Differential Expression of Isopentenyl Transferase and Cytokinin Oxidase/Dehydrogenase During Pod and Seed Development in Brassica

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Abstract

Consistency of yield and quality of seed are traits not yet optimised by the brassica seed industry in New Zealand. As of 2008, seed producers in Canterbury, New Zealand, exported approximately $18m of brassica seed. 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.

The two gene families targeted in this project play a key role in maintaining cytokinin homeostasis. Isopentenyl transferase (IPT) catalyzes the rate limiting step in the formation of cytokinins, and cytokinin oxidase/dehydrogenase (CKX) irreversibly inactivates cytokinins. The aim of this project was to identify those cytokinin gene family members expressing specifically during the early phases of pod and seed development. Initially this study used a rapid-cycling *Brassica rapa* (RCBr) because of its rapid life cycle, then, as the project developed, a commercial crop of forage brassica (*B. napus)* was studied.

Reverse transcriptase PCR (RT-PCR) and BLAST analysis was used to identify putative *IPT* and *CKX* genes from RCBr and *B. napus.* Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to measure the expression of individual gene family members during leaf, flower, pod and seed development. *BrIPT1, -3,* and *-5,* and *BrCKX1, -2, -3,* and *-5/7* and were shown to express differentially both temporally and spatially within RCBr root, stem, leaf, seed, and pod tissues. *BnIPT1, 3* and *7* and *BnCKX1, 2, 5* and *7* were also differentially expressed. Particularly strong expression was shown by *BrIPT3, BrIPT5* and *BrCKX2* in developing seeds. Both *Br-* and *BnIPT3* expressed strongly in maturing leaves.

In normal plant growth and development, biosynthesis and metabolism of cytokinin is tightly regulated by the plant. Increasing the levels of cytokinins during seed development, either by over expressing *IPT3* or *IPT5,* or decreasing the expression of *CKX2*, or both, could potentially increase both seed yield and seed vigour.

Acknowledgments

This work is dedicated to my father, Daniel, who lost his battle with stomach cancer in July 2010. Thank you for the example you were and the help you have given me over the years, may you rest in peace.

Mum, you have always been there for me, through thick and thin, thank you for all the help and support you have given me over the years, especially with Jessie and Jordan as they were growing up. I could not have raised them without you. Cherie, you kept my focused on my health, showed me what was important and where always there to help me when I was lacking enthusiasm.

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
ACO	ACC oxidase
ACS	ACC synthase
ACT	actin
bp	base pairs
cDNA	complementary deoxyribonucleic acid
°C	degrees Celsius
CKX	cytokinin oxidase/dehydrogenase
cm	centimeter
Ct	threshold cycle
c-t iso	*cis*-trans isomerase
cZ	*cis*-zeatin
d	days
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide-triphosphate
DMF	dimethylformamide
DZ	dihydrozeatin
Elf	Elongation factor
g	gram
g	gravity
GAP	glyceraldehyde-3-phosphate dehydrogenase
h	hours
iP	N^6-(D2-isopentenyl)adenine
IPT	adenosine phosphate isopentenyltransferases
M	Molar; moles per liter
mg	milligram
min	minute
ml	milliliter
mM	millimolar
ng	nanograms
nm	nanometer
PCR	polymerase chain reaction
PSAG12	promoter of SAG12
qRT-PCR	Quantitative reverse transcription polymerase chain reaction

RFU	Relative florescence units
Rnase	ribonucleases
RT	reverse transcription
s	second
TILLING	Targeting Local Lesions in Genomes
tRNA-IPT	tRNA isopentenyltransferases
tZ	trans-zeatin
µg	microgram
µl	microliter

Chapter 1

Introduction

1.1 Background

Consistent seed yield and assured seed quality are traits highly desired by the New Zealand seed industry (Johnson and Gallacher 2008). However, many forage plants are bred for vegetative characteristics which are often incompatible with physiologically efficient seed production and quality (Fairy and Hampton 1997). Forage plants of the Brassicaceae, as an example, have strongly indeterminate phenology and multi-aged reproductive structures which flower and set seed over an extended period of time, a low and variable harvest index, and seed which suffers physiological deterioration at a rate determined by both environmental and management factors.

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). The focus of this project, funded within the FRST Advanced Seeds Production Systems Programme, was to elucidate the role of the cytokinins in brassica seed development.

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 (Kakimoto 2001), and cytokinin oxidase/dehydrogenase (CKX), which deactivates cytokinin by cleaving the N^6 side chain (Mok and Mok 2001). These enzymes exist as gene families with individual family members expressing in specific tissues and/or at different developmental stages (Kasahara et al. 2004; Takei et al. 2004; Ashikari et al. 2005; Sakakibara 2006 and Brugière et al. 2008). The key objective in this project was to identify which cytokinin gene family members express specifically during pod and seed development of *Brassica rapa* and B. *napus*.

1.2 Brassica species

Brassica is an economically important genus of plants in the mustard family (Brassicaceae) containing around 85 species (Christey and Braun 2001). As a family it contains more agricultural and horticultural crops than any other family, but also a number of weeds. Generally known as either

cabbages or mustard, *Brassica* includes over 30 wild species and hybrids, and numerous additional cultivars and hybrids of cultivated origin. Originally a native of Western Europe, the Mediterranean and temperate regions of Asia the cultivated species are grown worldwide. There are six cultivated species, three diploid species (*B. oleracea, B. nigra and B. rapa*) of which interspecific hybridisations have resulted in three allopolyploid hybrids (*B. napus, B. juncea* and *B. carinata*) (Metz et al 1997). The Triangle of U (Figure 1.1) was first published by Korean botanist Woo Jang-choon in 1935. He postulated that the three ancestral species of Brassica naturally hybridised to create the three allotetraploids seen below. This has since been confirmed by genetic studies.

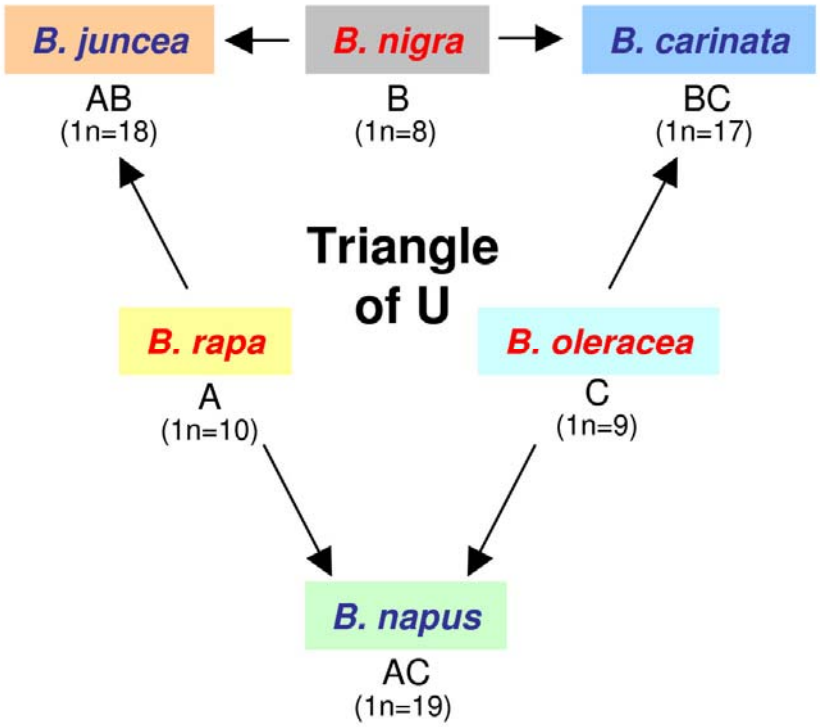


Figure 1.1 The 'Triangle of U' diplays the genetic relationship between Brassica species. The three diploid species (B. rapa, B. oleracea and B. nigra) are shown in red. The allotetraploid (B. juncea, B. carinata and B. napus) are shown in blue. (copied from www.plantmethods.com)

The Brassicaceae is a very versatile family: all parts of the plant have been developed for human foods, with some forms grown for ornament and others for human consumption or animal forage. Brassica vegetables e.g. broccoli, cabbage and cauliflower are grown on over 3500 ha in New Zealand annually and are highly regarded for their nutritional value. They contain high amounts of vitamin C and soluble fibre and contain multiple nutrients with potent anti-cancer properties including 3,3'-

Diindolylmethane, sulforaphane and selenium and a relatively high content of glucosinolates (Verhoeven et al. 1996)

New Zealand's brassica vegetable and forage seed production industry is worth approximately $32 million in export earnings (Johnson and Gallacher 2008). In Canterbury, vegetable seed was started to be grown in the 1980's. This seed was contracted to offshore companies and, as the experience of growers has increased, the demand has increased to the point where this is now a major part of Canterbury's arable industry (Johnson and Gallacher 2008). Brassica vegetable seed is centered in mid-Canterbury and is expanding into Southland and the North Island. Data collected by New Zealand Grain and Seed Trade Association (NZGSTA) (Appendix 1) indicates that approximately 3,400 ha of Brassica vegetable seed is grown in New Zealand with 54% grown in mid-Canterbury (between the Rangitata and Rakaia rivers) and a further 22% in South Canterbury and 21% in North Canterbury. Forage seed is also centered in mid-Canterbury, with only 40 ha recorded outside this region. This is not a complete summary as not all seed companies are members of the NZGSTA. Land use for all brassica seed crops is currently estimated at 8,600 ha. Information collected by Johnson and Gallacher (2008) led to projected total brassica seed production of 40,000 ha by 2018. Growing specialist seeds (e.g. Chinese brassicas, hybrid vegetable seeds) can return up to $20,000 per hectare to growers. However, it is a high risk industry that requires very exact management and is inherently sensitive to climatic variations around pollination and harvest times in particular. An example of the associated risk is shown in the M.A.F. review (http://www.maf.govt.nz/mafnet/publications/outside-in-review/page-09.htm retrieved 23 March 2009) which shows how seed production suffered in 2002 due to adverse climatic conditions with cloudy, wet weather and poor pollination.

1.3 Forage brassica

Forage brassicas are grown widely to supplement pastures in grazing animal production systems. In New Zealand, they comprise the largest area of cultivated crops, with about 250,000 ha grown annually. The forage brassicas have diverse growth types and encompass various species and inter-specific hybrids. They include crops that produce primarily root biomass (e.g. swede (Brassica napus) and turnip (B. rapa)), others that produce mainly leaf and stem (e.g. kale (B. oleracea) and rape (B. napus)), and some that produce much leaf but little stem or root (e.g. Pasja, a fast-growing, early-maturing turnip-Chinese cabbage hybrid). (www.cropscience.org).

Three *Brassica* species provide forage, *B. oleracea, B. napus* and *B. rapa* (Christey and Braun 2001). As reported by Thompson (1976), *B. oleracea* takes on many forms and provides both stem and leaf varieties of forage for animals as well as vegetables for humans. *B. napus* provides forage in the form

of leaf (rape) and root (swede) (McNaughton 1976). A CSIRO study presented by Moore et al. (2004) showed that inclusion of forage brassica into the rotation not only increased whole-farm gross margins but also decreased deep drainage of water. In a 1994 report by Weidenhoedft and Barton forage brassica was shown to extend the grazing season in higher latitudes.

In 1983 Jung et al. reported four major advantages of forage brassicas to livestock production: they suggest that cultivation of forage brassica led to reduced soil erosion; that plants increased the ability of soil to retain moisture; seeds can be drilled directly in to the soil mechanically and thus save time planting and, due to the structure and toughness of the plant, there is little crop loss to trampling during inclement weather.

The Advanced Seed Productions System Programme, which this research is part of, is a collaboration between Universities, CRIs and seed companies focusing on increasing the yield, both in terms of quantity and quality, for those farmers in the Canterbury region whose primary income is derived from growing seed, including clover, vegetable and forage brassica, process and field pea and ryegrass seeds. The focus of this project was forage brassica.

1.4 Rapid cycling brassica

Due to the long life cycle of forage brassica, a rapid cycling cultivar of *B. rapa (*syn. *campestris)* was selected for initial gene expression studies. Seed was obtained from Wisconsin Fast Plants®. The Rapid Cycling Brassica Collection (RCBC) was established in 1982 by Paul Williams as a means for distributing seed, and information, about various genetic stocks of six different species of rapid cycling brassicas. Initially known as the Crucifer Genetics Cooperative (GrGC) the collection now resides under the Wisconsin Fast Plants Program (www.fastplants.org; RCBC catalog dated 3/21/07 printed March 3 2008).

Rapid cycling *Brassica rapa* (RCBr) was derived from a global collection of *B. rapa* (L.) varieties (Williams and Hill 1986). Tomkins and Williams (1990) describe how plants were selected for the following six qualities: reduced size at maturity, minimum time from germination to flowering, uniformity of age at first flowering, greater flower production, rapid maturation of seeds, and lack of seed dormancy. Individuals that flowered fastest were used as the base population. These individuals were out-crossed to generate seeds. In the next generation, the 10% of the offspring population that flowered first were selected as parents. These plants were mass pollinated to produce the next generation of seeds. Artificial selection continued until the response to selection was stabilised (Williams and Hill 1986) resulting in plants that had a brief life cycle and a small adult size.

