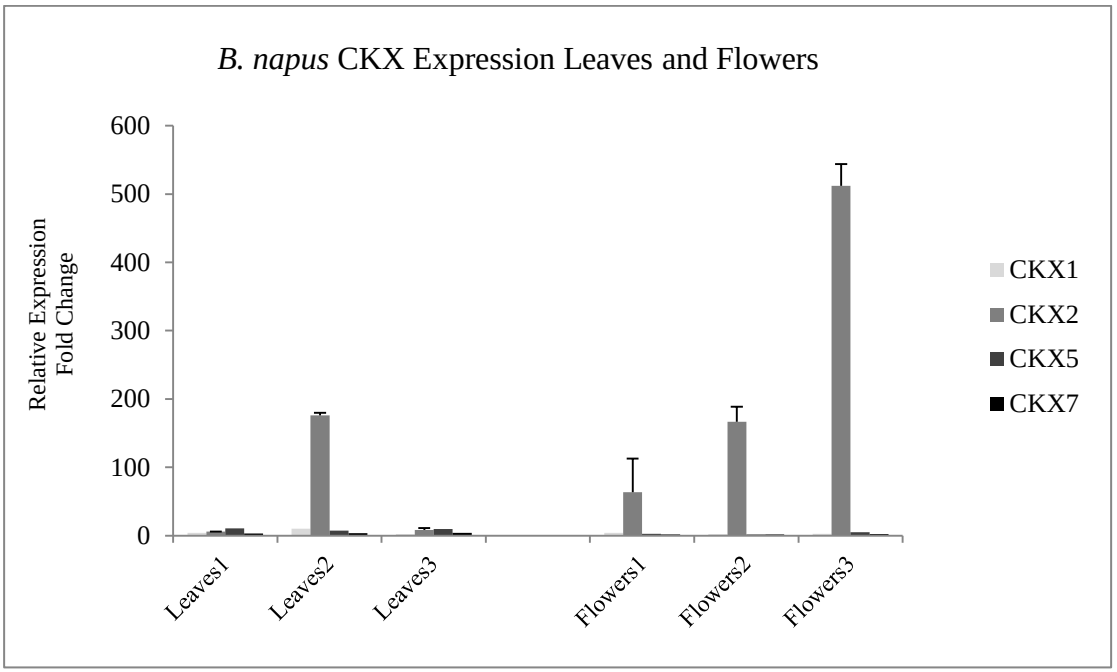

Figure 6: Expression of *BnCKX* gene family members during leaf and flower development determined by qRT-PCR. Leaves (leaves 1, 2, and 3 depicting youngest to oldest) and flowers (flowers 1, 2, and 3 depicting bud to fully open flower). Bars show SD of the mean of two biological and three technical replicates based on corrected CT number.

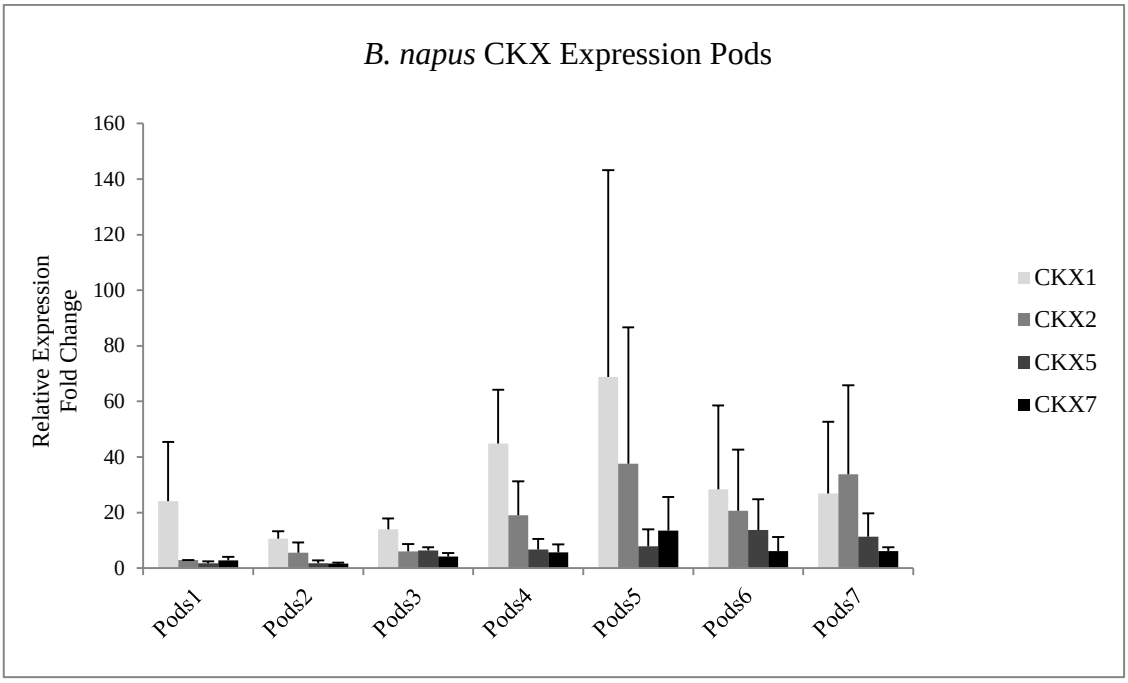

Figure 7: Expression of *BnCKX* gene family members during pod development determined by qRT-PCR. *Pods*1, 2, 3, 4, 5, 6 and 7 depicting earliest fertilised pods to fully expanded pods. Bars show SD of the mean of two biological and three technical replicates based on corrected CT number.