Rapid-cycling *Brassica* populations were initially developed as a model for probing the genetic basis of plant disease (Musgrave 2000). Musgrave (2000) reported that before development of rapid-cycling populations, the relatively long (0.5–2y) life cycles for most of the economically important brassicas was a negative element in this otherwise promising scenario for continuous plant improvement. It is this rapid life cycle that make RCBr ideal for use as a model plant. Under optimal laboratory conditions, RCBr flowers within 16 days of seed germination and has a life cycle of 35–40 days, from parental seed sown to offspring seed harvest (Williams and Hill, 1986). Compared to normal *B. rapa*, which can produce two generations in a year, under optimal conditions RCBr can produce 10 generations in a year. Because of their close relationship with economically important *Brassica* species, rapid-cycling *Brassica* populations, especially those of *B. rapa* (RCBr) and *B. oleracea,* have seen wide spread application in plant and crop physiology investigations. Another benefit of using RCBr is that the organs are larger than the model plant *Arabidopsis thaliana.* The flowers of RCBr are three times larger than flowers from *A. thaliana* (Weinig 2002 cited by Kelly 2006). Brassicas are also well suited for molecular studies. Williams and Hill (1986) demonstrated how most species are able to be transformed by *Agrobacterium tumefaciens* and *A. rhizogenes*. Musgrave (2000) identified a chief value of the RCBr model would be the possibility of combining genetic research into the investigation and improvement of seed storage reserves in *Brassica.*

It is important to recognise that RCBr retains considerable isozymic variation (Williams and Hill 1986). Evans (1991) noted that when inbreeding was forced, fitness was significantly reduced. Both outcomes suggest, according to Kelly (2006), that *B. rapa* did not pass through a genetic bottleneck in order to establish the rapid cycling lines. According to Musgrave (2000), the RCBr system has a unique standing as a model plant because of its close relationship with crop plants and its close phylogenetic relationship with *Arabidopsis.*

1.5 Cytokinins

Plant hormones are small organic molecules that affect diverse developmental processes (Bishopp et al. 2006)**.** The cytokinins are plant hormones that regulate many stages of plant growth and development including control of root/shoot balance, delay of senescence, nutritional signalling (Sakakibara 2006), and release from apical dominance (Sachs and Thimann 1967). Cytokinins also control the regulation of cell division and enhancement of sink strength (Werner et al. 2008), both of which are important processes in seed development and embryonic growth. Their biosynthesis and homeostasis is finely controlled by internal and external factors such as other phytohormones, inorganic nitrogen sources and other environmental factors (Silverstone and Sun 2000; Ashikari et al. 2005; Bishopp et al.2006; Hirose 2007 and Zhao 2007).

1.5.1 Cytokinin structure

The naturally occurring cytokinins are N^6-substituted adenine derivatives that carry either an isoprene-derived or an aromatic side chain at the N^6 terminus; conventionally these families are called isoprenoid cytokinins and aromatic cytokinins, respectively (Figure 1.2). Each of these can occur in the free base, riboside, or ribotide form. Each cytokinin molecule is distinguished by characteristics of the side chain, namely the presence or the absence of a hydroxyl group at the end of the prenyl chain and the stereoisomeric position (Sakakibara 2010). In addition to the naturally occurring isoprenoid and aromatic derivatives, the synthetic diphenylureas exhibit strong cytokinin activity. The naturally occurring isoprenoid cytokinins, *trans*-zeatin (tZ), isopentenyladenine (iP), *cis*-zeatin (cZ), and dihydrozeatin (DZ), and their nucleosides and nucleotides are all widely found in higher plant species and in greater abundance than the aromatic cytokinins (Mok and Mok 2001; Sakakibara 2006). Zeatin occurs in two isomers, with *trans*-zeatin being accepted as the biologically active form and *cis*-zeatin reportedly having little or no activity. A recent study by Gajdošová et al. (2011) found that in some species *cis*-zeatin cytokinins were more prevalent than trans-zeatin and that the *cis* isomer appears during periods of slow or low growth in the plant's life cycle. Emery et al. (1998) found the *cis*-isomer was the predominant cytokinin in developing chickpea. Emery et al. (1998) concluded that the hydrolysis of tRNA could not account for the presence of the cZ and that either there may have been an undiscovered *cis-trans* isomerase (subsequently shown by Mok et al. 2001) or possibly some other non-enzymatic activity.

Cytokinins act as long range signallers and are very mobile. Kudo et al. (2010) reported that the major form of cytokinin is tZR in xylem sap and the major forms in phloem sap, are iP-type cytokinins, such as iPR and iP-ribotides. These cytokinins travel up or down the plant co-ordinating root-shoot development and communicating environmental conditions e.g. stresses such as drought (Stirk and Staden 2010). Hwang and Sakakibara (2006) report that the main transport form of cytokinin is t*rans*-zeatin riboside.

Glycosylation of cytokinins has been found in many plant species. Although not well characterised, this modification to the side chain inactivates the cytokinin (Wang et al. 2011). Irreversible glycosylation can occur at N^3, N^6 and N^9positions of the purine ring forming an N-glucoside. Reversible glycosylation occurs at the hydroxyl group of the zeatin and dihydrozeatin side chain, forming an *O*-glucoside. Once converted to an N-glucoside the cytokinin cannot be reactivated, but an *O*-glucoside can be reactivated by β-glucosidases and it is considered that *O*-glycosylation represents the storage form of active cytokinin (Wang et al. 2011).

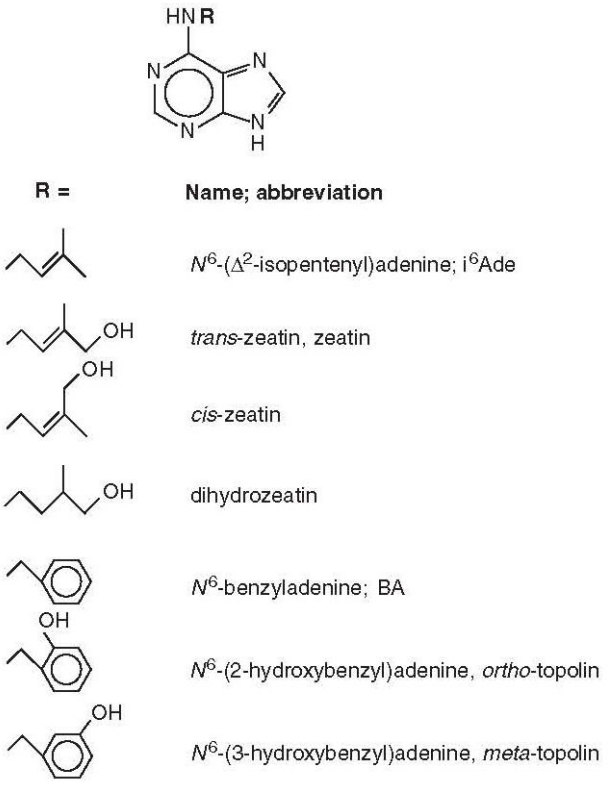


Figure 1.2 Structures of isoprenoid and aromatic cytokinins. Sourced from Mok DWS and Mok MC (2001).

1.5.2 Biosynthesis of cytokinin

From the time when endogenous cytokinins were first identified in plants, these hormones have been shown to accumulate during the phase of cell division in developing fruit and seeds (Letham, 1963; and Jameson et al. 1982). The origin of this cytokinin was initially proposed to be from the roots (see Nooden and Leopold, 1978). For many years there was much debate about whether the 'free' cytokinins originated by *de novo* synthesis or by the release of cytokinin following alkaline hydrolysis of tRNA (Jameson 2003).

1.5.3 Cytokinin biosynthesis in bacteria

Following the identification of isopentenyl transferases on the Ti plasmid of *A. tumefaciens* there are several gall forming bacteria that possess genes that encode the DMAPP:AMP isopentenyltransferases e.g., *tzs* and *ipt* (same as *tmr*) of *A. tumefacians*, *ptz* of *Pseudomonas savatanoi*, *ipt* of *Rhodococcus fascians*, and *ipt* (same as *etz*) of *Erwinia herbicola* (Jameson 2003). The obligate biotroph *Plasmodiophora brassicae* induces club formation on roots of various species of the *Brassicaceae*

family also by the same pathway (Ando et al. 2005). The expression of *tzs* in *A. tumefaciens* results in the secretion of cytokinins, whereas *ipt* in *A. tumefaciens* is integrated into the plant genome using the host plants to produce cytokinins and form tumors.

1.5.4 Cytokinin biosynthesis in plants

For many years, it was believed that the key step in cytokinin biosynthesis in plants would be the same as that in bacteria. However, identification of this gene proved difficult. With the *Arabidopsis* genome completely sequenced, it became possible to use molecular genetics to identify candidate genes in plants. Nine genes identified in *Arabidopsis* (*AtIPT1* to *AtIPT9*) showed similarity to bacterial *IPT* (Sakakibara 2004; Kakimoto 2001). Takei et al. (2001) reported that while the AtIPTs did not show close similarity to tRNA IPTs and bacterial IPTs at the amino acid level, some common structural features were found. The putative motif for DMAPP binding, which is similar to the ATP/GTP-binding motif at the amino-terminal region, was conserved in both types of isopentenyltransferase.

Applications of exogenous cytokinins induce cell division and shoot formation on calli in the presence of an auxin (Skoog and Miller 1957). The same results were observed by the over expression of *AtIPT4* under the control of the 35S-promoter (Kakimoto 2001). Even in the absence of exogenous cytokinins, calli transformed with 35S::AtIPT4 regenerated shoots. Takei et al. (2001) used transgenic *E. coli* to express *AtIPT1* and *AtIPT3*-*AtIPT8* and found iP and tZ accumulated in the culture medium. It was also observed that expression of *AtIPT1*, *AtIPT4*, *AtIPT7* and *AtIPT8* resulted in relatively higher tZ than *AtIPT3*, *AtIPT5* and *AtIPT6*. Kakimoto (2001) used *E. coli* extracts expressing *AtIPT4* or *AtIPT5* to display DMAPP::AMP IPT activity, whereas extracts expressing *AtIPT2* did not. Unlike bacterial enzymes, which catalyze the transfer of the isopentenyl moiety from dimethylallyldiphosphate (DMAPP) to the N^6 position of AMP, plant enzymes catalyze the transfer of the isopentenyl moiety from DMAPP preferentially to adenosine 5'-diphosphate (ATP) and to adenosine 5'-triphosphate (ADP) (Takei et al. 2001) (Figure 1.3). In rice, Sakamoto et al. (2006) reported eight IPT genes (OsIPT1–OsIPT8) identified as being involved in the N-prenylation step of CK biosynthesis. IPTs are considered the rate-limiting enzyme of cytokinin biosynthesis because overexpression of IPTs in transgenic *Arabidopsis* caused increased cytokinin levels and phenotypes indicative of cytokinin overproduction (Ando et al. 2003).

In some species (*AtIPT6* and *OsIPT6*) appear to be pseudo-genes due to a frame shift caused by a nucleotide deletion (Kakimoto 2001). AtIPTs and OsIPTs prefer ATP or ADP to AMP as the prenyl

acceptor, and utilize DMAPP as the prenyl-donor. Using isotope-labeling experiments in *Arabidopsis* seedlings, Kasahara et al. (2004) demonstrated how the prenyl group of tZ and iP is mainly produced by the MEP pathway, suggesting that plastids play an important role in the initial step of cytokinin biosynthesis in *Arabidopsis*. In concurrence with this result, Kasahara et al., (2004) localised four AtIPTs (AtIPT1, AtIPT3, AtIPT5, AtIPT8) in plastids. In contrast, Kasahara et al. (2004) found AtIPT4 and AtIPT7 localised to the cytosol and mitochondria, respectively.

AtIPT2 and AtIPT9 show homology to an Agrobacterial tRNA modifying enzyme (Davies 2004) known as a tRNA-isopentenyltransferase. It has been shown that these tRNA IPT genes are expressed ubiquitously in *Arabidopsis* (Kakimoto et al 2006). Levels of iP-type and tZ-type cytokinins were shown to be unaffected in either the *AtIPT2* or *AtIPT9* mutants or the *AtIPT2 9* double mutant, but in these mutants the *cis* isomers of Z, ZR and ZRMP were greatly reduced or undetectable.

1.5.6 IPT expression is tissue specific

In mature plants, the AtIPTs are differentially expressed in various tissues including the roots, leaves, stems, flowers and siliques. Analyses of spatial expression patterns of AtIPTs using promoter::reporter genes revealed tissue- and organ-specific patterns of cytokinin synthesis by IPT. AtIPT1 was expressed in xylem precursor cell files in root tips, leaf axils, ovules, and immature seeds; AtIPT3 was expressed in phloem companion cells; AtIPT4 and AtIPT8 were expressed in immature seeds with highest expression in the chalazal endosperm (Takei et al. 2004; Sakakibara 2006). AtIPT8 had a slightly more extensive expression pattern in the endosperm and persisted until late heart stage, whereas AtIPT4 was very specific to the chalazal cyst and the activity in IPT4-GUS transformants disappeared prior to the heart stage (Miyawaki et al. 2004). AtIPT5 was expressed in lateral root primordia, columella root caps, upper parts of young inflorescences, and fruit abscission zones; AtIPT6 was expressed in siliques; AtIPT7 was expressed in phloem companion cells, the endodermis of the root elongation zones, trichomes on young leaves, and occasionally in pollen tubes. (Takei et al. 2004; Sakakibara 2006).

Recent studies with other species have found that individual *IPT* (Kasahara et al., 2004; Brugière et al. 2008; Takei et al. 2004; Sakakibara 2006) gene family members were differentially expressed in various tissues.

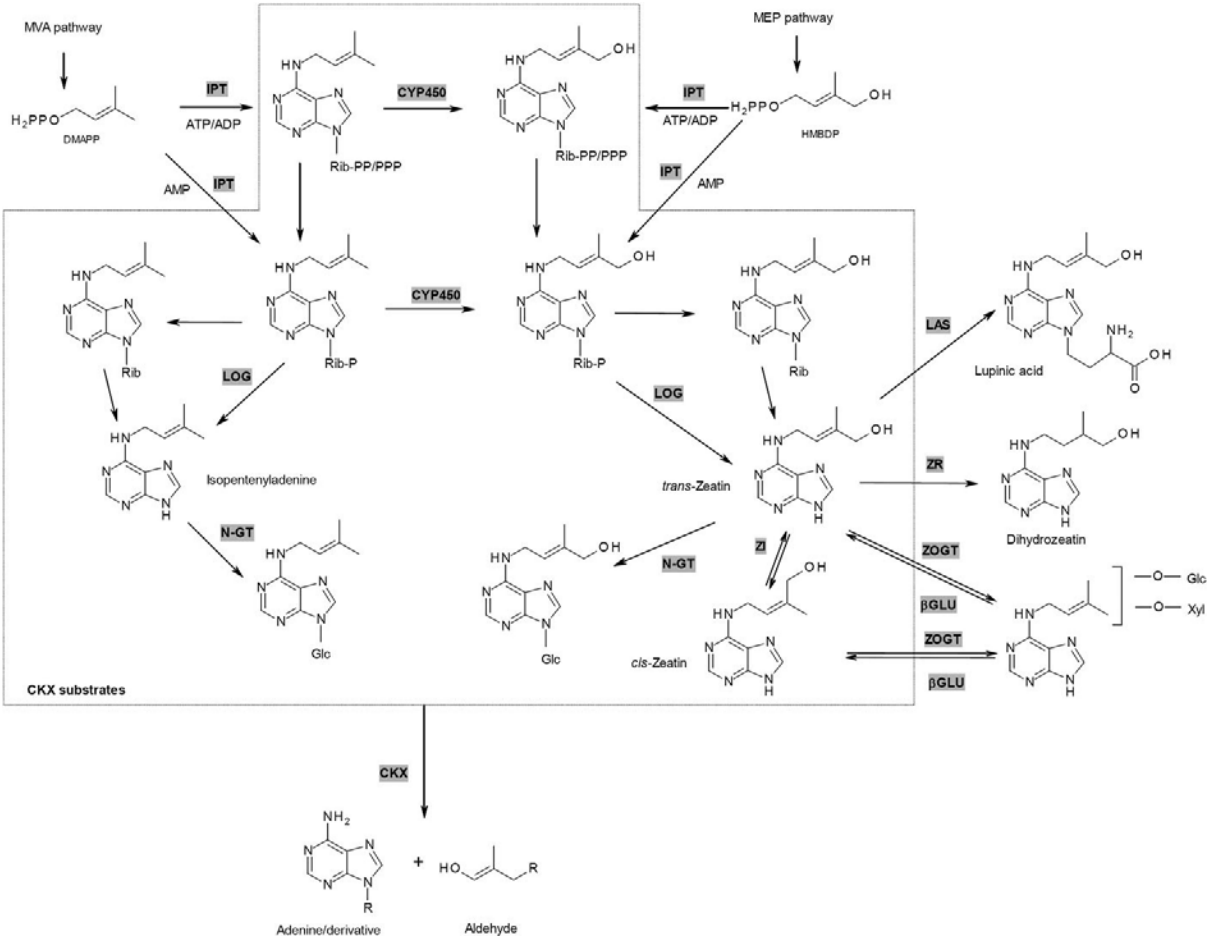


Figure 1.3 The key enzymes of cytokinin biosynthesis and conversion. Copied from Frébort et al. (2011)

1.5.7 Cytokinin oxidase/dehydrogenase

The degradation of cytokinins by a cell free system was first shown by Paces et al. (1971). They reported that IPA was degraded to adenosine by an enzyme preparation from tobacco callus tissue. The term cytokinin oxidase was first used by Whitty and Hall in 1972 (cited by Armstrong 1994) to describe enzyme activity obtained from corn kernels.

Subsequently it has been shown that cytokinin oxidase/dehydrogenase (CKX) enzymes inactivate cytokinins irreversibly in a single enzymatic step by cleaving the N^6-side chain (Figure 1.4) from the adenine/adenosine moiety, converting active cytokinins such as zeatin and iP to adenine (Galuska et al. 2000; Mok and Mok 2001; Eckardt 2003; Massonneau et al. 2004). Susceptibility to CKX differs among cytokinins. Generally, iP is more susceptible to CKX than tZ; cZ and DZ are resistant to CKX (Bilyeu et al. 2001; Galuszka et al. 2007). Cytokinin bases, ribosides and nucleotides are susceptible to CKX activity but not the *O*-glucosides (Mok and Mok 2001). CKX has been found to be widely

distributed in plants (Jones et al. 1997). *CKX* genes are found in both plants and bacteria. Phylogenetic analysis indicates that plants acquired the *CKX* genes through a lateral transfer from bacteria to plants via the chloroplast, which is of cyanobacterial origin (Eckhardt 2003; Schmülling et al. 2003). The *CKX* gene families of *Arabidopsis* and rice comprise seven and at least ten members, respectively (Schmülling et al. 2003).

Activity of CKX within the plant is governed by a complex series of positive and negative feedbacks. The regulatory mechanisms that control cytokinin degradation and the levels of CKX activity present in plant tissues are known to be sensitive to cytokinin supply (Massonneau et al. 2004; Brugière et al. 2003). Rapid turnover of cytokinin has been reported, and catabolism by CKX enzymes is believed to play a critical role in regulating cytokinin activity in vivo (Schmülling et al., 2003).

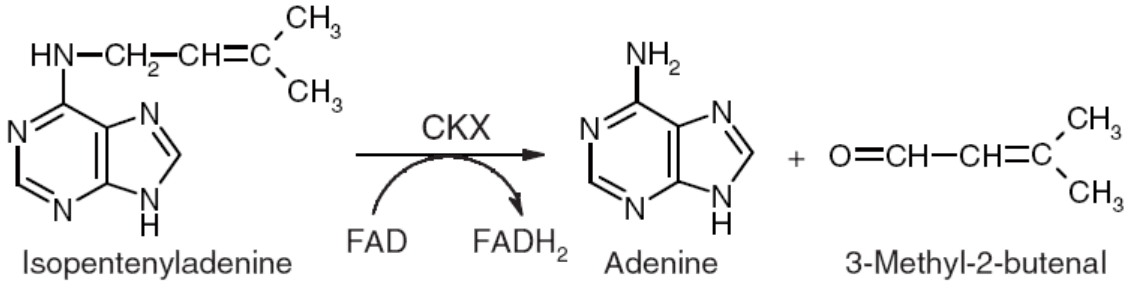


Figure 1.4 The reaction catalysed by the cytokinin oxidase/dehydrogenase enzyme. From Schmülling et al. (2006)

1.5.8 Cytokinin homeostasis

Cytokinin levels in plant tissues are determined by the rate (and location) of biosynthesis, the formation of inactive conjugates (mainly glycosides), and the rate of catabolism (Eckhardt 2003). The levels of physiologically active cytokinins (free bases) are down regulated in plants mainly by side-chain cleavage with cytokinin oxidase/dehydrogenase (CKX) and by conjugation to glucose, either reversible (yielding O-glucosides and N^3-glucosides) or irreversible (yielding N^7- and N^9-glucosides) (Jameson 2003). Based largely on hormone over-expression studies, it has been suggested that cytokinin acts to reduce the pool of active auxin and vice versa (Coenen and Lomax, 1997).
There are two ways of increasing endogenous cytokinin. One way is to over express an *IPT* gene family member or, conversely, to decrease the activity of a *CKX* family member. It has been demonstrated that reduced expression of *OsCKX2* in rice (Ashikari et al. 2005) increased grain number, and silencing *HvCKX1* increased grain size and number in barley (Zalewski et al. 2010). Constitutive over-expression of some of these genes (*AtCKX1*, *AtCKX3*, and *AtCKX5*) resulted in a marked reduction of endogenous cytokinins in both root and shoot meristems (30–60% of that of wild

type), strongly affecting root and shoot development (Ioio et al. 2007). In these lines, growth of the aerial parts of the plant was severely retarded: leaf and shoot apical meristem size decreased while the total root mass increased. Cytokinins might therefore act as positive regulators of shoot apical meristem development and as negative regulators of root apical meristem development (Ioio et al. 2007). This homeostatic regulation ensures normal plant growth. In their study into *IPT* over-expression Sýkorová et al. (2008) delayed senescence in the aim of increasing grain yield. However, this delay meant the leaves did not open to release the panicle, the grain did not fill, and the yield was not increased.

1.6 Cytokinins and seeds

While cytokinin biosynthesis was originally proposed to be limited to the roots, it was recognised in early metabolism studies that root-supplied cytokinin does not provide sufficient cytokinin for seed development (Letham 1994). Radiolabelled cytokinins, supplied to the xylem stream of leguminous plants, showed no intact cytokinin moieties in the embryo while they were detected in the pod and seed coat (e.g. Jameson et al. 1987, Singh et al. 1988), even though labelled adenosine supplied in a similar manner was detected reaching the seed (Nooden and Letham 1984).

Many studies have emphasised the role of cytokinins in determining sink strength by increasing cell division (Micheal and Seiler-Kelbitsch 1972; Yang etal. 2002 and Gupta et al. 2003). Miyawaki et al. (2004) reports that other studies (Emery et al. 2000; Morris et al. 1993; Yang et al. 2000) have found a large increase in seed cytokinin content within a specific developmental window of seed formation in cereals and beans. Indeed, increased cytokinin content during early fruit and seed development has been a common finding over many studies. Jameson et al. (1982) found in wheat that cytokinin peaked four days after anthesis; Morris et al. (1993) showed in maize a peak nine days after pollination and in rice the cytokinin peak was detected 4-5 days after pollination (Morris et al. 1993). Yang et al. (2001) confirmed this by showing that cytokinins and indole-3-acetic acid contents in rice grains transiently increased at the early filling stage and coincided with the rapid increase in grain-filling rate. It was also noted that changes in Z and ZR in superior and inferior spikelets directly defined the pattern of grain filling. Rice varieties with high percentage grain filling and rapid filling rates have high cytokinin content (Yang et al. 2000).

Cytokinin application has also been shown to increase grain yield. Nagel et al. (2001) treated raceme tissues of soybean (*Glycine max* L. Merr.) with exogenous cytokinin, causing greater flower production and a 79% increase in seed yield over controls. Experiments by Hosseini et al. (2007) studying the relationship between cytokinins and source and sink strength in barley using the

exogenous application of a synthetic cytokinin, 6-benzylaminopurine (6-BAP), showed an increased grain yield. By applying 6-BAP to either the whole plant or only the ears there was an increase in ear weight, grain yield and 1,000-grain weight. Grain size was found to be related to the number of endosperm cells. These increased with the application of 6-BAP. Hosseini et al. (2007) showed that plant responses also differed to exogenous application of synthetic cytokinin dependent upon which tissue was sprayed and at what time during the growth cycle the application was made. Studies using the cytokinin response receptors support this, with different receptors expressed in different tissues and at different levels (Muller and Sheen 2006).

Spraying plants in the field with cytokinin could potentially be used to increase plant production. This currently is an expensive method, requiring precise timing of application and at a commercial level probably impractical and not cost effective. The application of cytokinin or the modification of cytokinin activity needs to be targeted and delivered in a specific and tightly controlled manner. With the advances in genetic studies this was able to be achieved using transgenics. Ma et al. (2007) fused an *IPT* gene onto a seed specific lectin promoter and this resulted in significant increases in embryo diameter, followed by increases in soluble protein content and dry weight of tobacco seeds. The resulting transgenic seedlings grew quickly and, compared with the controls, obtained an observable increase in fresh weight at 20 (14%) and 35 (8%) days after germination.

Daskalova et al. (2006) used transgenic tobacco to study the activity of IPT in seeds. They used the 2.6 kb wheat high molecular weight (HMW) glutenin subunit 12 promoter fused to either the *gus* reporter gene (*HMW::gus* construct) or to the *Agrobacterium ipt* gene (*HMW::AtIPT* construct). Results indicate that the highest level of cytokinin activity during seed development coincided with the period of most intensive cell division.

Rivero et al. (2007) found that, under drought conditions, transgenic tobacco over expressing *IPT* retained significantly more biomass and produced a larger seed yield than wild type plants. Under these drought conditions no delay was observed in flowering and seed set, indicating that even under drought stress the source/sink relationship was maintained in plants expressing higher levels of IPT.

However, as exemplified by the results of Sýkorová et al. (2008), when delayed senescence in *IPT* over-expressing plants meant the panicle was not released and yield was in fact reduced, *IPT* over-expression should be targeted to those organs governing yield components and the technology exists to now identify gene family members that do not express elsewhere in the plant.. As Miyawaki et al. (2004) showed AtIPT4::GUS and AtIPT8::GUS were expressed soon after fertilisation in the developing seeds with highest expression in the CZE (Miyawaki et al. 2004). *AtIPT4* and *AtIPT8* may

play a significant role in these processes in developing seeds and are therefere gene family members that might be appropriate targets.