Discussion

The phylogenetic tree shows four major clades of *IPT* genes (Figure 2). The monocot and eudicot *IPT* gene families show their divergence after the monocot eudicot split (Kakimoto, 2001; Sakamoto *et al*., 2006) while the eudicot genes have split further. Amongst the eudicot genes there are two clades, one containing *AtIPT1*, *AtIPT4*, *AtIPT6* and *AtIPT8* and a second clade containing *AtIPT3*, *AtIPT*5 and *AtIPT*7. The majority of the monocot *IPT* genes including *OsIPT1-5*, *OsIPT7*, *OsIPT8*, *ZmIPT2* and *ZmIPT4-8* formed a separate clade with one further clade made up of the two tRNA *IPT* genes, *AtIPT2* and *AtIPT9*, along with two monocot *IPTs*, *ZmIPT1* and *OsIPT6*. The *Brassica* gene family members showed the expected close relatedness to each other and to *Arabidopsis* (Figure 2).

As shown with *BrIPT1* (O'Keefe *et al*., 2011), *BnIPT1* expression occurred in most tissues, but with minimal expression in pods relative to other *IPT* family members. It is clear that, in both *B. rapa* (O'Keefe *et al*., 2011) and *B. napus*, *IPT3* was expressed during pod development. When pod walls and seed were extracted separately, *BrIPT3* showed strong expression in the developing seed (O'Keefe *et al*., 2011). It is essential to confirm this interesting result with *B. napus*.

In both *B. rapa* (O'Keefe *et al*., 2011) and *B. napus*, *IPT3* expression increased during leaf development and was most highly expressed in mature leaves with maximum chlorophyll content. This is interesting because cytokinin is normally associated with delaying senescence and unloading from the phloem into sink tissues (Lara *et al*., 2004). As the pods and seeds were developing at this stage, these leaves

would be expected to function as source leaves, so this high level of activity of one *IPT* gene family member warrants further investigation. Increased longevity of leaves is considered a target for increasing seed yield (Ma *et al*., 2008), but source leaves do need to senesce to provide resource for the developing pods and seeds. Consequently, selecting plants with increased *IPT3* expression may be counter-productive.

O'Keefe *et al*. (2011) showed *BrIPT5* was most highly expressed in roots and developing pods. However, when pods and seeds of RCBr were separated it is clear that *BrIPT5* expression was focused in the developing seed. As it is also expressed in the roots, selection for increased expression of this gene family member may result in plants with reduced root development.

BnIPT7 expression increased markedly during the second stage of flower devlopment. There was also a gradual increase in expression during pod development, peaking at Pod 4 and then declining to Pod 7. Leaves showed a very low level of *BnIPT7* expression. Pods and seeds of *B. napus* have yet to be analysed separately, so the specificity of this expression has still to be determined.

Overall, there was relatively low expression of the identified *IPT* gene family members in developing pods of *B. rapa* and *B. napus*, indicating that maternally supplied cytokinin may be required during early pod development. None of *IPT1, 3, 5* or *7* expressed specifically in developing pods and/or seeds, so may not be ideal targets for breeding.

Metabolism of cytokinins is complex and involves irreversible deactivation by CKX as well as a complex array of glucose conjugations. As reduced *CKX* expression has been implicated in increased seed yield in cereals (Ashikari *et al*., 2005; Zalewski *et al*., 2010), this gene family was also studied. The phylogenetic tree shows that *CKX* genes are more divergent than the *IPT* genes. As with the *IPT* genes, the *Brassica CKX* genes show very close homology to their *Arabidopsis* counterparts (Figure 3).

BrCKX1 was expressed in a range of tissues (O'Keefe *et al*., 2011) including developing pods and, clearly, *BnCKX1* is also expressed in developing pods (Figure 7). *BrCKX2* was shown to be highly expressed in developing seeds (O'Keefe *et al*., 2011). In the *B. napus* study, *BnCKX2* increased during pod development but independent seed analysis has yet to be completed. *BrCKX3* showed increased relative expression in 7 DAP pods and early and late stage leaves (O'Keefe *et al*., 2011). Developmental expression analysis of *BnCKX3* is yet to be completed.

In the phylogenetic analysis, *BnCKX7* located close to *AtCKX7* and to *BrCKX5*. *BrCKX5* may well have been misidentified when submitted to GenBank. *BrCKX5/7* was shown to express very highly in early and late stages of leaf development, and at a low level, relative to *BrCKX2*, in seeds. *BnCKX7* showed closest homology to *AtCKX7*. While *BnCKX7* showed an increase in relative expression in Pod 5 compared to the other pod stages, its expression in the pod was insignificant compared to other gene family members (Figure 7). Likewise, *BnCKX5* showed a small increase during pod development and its expression was relatively low across all other tissues.

Overall, the *Brassic*a *CKX* genes expressed in a variety of tissues which is in line with other species. To date, the most active *CKX* gene family member detected during seed development of Brassica species is *BrCKX2*, which aligns with the Gu *et al*. (2010) suggestion that Clade III

(to which *BrCKX2* is closely associated) and VI family members preferentially express in reproductive tissues.

Conclusions

The sequencing and phylogenetic analysis clearly show the expected close relatedness between the *IPT* and *CKX* gene family members of Rapid Cycling *B. rapa* and forage brassica (*B. napus*) The qRT-PCR data presented here involved simultaneous monitoring of three members of the *BnIPT* gene family and four members of the *BnCKX* gene family. Similarities in expression patterns were shown between RCBr and *B. napus* (O'Keefe *et al*., 2011). Although a full seed analysis has yet to be completed for *B. napus*, the similarities between RCBr and forage brassica indicate that targeted modification of cytokinin gene activity in RCBr is an appropriate model for forage brassica.

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