In a contrasting approach, Ashikari et al. (2005) used an *OsCKX2* deficient mutant to display cytokinin accumulation in inflorescence meristems of rice which increased the number of reproductive organs, resulting in enhanced grain yield. While it may be logical to expect increased cytokinin levels by lowering the enzymes that metabolise them, it is of particular interest to observe the effect of increased cytokinin on grain yield. The panicle structure of their *OsCKX2* mutant (5150) was significantly larger than the two common rice cultivars (Kosihikari and Habataki). This was also reflected in the actual grain count per panicle of the three cultivars with CKX mutant having an increased grain count per panicle compared to Habataki and Koshihihkari. Furthermore, when Zalewski et al. (2010) used RNAi to silence the *CKX1* gene in barley, the resulting phenotype displayed a positive correlation between activity of the CKX1 enzyme and plant yield, with both seed number and seed size increased in the transgenic plants. Consequently, in this project identification of a CKX gene family member expressing specifically during seed development may provide a target for selection.

1.7 Aims and Objectives

The Advanced Seed Production Systems Programme has two objectives. Objective 1 focused on crop manipulation in the field, whereas Objective 2 is targeted to identifying gene family members of *IPT* and *CKX* expressing at critical stages of seed and pod development in brassica and pea.

The purpose of this thesis was the identification, in rapid cycling brassica (*B. rapa*) and forage brassica (*B. napus*), of the key *CKX* and *IPT* gene family members involved in seed development. The first aim was to develop a set of primers for as many *CKX* and *IPT* gene family members as possible. This was done *in silico*, interrogating online gene bank databases. The objective was to then use these primers were used in subsequent gene expression studies.

The second aim was to investigate the expression levels of individual gene family members. This was done using qRT-PCR. To achieve this, Rapid Cycling *B. rapa* plants were grown in a contained growth room and tissue samples were taken from roots, stem, flowers, seed pods, seeds and leaves of *B. rapa* as the plants developed to determine expression of *IPT* and *CKX* gene family members. Once this was completed, the study was extended to forage brassica (*B. napus*) with tissue collected from a commercial property in Tinwald, South Canterbury.

Chapter 2

Materials and Methods

2.1 Plant Material

2.1.1 Rapid Cycling *Brassica rapa* (Wisconsin Fast Plants® Standard Brassica rapa seed)

A packet of 200 seeds of RCBr was obtained from Wisconsin Fast Plants (www.fastplants.org). Seeds were imbibed for 4 h and planted in a tray of 60 small pottles filled with general purpose potting mix. As per the suppliers instructions the seeds were kept in a controlled growth room set at 25°C. Plants were supplied with 24 h lighting and continuous water. Plants were hand pollinated. Three samples were taken at each selected stage of plant development. The stages sampled included 1-2 day old leaves, expanded leaves, and mature leaves; unopened young flowers, flowers where the petals were starting to be visible and fully opened flowers; and four stages of pod and seed development (1 day after pollination (DAP), 2 DAP, 7 DAP and 14 DAP) (Figure 2.1). Root tips and stems from a mature plant were also harvested. To collect roots of *B. napus*, plants were carefully removed from the pottles and washed free of soil and patted dry. If tissue was not completely dry prior to freezing then RNA extraction was severely impacted as the water crystals impeded grinding of the tissue. Two independent experiments were run: from the first experiment whole pods were extracted starting at 1 DAP as well as leaves, stems and roots; from the second, seeds were separated from pods starting from 4 DAP and analysed independently.

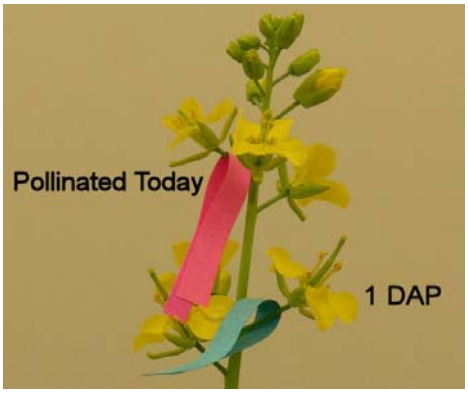


Figure 2.1 Tracking hand pollination of *B. rapa*. Coloured strips enabled easy identification of individual flowers/pods. Each flower was labelled after being brushed with pollen.

2.1.2 Brassica napus

Brassica napus was sourced from commercial property in Tinwald, South Canterbury (December 2010) (Figure 2.2). Six similar plants were identified and three stages of leaf and flower and seven stages of pods were collected.

Figure 2.2. Field of forage brassica. Tinwald, South Canterbury. December 2010

For the most part tissue samples were collected into 1.7 ml centrifuge tubes and immediately frozen in liquid nitrogen and stored at -80°C. On occasion larger tissues e.g. stems were initially placed into 15 ml tubes. Leaves were excised from their petiole with three stages collected. Leaf 1 was identified as being small, very young leaves nearer the top of the extending plant. Leaf 2 larger, older leaves nearer to the middle of the plant with Leaf 3 being the oldest leaves nearest the base of the plant. Flower 1 was young, closed flowers with no yellow of the petals visible. Flower 2 was still just closed but the yellow of the petals were visible. Flower 3 was newly opened flowers ready to be pollinated. The seven stages of pod were taken from 1 day after pollination through to mature pods (Figure 2.3). In the field it was not possible to take root samples due to the requirement for washing and freezing.

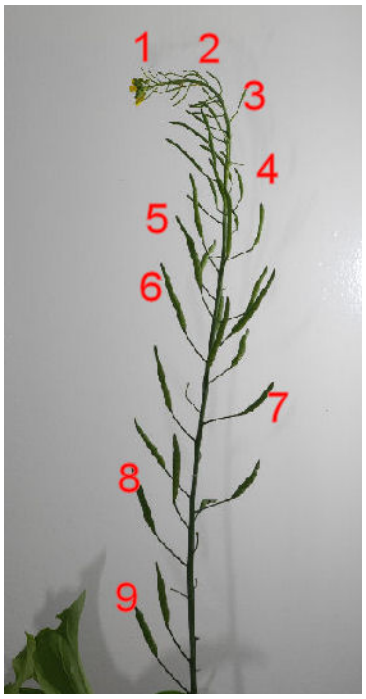


Figure 2.3 Secondary flowering stem *B. napus*. In the field, mature plants were selected and a secondary stem was isolated from the rest of the plant. Working from the top down the three stages of flowers were collected then seven stages of pods were taken.

2.2 Sample processing

In preparation for RNA extraction, tissue was reduced to a fine homogeneous powder by hand grinding with a plastic pestle in 1.7 ml centrifuge tubes cooled with liquid nitrogen. To ensure sterility pestles were treated with 3% H_2O_2 for 10 m and rinsed with ultra pure water. To enable chlorophyll and RNA extractions to be carried out on the same leaf, excess material was ground and material was taken as required.

On the rare occasion when material was too tough to grind in the plastic pestle it was crushed in the stainless steel 'tissue crusher' (Figure 2.1). The crusher was treated with 3% H_2O_2 for 10 mim and rinsed with ultra pure water. It was then filled with liquid nitrogen and along with the piston placed in a liquid nitrogen 'bath'. When completely chilled, tissue was placed in the cylinder and the piston repeatedly hit with a hammer to crush the tissue. The wafer of crushed tissue was carefully transferred to a 1.7ml Eppendorf tube. Once completed the crusher was thawed, cleaned carefully, dried, ready to be used again.

2.3 RNA isolation and cDNA synthesis

RNA was extracted from brassica tissues using three different methods. For the RcBr work TRIzol® / TRI Reagent® (Section 2.3.1) and BP-10 spin column total RNA Miniprep (Section 2.3.2) were used. For the *B. napus* work the Total RNA Purification from Plant kit (Section 2.3.3) were used. The

integrity and quality of isolated RNA was assessed by running 1 µl of the samples on a 1% (w/v) agarose gel (Section 2.7). The concentration and purity of the RNA was assessed by nanodrop spectrophotometry (Section 2.8)

RNA is very susceptible to degradation. The RNA degrading enzymes, ribonucleases (RNase) are prevalent throughout the environment and within plant tissues and the utmost care must be taken to prevent contamination of RNA samples. To prevent RNA degradation all tissue samples were immediately frozen in liquid nitrogen and stored at -80°C. Liquid nitrogen was used to prevent samples from defrosting during grinding or pulverising. To eliminate RNase from the work environment, work surfaces and pipettes were treated with RNA zap. Hydrogen peroxide (3%) was used to treat plastic and metal equipment. Glass and some metal equipment was baked at 300°C for 4 h. Commercially supplied RNase free pipette tips were used for all RNA work.

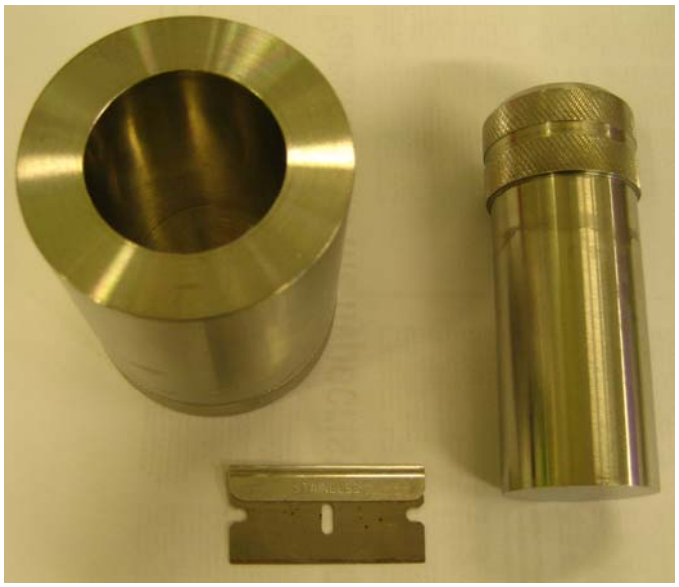


Figure 2.4 The stainless steel tissue crusher V.1: Due to mechanical wear and tear V.2 of the crusher featured a bottom that was welded, not screwed on. (Picture courtesy Thomas Evans)

2.3.1 TRIzol® / Tri Reagent® RNA Extraction and cDNA synthesis

TRIzol® and Tri reagent® RNA extractions were used to extract RNA from all plant tissues except flowers. This extraction is a phenol-chloroform based extraction procedure. Phase separation of the aqueous phenol and organic chloroform containing fraction separates RNA into the aqueous fraction, leaving DNA on the interface and proteins in the organic fraction (Chomczynski and Sacchi, 1987).

A maximum of 100 mg of frozen and ground, or pulverised, tissue was added to 1ml of TRIzol® (Invitrogen) or Tri reagent® (Ambion) in a 1.7 ml centrifuge tube and incubated for 3 min at room temperature. The tubes were then centrifuged at 11000 rpm, 4°C for 5 min. The supernatant was transferred to a new tube, 100 µl of chloroform was added and the mixture shaken vigorously for 15 s, incubated at room temperature for 10 min and then centrifuged for 15 min at 11000 rpm, 4°C. The upper fraction was transferred to a new tube and left to stand for 5 min at room temperature. RNA was precipitated with isopropanol (500 ml), mixed well and incubated at room temperature for 10 min, then centrifuged at 11000 rpm, 4°C to pellet the RNA. The supernatant was discarded and the RNA pellet washed with 1 ml 75% (v/v) ethanol, centrifuged at 11000 rpm, 4°C for 5 min, the supernatant discarded and the wash step was repeated. The remaining ethanol was aspirated and the pellet air dried for 5 min. The RNA was resuspended in 50 µl of DEPC water (Appendix 5.2.8) with 1x RNA Secure (Invitrogen). The samples were then incubated at 65°C for 15 min to deactivate any RNase and stored at -20°C.

Extracted RNA was converted to cDNA by reverse transcription. Because of the high content of degraded RNA collected using the Trizol extraction method, approximately 2 µg of RNA was mixed with 1 µl (100 pmole) of random primers (pDN6) and 1 µl (500 pmole) oligo (DT) primers and made up to 10 µl with DEPC treated water (Appendix 5.2.8). The solution was incubated at 65°C for 10 min and then placed on ice. RT master mix (10 µl) (Section 0) was added to the RNA primer mix and incubated initially at 42°C for 240 min, and then at 70°C for 15 min to deactivate the enzyme. The cDNA was diluted 10 fold with nanopure water and stored at -20°C.

2.3.2 BP-10 Spin Column Total RNA Minipreps Super Kit and cDNA synthesis

The BP-10 Spin Column Total RNA Minipreps Super Kit (Bio Pioneer) utilises a spin column with an embedded membrane that selectively binds RNA. Nucleotides, proteins, salts and other impurities are spun from the sample via a series of washes. Pure water was then used to elute RNA from the membrane which was then spun into a collection tube.
Total RNA was extracted from samples of up to 100 mg of frozen and ground, or pulverised, tissue. Extractions were carried out according to the manufacturer's instructions with the following modifications: To increase final RNA concentration, two separate final rinses were performed with 1x RNA Secure (Invitrogen) and the products combined. Samples were incubated at 65°C for 15 min, then frozen at -20°C.

Extracted RNA was converted to cDNA by reverse transcription. Approximately 1 µg of RNA was mixed with 1 µl (100 pmole) of random primers (pDN6) and 1 µl (500 pmole) oligo (DT) primers and

made up to 10 µl with DEPC treated water (Appendix 5.2.8). The solution was incubated at 65°C for 10 min and then placed on ice. RT master mix (10 µl) (Section 5.2.6) was added to the RNA primer mix and incubated initially at 42°C for 240 min, and then at 70°C for 15 min to deactivate the enzyme. The cDNA was diluted 10 fold with nanopure water and stored at -20°C.

2.3.3 Total RNA Purification from Plant Nucleospin® RNA Plant kit and cDNA synthesis

The Nucleospin® RNA Plant kit is also a spin column kit. Unlike the BP-10 Spin Column kit this is a two column kit with an incorporated DNAse treatment step. Total RNA was extracted from samples of up to 100 mg of frozen and ground, or pulverised, tissue. Extractions were carried out according to the manufacturer's instructions. The final elution was 60 ul ultrapure water and samples were transferred to -20°C immediately.

Extracted RNA was converted to cDNA by reverse transcription. Approximately 1 µg of RNA was mixed with 1 µl AP3 primers and made up to 10 µl with DEPC treated water (Appendix 5.2.8). The solution was incubated at 65°C for 10 min and then placed on ice. RT master mix (10 µl) (Appendix 5.2.6) was added to the RNA primer mix and incubated initially at 42°C for 240 min, and then at 70°C for 15 min to deactivate the enzyme. The cDNA was diluted 10 fold with nanopure water and stored at -20°C.

2.4 DNase treatment

Contaminating DNA was removed from RNA by treating RNA with DNase. DNase (2 µl), 10 × DNase (2 µl) and RNA (16 µl) were incubated at 37°C for 10 min to digest DNA. To degrade the DNase (2 µl) 25mM EDTA was added, the mixture was incubated at 65°C for 15 min, then frozen at -20°C.

2.5 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was used to amplify DNA fragments. PCR reactions were performed using Taq PCR reagents from Roche (Section 5.2.7). Reactions were carried out following the manufacturer's instructions. Where multiple samples were being used, a master mix without the template and/or primers was prepared.

The standard PCR program consisted of 35 cycles of 94°C for 40 s, 50-60°C for 30 s, 72°C for 30 s followed by one cycle of 72°C for 5 min then held at 4°C. When cDNA was being used, an initial cycle of 94°C for 5 min, 40°C for 5 min, 72°C for 5 min was performed to complete synthesis of the

second DNA strand. Thermo-cycling was performed on either a BIOrad DNA Engine Peltier Thermal Cycler or a MJ Research PTC-200 Peltier Thermal Cycler.

2.6 PCR primer design

Primers for PCR were designed using Primer Premier™ 5.00. This software displayed forward and reverse primers, product length and provided a useful GUI to display potential primer dimers, false priming and hairpin structures. Primers were generally designed with lengths between 18 and 25 nucleotides and melting temperatures (Tm) between 55°C and 65°C.

2.7 DNA/RNA Quantification

To determine the weight, integrity and purity of DNA and RNA samples both agarose gel electrophoresis and spectrophotometry were used. Spectrophotometry provides an accurate measurement of the concentration of DNA/RNA and the purity of the sample. Agarose gel electrophoresis was used to assess the integrity and concentration of the samples.

2.8 Agarose-gel electrophoresis

DNA/RNA agarose-gels were used throughout the project for quantifying and assessing the quality of RNA extractions and PCR products, and for separating and purifying DNA. Generally 1% (w/v) agarose gels were used except when attempting to separate two very similar products when a 2% (w/v) agarose gel was used. The gels were made by mixing the appropriate weight of agarose in 25x TAE buffer (Appendix 5.2.1) and heating in the microwave until all the agarose had dissolved. Then 2µl of SYBR Safe™ DNA Gel Stain per 30 ml agarose was added and swirled to mix. Once the solution had cooled to around 60°C it was poured into a cradle with the appropriate comb and allowed to set.

Gels were run between 50V and 100V for a sufficient time for the DNA/RNA to run 2/3 of the way down the gel. Samples were loaded with 6x agarose-gel loading dye (Appendix 5.2.5). Bioline HyperLadder 1 (2 µl) was used for quantifying the size and weight of bands. Gels were visualised using a Safe Imager™ blue-light transilluminator and/or a Chemi Genius2 BioImaging System (Syngene). Images were visualised, recorded and analysed using GeneSnap image acquisition software (Synoptics Ltd).

The integrity of RNA was assessed by the structure and distribution of bands (Figure 2.5). Integrity and molecular weight of DNA was determined by assessment of band structure and by comparison with the ladder.

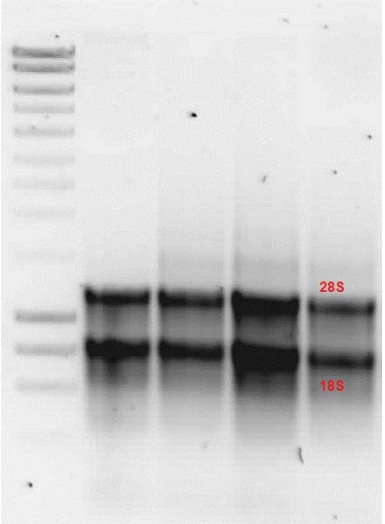


Figure 2.5 RNA integrity gel demonstrating extracted total RNA

A "NanoDrop™ " spectrophotometer (Thermo Fisher Scientific Inc.) was used to measure the quantity and purity of DNA/RNA in Samples (Evans et al in Press). The NanoDrop utilises the absorbance peak of DNA/RNA (260 nm) to calculate concentrations and provides an estimate of purity by assessing the 260/280 nm and 260/230 nm ratios. Samples with, 260/280 ratios of between 1.8 – 2.0, and 260/230 ratios are generally accepted as pure (NanoDrop, 2005). Pure RNA has an A260/A280 of 2.1. (http://biomedicalgenomics.org/RNA_quality_control.html)

2.9 DNA Purification

A pure DNA template is required for optimal sequencing and second round PCR reactions. Gel purification and silica bead DNA purification were used to clean up PCR products.

Gel Purification: DNA was run in an agarose gel until bands had well separated. A Safe Imager™ blue-light transilluminator was used to visualise the DNA while bands were cut out. DNA was extracted from the gel using silica bead DNA purification.

Silica bead DNA purification: The UltraClean™15 DNA Purification Kit and Roche Agarose Gel DNA extraction kit were used to purify DNA from agarose gel and PCR products. These kits utilise silica beads that bind DNA in the presence of a chemotropic salt. DNA bound to the silica is washed free of impurities and then resuspended in water or buffer. The kits were used as per the manufacturer's instructions. Purified DNA was eluted in TE buffer.

2.10 Sequencing

The initial RCBr DNA sequencing was performed at Canterbury Sequencing (School of Biological Sciences, University of Canterbury) with a capillary ABI3100 Genetic Analyzer from Applied Biosystems Inc., using a procedure based on the Sanger chain-termination protocol. Purified DNA (Section 2.9) was either sent directly for sequencing or the "BigDye Terminator" sequencing reaction was performed and the product sent for analysis.

The Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit was used to perform the sequencing reaction following the manufacturer's instructions. BigDye Master Mix (Section 5.2.9) was adjusted for template concentration and the extension temperature matched to specific primers. Sequencing product was purified through Sephadex resin (Appendix 5.2.10). Sephadex (500 µl per sample) was added to a Whatman Ultrafilter plate and spun at 750g, 5 min. Flow through was discarded and the plate spun at 750g, for 3 min. The sequencing sample was added to the column and spun through to a collection tube at 750g, for 5 min.

B. napus sequences were sent to Macrogen Inc, Korea (www.macrogen.com) for sequencing.

2.11 Quantitative reverse transcriptase polymerase chain reaction

Relative gene expression was measured using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). This reaction utilises SYBR green which binds double strand DNA and fluoresces allowing the quantification of double stranded DNA. The accumulation of the PCR product is measured in terms of Relative Florescence Units (RFU).

Reactions volumes of 20 µl were used, consisting of 7 µl nanopure water, 10 µl 2×SYBR green qRT-PCR reaction buffer (Section 5.2.11), 1 µl cDNA and 1 µl of both the forward and reverse primers. The thermo-cycle consisted of an initial heating step of 3 min at 95°C which was followed by 40 cycles of 95°C for 30 s, 53°C - 60°C for 15-30 s, 72°C for 15-30 s and 78°C-84°C for 30 s. Fluorescence was measured at 78°C-84°C to eliminate contaminating fluorescence from dimers. Following the amplification cycles a melting curve was generated by first heating the sample to 95°C for 1 min, 55°C for 30 s then raising the temperature to 95°C, measuring the fluorescence every 0.5°C. All reactions were carried out using a Stratagene Mxpro-Mx5005P Thermocycler. Quantitative reverse transcriptase PCR was used to measure gene expression of the individual family members across the various plant tissues as they developed. Primers were designed and tested for three reference genes (actin (*ACT*), elongation factor (*Elf1*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)), and each *IPT* and *CKX* family member that had been isolated. Products

for each primer pair were sequenced to confirm homology to genes already identified in various gene banks (e.g. NCBI) and the results included in Appendix 5.1.10, 5.1.11 and 5.1.12. Primers were then tested at different temperatures to determine maximum efficiency. Reference genes were used to normalise each cDNA sample so relative expression across all tissues could be calculated.

The data was analysed based on the methods of Pfaffl (2001) where the expression of target genes is calculated relative to the constitutively expressed reference genes (Le Bail et al. 2008; Gonzalez-Verdejo et al. 2008; Nicot et al. 2005; Kim et al. 2003). Threshold cycle (*Ct*) values were measured at 2000 fluorescent units. This fluorescence threshold was above the background noise levels and centrally located in the exponential amplification zone.

$$R = \frac{\left(E_{target}\right)^{\left(\overline{X}(C)_{target} - \overline{X}(S)_{target}\right)}}{\left(E_{refrence}\right)^{\left(\overline{X}(C)_{refrence} - \overline{X}(S)_{refrence}\right)}} \qquad 2$$

Where $\bar{x}(C)_{target}$ and $\bar{x}(C)_{refrence}$ are the average Ct values of the control for the target and reference genes, $\bar{x}(S)_{target}$ and $\bar{x}(S)_{refrence}$ are the average Ct values of the subject for the target and reference genes and *E* is the respective PCR efficiency for the target and reference genes.

2.12 Bioinformatics

The sequenced *Arabidopsis* genome provided template *IPT* and *CKX* genes to interrogate the National Center for Biotechnology Information (NCBI) database. The Basic Local Alignment Search Tool (BLAST) was used to search protein, nucleotide and EST databases for sequences homologous to the Arabidopsis sequences. 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 the Neighbour Joining and Maximum Parsimony methods.

2.13 Chlorophyll analysis

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

Chapter 3

Results

The sequencing of the *Arabidopsis* genome, completed in 2000, provided a genetic baseline for dicotyledonous plants. The Brassicaceae are closely related to *Arabidopsis* and so it was logical to use genes identified in the *Arabidopsis* genome as templates for the interrogation of gene banks. Protein, nucleotide and EST BLAST searches were performed for each target gene with the aim of identifying these genes in *B. rapa* and *B. napus*.

3.1 IPT gene identification

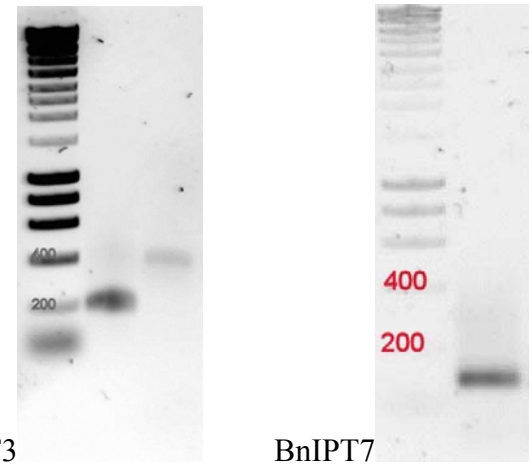
A thorough search of the NCBI sequence databases provided several EST and nucleotide sequences for several but not all *IPT* sequences. As mentioned in Chapter 1, previous studies have identified two *IPT* genes that appear to be directly involved in seed development in *Arabidopsis*, *IPT4* and *IPT8*. Initially these two genes were the major focus but at the time of writing no brassica EST or nucleotide sequences with close homology had been uploaded to NCBI. Other gene family member sequences were identified. Across both the *IPT* and *CKX* gene families there are conserved regions that can confirm a potential sequence as being a target, but not identify it specifically.

Initially degenerate PCR primers were designed using Primer Premiere™ 5.0. PCR products that were of the expected size were purified and sequenced. From these sequences more 'specific' primers were designed (Table 3.1)

Primer pairs were tested by running a reverse transcriptase PCR reaction and displaying the products on electrophoresis gels (Figure 3.1). Due to the tissue specific nature of the members of the *IPT* and *CKX* gene families, a mixture of cDNA from various tissues was used for primer design and PCR testing. In most instances a mixture of three stages of pods, two stages of leaves and two stages of flowers was used.

Table 3.1 IPT PCR primers

Gene	Primer name	
BrIPT1	BrIPT1F:	5'- CGATAAGGCGGTGGAGGAT
	BrIPT1R:	5'- CTGGAAAATGGCTGGGAAAT
BrIPT3	BrIPT3F:	5'- CAAGAAGTGGTGCATTCAGAGAT
	BrIPT3R:	5'- TTGAGTCCAAAAAAGTAGAAGATAATA
BrIPT5	BrIPT5F:	5'- AGAAAGAACATAACAAGAACCGA
	BrIPT5R:	5'- GATAACTCTAACCTAATCCACCGT
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



BnIPT1 BnIPT3 BnIPT7

Figure 3.1 PCR results *B. napus* IPT primers.

Reactions were performed with a Master mix of several cDNAs derived a mixture of three stages of pods, two stages of leaves and two stages of flowers. BrIPT1F/BrIPT1R (lane 2) produced a single band at the expected 270 bp. BrIPT3F/BrIPT3R (lane 3) produced a single band at the expected 490 bp. BnIPT7F/BnIPT7R (lane 4) produced a single band at the expected 180 bp.

The PCR products (Fig 3.1) were sequenced to confirm the identity of the target gene. The sequence results for *BrIPT1* (Appendix 5.1.1), *BrIPT3* (Appendix 5.1.2), *BrIPT5* (Appendix 5.1.3), *BnIPT1* (Appendix 5.2.1), *BnIPT3* (Appendix 5.2.2), *BnIPT5* (Appendix 5.2.3) and *BnIPT7* (Appendix 5.2.4) were aligned with *Arabidopsis* homologues.

3.2 CKX gene identification

An extensive search of the NCBI databank identified many CKX homologues in the protein, nucleotide and EST databases. Using these sequences a set of degenerate primers was designed for the *B. rapa* studies and later a set of more specific primers was developed for *B. napus* (Table 3.2).
The PCR products (Fig 3.2) were sequenced to confirm the identity of the target gene. The sequence results are presented in the Appendices for *BrCKX1* (Appendix 5.3.1), *CKX2/4* (Appendix 5.3.2), *CKX 3* (Appendix 5.3.3), *CKX5* (Appendix 5.3.4), *BnCKX1* (Appendix 5.4.1), *BnCKX2*(Appendix 5.4.2), *BnCKX3*(Appendix 5.4.3),*BnCKX5* (Appendix 5.4.4) *and BnCKX7* (Appendix 5.4.5).

Table 3.2 CKX PCR primers

Gene	Primer name	
BrCKX1	BrCKX1F:	5'- CTCTTGRACCAGCACCRCATA
	BrCKX1R:	5'- TGTGGAACCTCCCATAAACCCTT
BrCKX2/4	BrCKX24F:	5'- ACGTTATGGGTKGAYGTGCT
	BrCKX24R:	5'- TGATACYRTGTTGYTTCACTAAAT
BrCKX3	BrCKX3F:	5'- GGTTTCTTGGACGGAYTATTTG
	BrCKX3R:	5'- GRTGTCGTTTKAYCATTGAGRC
BrCKX5	BnCKX5F	5'- GTCTTGGYCARTTYGGTATCATCA
	BnCKX5R	5'- ATCCAYTCCTCTYGAGACTTGTA
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

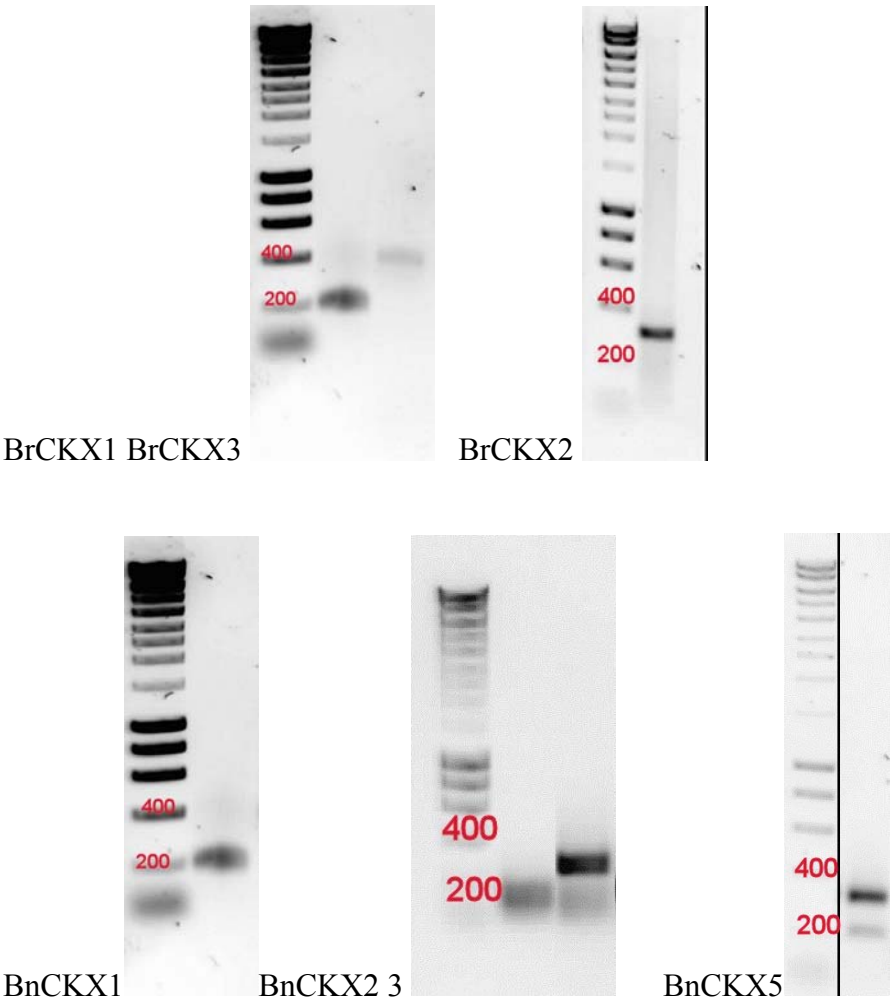


Figure 3.2 PCR results *B. rapa* and *B. napus CKX* gene family members. Reactions were performed with a Master mix of several cDNAs derived from a mixture of three stages of pods, two stages of leaves and two stages of flowers. BrCKX1F/BrCKX1R produced a single band at the expected 270 bp. BrCKX3F/BrCKX3R produced a single band at the expected 490 bp. BnCKX2F/BnCKX2R produced a single band at the expected 120 bp. BnCKX1F/BnCKX1R produced a single band at the expected 260 bp. BnCKX2F/BnCKX2R produced a single band at the expected 120 bp. BnCKX3F/BnCKX3R produced a single band at the expected 330 bp. BnCKX5F/BnCKX5R produced a single band at the expected 335 bp.

3.3 Reference genes

Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*): Using known GAPDH sequences as a template for a BLAST search of the NCBI database several homologous brassica EST's were found. Primers (Table 3.3) were designed from EST sequences, tested by PCR and sequenced. Sequencing results confirmed the specificity of the primers and the product as GAPDH.

Actin (*ACT*): As with GAPDH a search of the NCBI databases identified several EST's homologous to actin. Primers (Table 3.3) were designed using EST sequences and these were tested by PCR and sequenced. Products were confirmed to be actin.

Elongation Factor (Elf): A search of the NCBI databases identified several EST's homologous to Elf. Primers (Table 3.3) were designed using EST sequences and these were tested by PCR and sequenced. Products were confirmed to be Elf.

Table 3.3 PCR primers for the reference genes

Gene	Primer name	
Actin	BrACTF:	5'-CAGCAACTGGGACGACATGGA
	BrACTR:	5'-CAGCCTGRATRGCVACATACAT
GAPDH	BrGAPF	5'-CTAGTCGTACCGCTAACTGCCTT
	BrGAPR	5'-CRGTGCTGCTGGGAATGA
Actin	BnACTF:	5'-TGTGACAATGGAACTGGAATGGT
	BnACTR:	5'- ACGGAGGATAGCGTGAGGAAG
GAPDH	BnGAPF:	5'-GATCCCTTCATCACCACCGAGTA
	BnGAPR:	5'-GGGGAGCAAGGCAGTTAGTG
Elf1	BnELFF:	5'-AGGAGGCTGCTGAGATGAACAA
	BnELFR:	5'-CCATCTTGTTACAGCAGCAAATCA

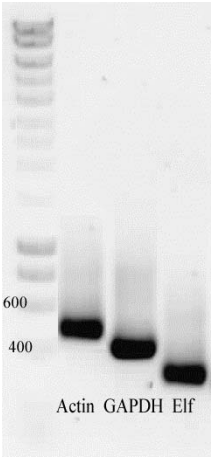


Figure 3.3 The PCR products for *actin, GAPDH* and *ELF*. All three reactions were performed with a Master mix of several cDNAs derived from a mixture of three stages of pods, two stages of leaves and two stages of flowers was used. the first of each tissue type (leaf, flower, pod).

PCR with actin primers produced a product at the expected size (530 bp). The *GAPDH* primers produced a product at the expected size (400 bp) and the *Elf* primers produced a product of the expected size (335 bp). All three were confirmed by sequencing the PCR products.

3.4 Phylogenetics

3.4.1 IPT Phylogeny

The phylogenetic relationship between the putative *B. rapa* and *B. napus IPT* genes and *Arabidopsis, O. sativa, Z. mays* and other selected *IPT* genes (Appendix 5.3) was determined using "Maximum Parsimony". Translated sequence data were aligned (Appendix 5.1.1-5.1.4 and 5.1.17-5.1.19) and a phylogenetic tree estimated using the Maximum Parsimony function in MEGA4 (Figure 3.4). Monocot and dicot *IPT* genes formed two distinct clades. *BrIPT1* and *BnIPT1* grouped together with *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. *BrIPT3 and BnIPT3* grouped together with *AtIPT3, GmIPT* and a published *BrIPT3* sequence (AB186133.1). *BrIPT5 and BnIPT5* grouped together with *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. *BrIPT3 and BnIPT3* are most closely related to the *BnIPT7* clade. As only short fragments, >500 bp, were used for each gene and the sequences are from different regions of the gene there may be little or no overlap. This has affected resolution of the phylogenetic tree. To ensure that this phylogenetic tree was a valid representation, the entire gene of the previously sequenced species eg. Arabidopsis were used. The results show that the fragments identified in sequencing are specific to the named gene family members and are valid for these gene expression studies. This was also required as the different gene family member segments identified during primer design were not all from the same location on the cDNA, making side by side comparison impossible.

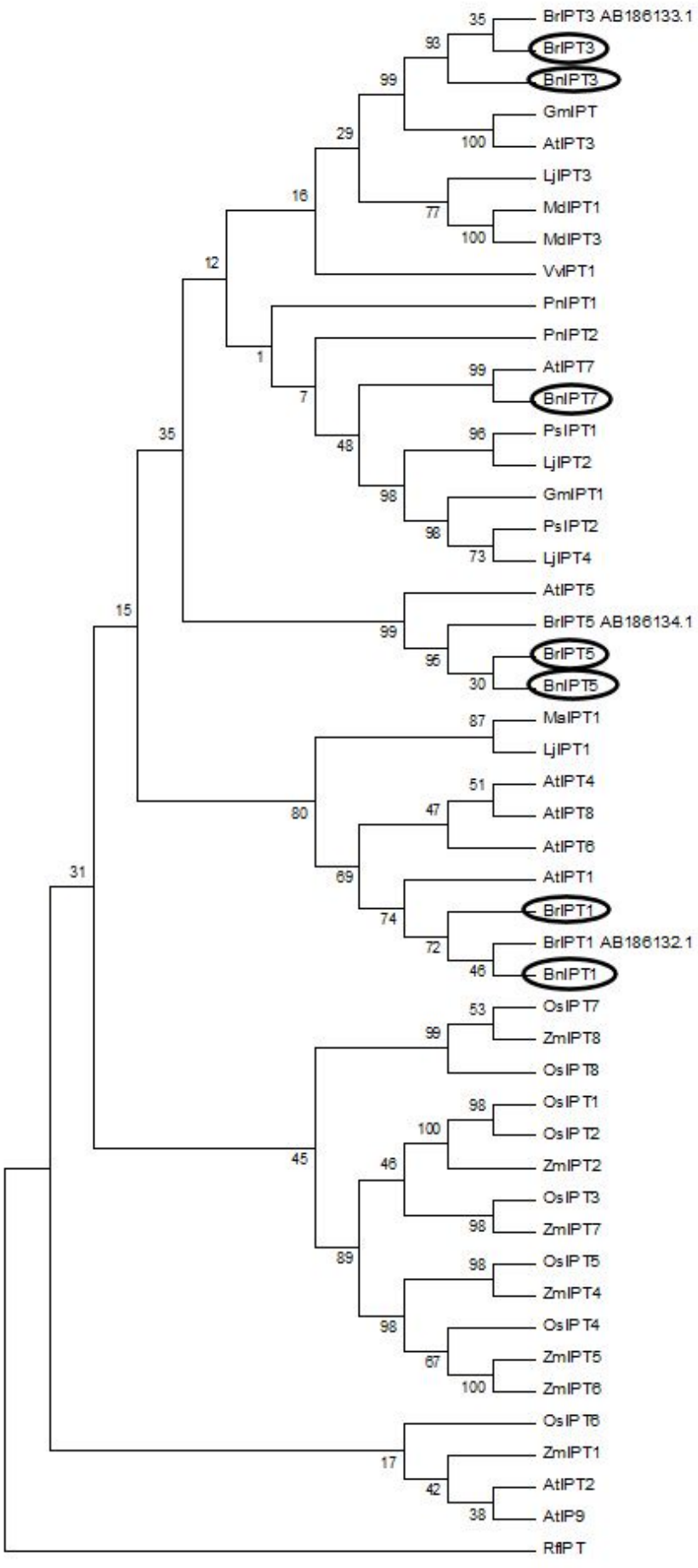


Figure 3.4

IPT Phylogenetic tree
Tree showing the relationship between *B. rapa* and *B. napus IPT* genes and the *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays* and various other *IPT* genes. Gene family members identified in this study are circled in black. Maximum parsimony tree was rooted with *Rhodococcus fascians IPT genes*. Bootstrap tree branch support values shown (10 000 bootstrap replications).

3.4.2 CKX Phylogeny

The phylogenetic relationship between the putative *B. rapa* and *B. napus CKX* genes and *Arabidopsis, O. sativa, Z. mays* and various other *CKX* genes (Appendix 5.4) was determined using "Maximum Parsimony". Translated gene sequences were aligned (Appendix 5.1.5-5.1.9 and 5.1.13-5.1.16) and a phylogenetic tree estimated using the Maximum Parsimony function in MEGA4 (Figure 3.5). *BrCKX1* and *BnCKX1* grouped with *AtCKX1*, *BoCKX1* (AB331918), *BrCKX1* (AB331924) along with many other CKX genes from various species. Br*CKX2* and *BnCKX2* grouped together with *AtCKX2* and *AtCKX4* and this group is very closely related to *BrCKX3, BnCKX3* and *AtCKX3*. *BrCKX5* may actually be *BrCKX7* as it is in the same clade as *BnCKX7* and *AtCKX7*. This is probably due to some confusion with sequences on NCBI. *BnCKX5* is in a clade of its own with *AtCKX5*. As with the IPT tree, only short fragments >500 bp were used for each gene, and as the sequences are from different regions of the gene there may be little or no overlap. This has affected resolution of the phylogenetic tree.

3.5 Gene expression

SYBR green detects any double stranded DNA by tightly binding to the minor groove of dsDNA and fluorescing brightly. Quantitative RT-PCR with SYBR in the reaction uses this fluorescence to measure the expression of genes. Primers were selected for qRT-PCR from the primers successfully used for sequencing gene fragments. The following primers were selected for the *B. rapa* target genes: *BrIPT1*: BrIPT1F1/BrIPT1R1; B*rIPT3*: BrIPT3F/BrIPT3R; *BrIPT5*: BrIPT5F/BrIPT5R (Table 3.1); *BrCKX1*: BrCKX1F/BrCKX1R; B*rCKX2*: BrCKX2F/BrCKX2R, *BrCKX5*: BrCKX5F/BrCKX5R (Table 3.2); and for the reference genes *GAPDH*: BrGAPF/BrGAPR; and *ACT*: BrACTF/BrACTR (Table 3.3).

The following primers were selected for the *B. napus* target genes: *BnIPT1*: BnIPT1F1/BnIPT1R1; B*nIPT3*: BnIPT3F/BnIPT3R; *BnIPT5*: BnIPT5F/BnIPT5R; *BnIPT7*: BnIPT7F/BnIPT7R (Table 3.2); *BnCKX1*: BnCKX1F/BnCKX1R; B*nCKX2*: BnCKX2F/BnCKX2R; B*nCKX3*: BnCKX3F/BnCKX3R; BnCKX5: BnCKX5F/BnCKX5R; B*nCKX7*: BnCKX7F/BnCKX7R (Table 3.3); and for the reference genes *GAP*: BnGAPF/BnGAPR; *Act*: BnACTF/BnACTR; and *Elf*: BnElfF/BnElfR (Table 3.4). The primers were then tested for their suitability for qRT-PCR with a range of cDNAs.

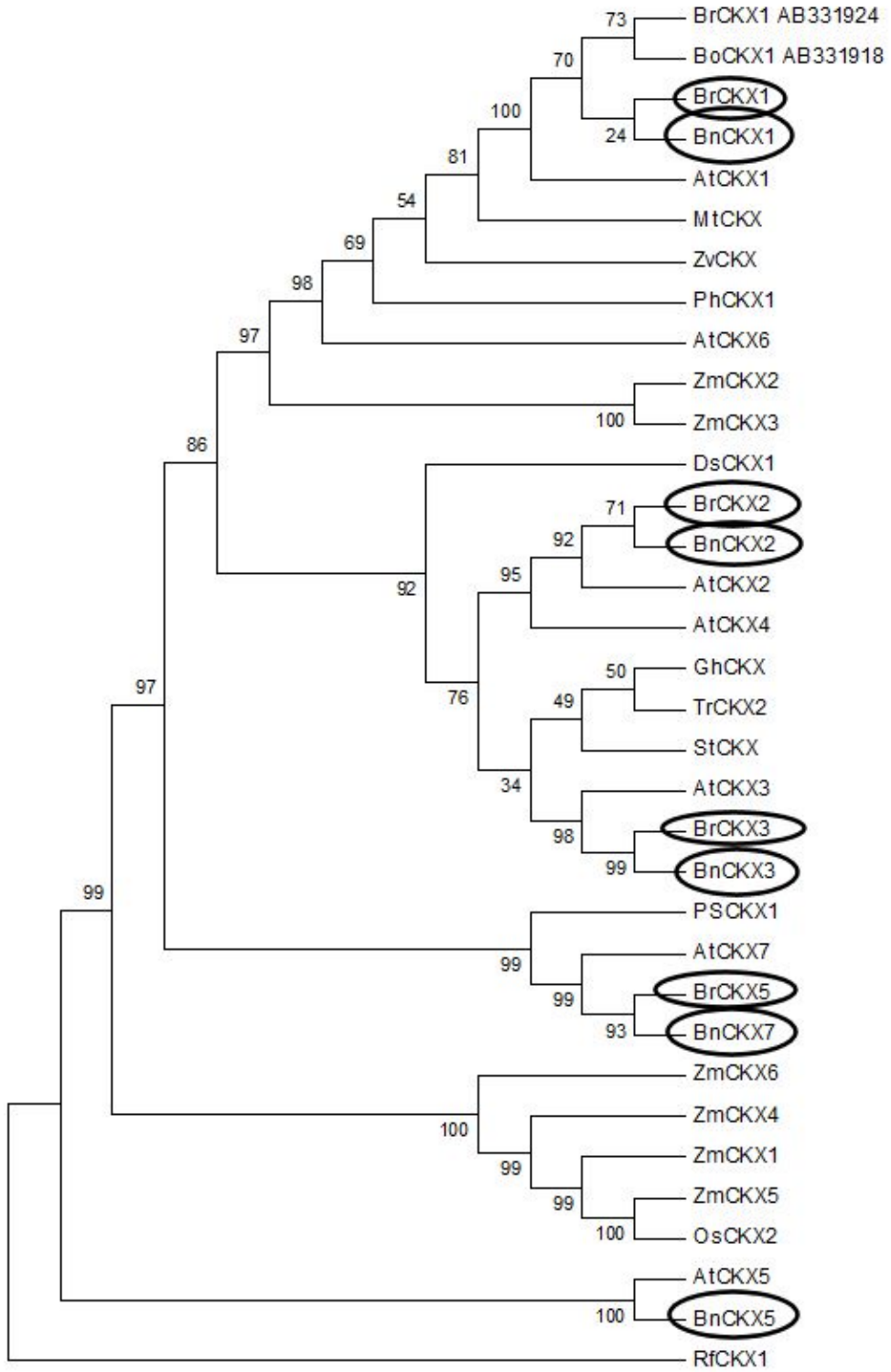


Figure 3.5 CKX Phylogenetic tree

Phylogenetic tree for *CKX* showing the relationship between *Brassica rapa* and *Brassica napus CKX* genes and *Arabidopsis thaliana* , *Zea mays* and legume *CKX* genes. Maximum parsimony tree was rooted with *Rhodococcus fascians CKX* gene. Bootstrap tree branch support values shown (10 000 bootstrap replications). Gene family members identified in this study are circled in black.

3.6 Optimising the qRT-PCR

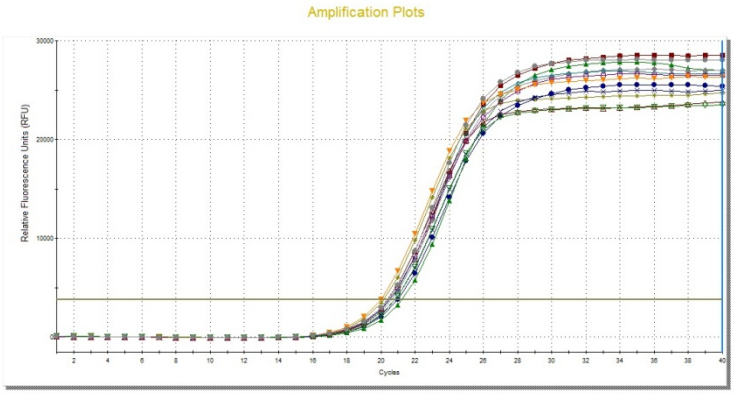
The specificity of PCR primers can be gauged by the melting curves. A single PCR product will display one sharp peak. The reference genes, *ACT*, *GAP* and *Elf,* typically produced Ct values of between 18 and 22 (Figure 3.6, Figure 3.7 and Figure 3.8). The melting curves for *ACT* (Figure 3.6), *GAP* (Figure 3.7) and *Elf* (Figure 3.8) are shown. PCR products produced sharp peaks at 85°C, 84°C and 85°C respectively.

A dissociation curve that contains multiple peaks or irregular melting indicates either non-specific products and/or primer dimers. For the most part primers that displayed these traits were not used in these expression studies. Melting curves for the target gene PCR product produced mixed results with some reactions producing multiple or irregular peaks. Notably *BnIPT5* (Figure 5.5.2) produced double peaks with some cDNAs. To avoid these products the read temperature was set to 81°C during the PCR run. This ensured that the dimers were not included in the results.

Following on from the initial primer testing, qRT-PCR was performed with specific tissue cDNAs to measure expression throughout plant growth and pod and seed development. Ct values for the reference genes *ACT, GAP* and *Elf* varied due to the ease of RNA extraction and amount of RNA collected. In later stages of pod development, the amount of RNA able to be collected diminished, possibly due to high levels of starch. In later stages of leaf development the levels of RNA deceased as well. The highest quantities of RNA came from young leaves and pods. To enable the Ct values to be closely aligned, the amount of RNA used for the cDNA synthesis reaction was measured using the Nanodrop and each cDNA reaction used ca.1ug RNA.

Serial dilutions of template DNA were performed to determine the primer specific PCR efficiencies and to test the range of template concentration each qRT-PCR assay could detect. These results are unavailable due to loss of the hard drive on the Stratagene computer after it fell off the lab bench during the September 2010 7.1 earthquake.

(A)



(B)

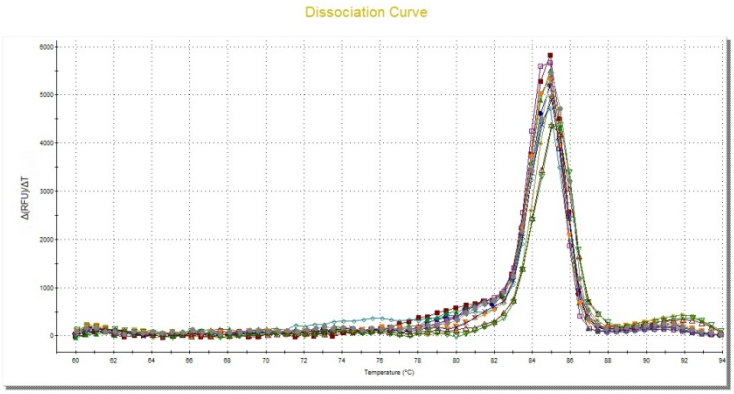
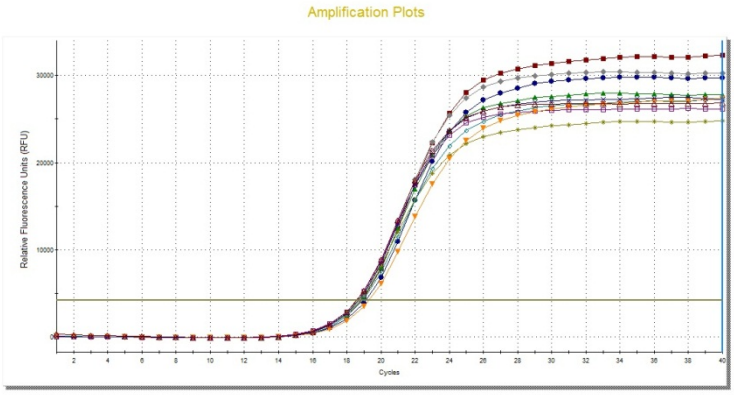


Figure 3.6 ACT amplification plot

ACT amplification plot (A) and melting curve (B). qPCR performed with leaf and flower cDNAs and the *ACT* primers BnACTF/BnACTR.

(A)



(B)

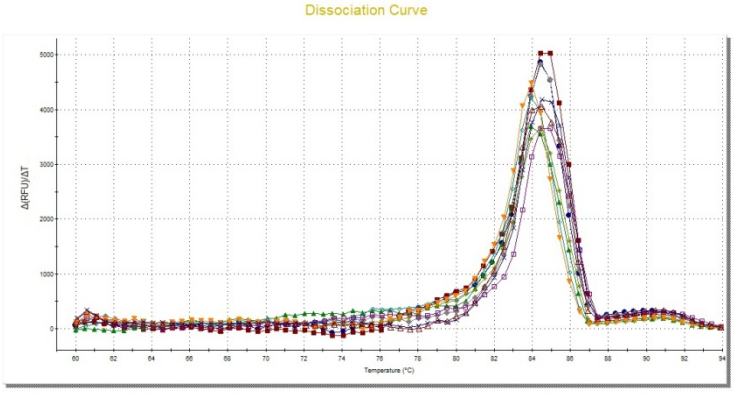
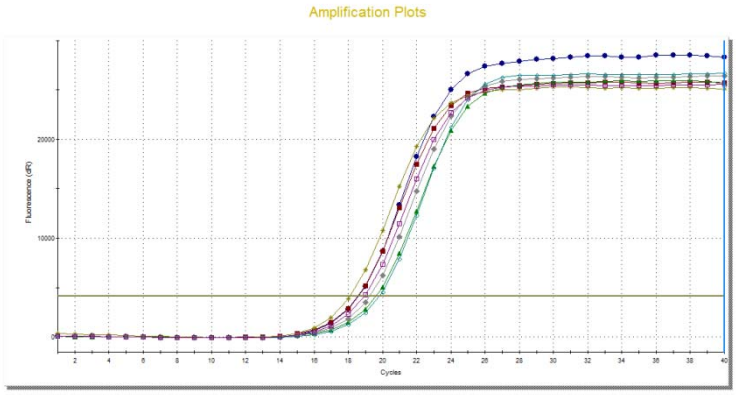


Figure 3.7 GAP amplification plot

GAP amplification plot (A) and melting curve (B). qPCR performed with leaf and flower cDNAs and the *GAP* primers BnGAPF/BnGAPR.

(A)



(B)

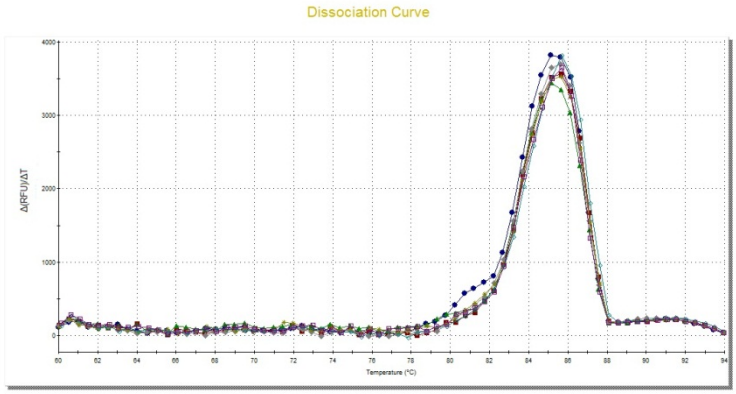
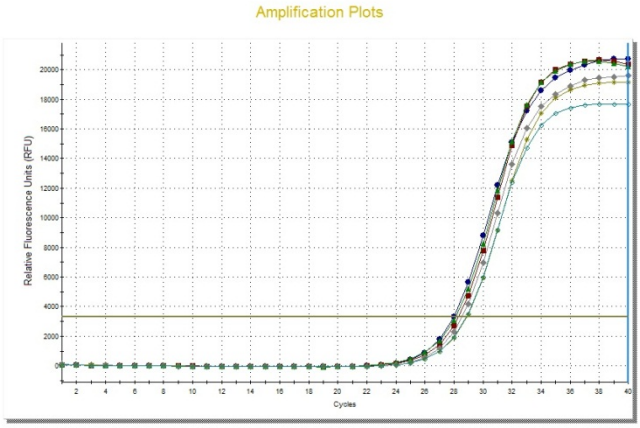
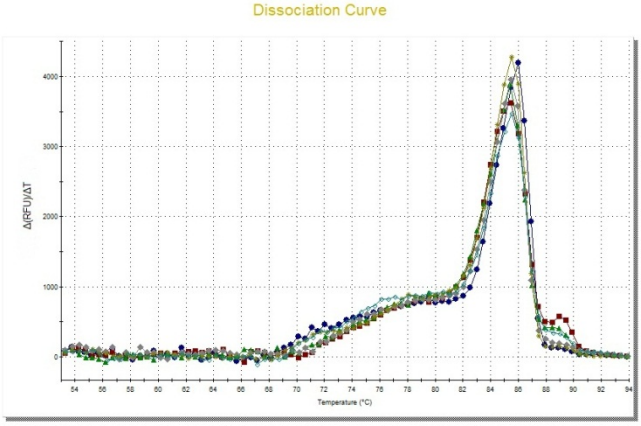


Figure 3.8 Elf amplification plot

Elf amplification plot (A) and melting curve (B), qPCR performed with leaf and flower cDNAs and the *Elf* primers BnELFF/BnELFR.



(A)



(B)

Figure 3.9 BnIPT1 amplification plot
BnIPT1 amplification plot (A) and melting curve (B), qPCR performed with leaf and flower cDNAs and the *BnIPT1* primers *BnIPT1F*/ *BnIPT1R*.

3.7 Expression of *IPT* and *CKX* gene family members during development

Relative expression was determined for each identified gene family member across each tissue type. *BrIPT1* was most highly expressed in maturing pods and mature leaves (Figure 3.10), but less so in young developing pods. *BrIPT1* showed similar levels of expression in the first two stages of flowers, early leaves, roots and stems. The third stage of flowers, the second stage of leaves and early pods showed the lowest relative levels of *BrIPT1* expression. *BrIPT3* expressed highly in the oldest leaves relative to expression in all other tissues (Figure 3.10). Expression of *BrIPT3* was apparent in pods from 2 DAP through to 14 DAP. *BrIPT3* showed relatively lower levels of expression in other tissues. *BrIPT5* was most highly expressed in roots (Figure 3.10). Expression is also apparent in late

stage pods 7 and 14 DAP. *BrIPT5* showed very low relative expression in all stages of flowers, middle stages of leaves and stem tissue.

Seed were separated from pod cases and the tissues analysed separately. Both *BrIPT3* and *BrIPT5* show marked increases in expression at 14DAP. *BrIPT3* expression increases dramatically in seeds relative to the pod case (Figure 3.11). On the other hand, *BrIPT1* expression is at a much lower level relative to the other two gene family members in seeds relative to pod cases at 14 DAP (Figure 3.10).

In *B. napus*, *BnIPT1* showed minimal expression in flowers or leaves (Figure 3.12); low expression was apparent in mid stages of pod development (Figure 3.12). *BnIPT3* expression increased during leaf development and was most highly expressed in mature leaves (Leaf 3) (Figure 3.12). There was a spike of *BnIPT3* expression at Pod 4 (Figure 3.12) but otherwise relative expression was low in all other tissures. *BnIPT7* expression increased markedly during the second stage of flower devlopment (Flower 2) (Figure 3.12). There was also a gradual increase in expression during pod development, peaking at Pod4 and then declining to Pod7 (Figure 3.12). Leaves showed a very low level of *IPT7* expression.

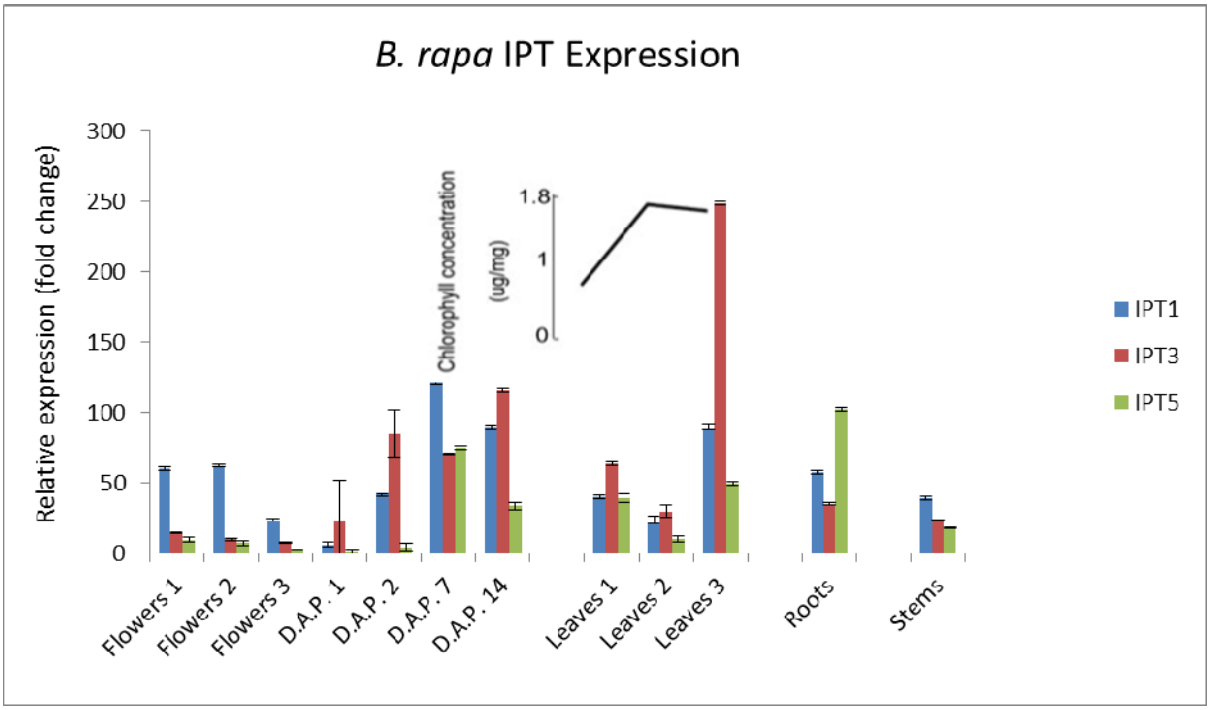


Figure 3.10 ***B. rapa*** **qRT-PCR gene expression IPT gene family members** from buds and flowers (flowers 1, 2, and 3, depicting bud to fully open flower); pods from 1-, 2-, 7-, and 14-DAP; leaves (leaves 1, 2, and 3, (depicting youngest to oldest); root tips and stems. Bars show ±SD of the mean of two biological and three technical replicates based on corrected CT number

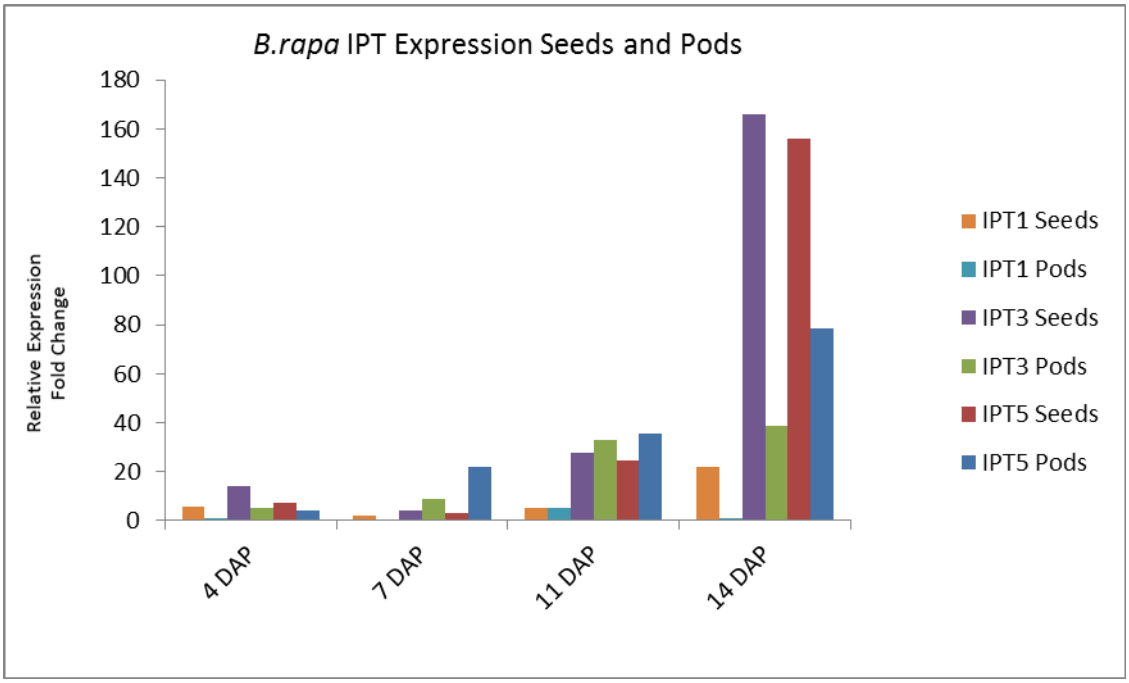


Figure 3.11 qRT-PCR gene expression of IPT gene family members in pod cases and whole seeds. Pod cases and whole seeds, 4, 7, 11, and 14 DAP.

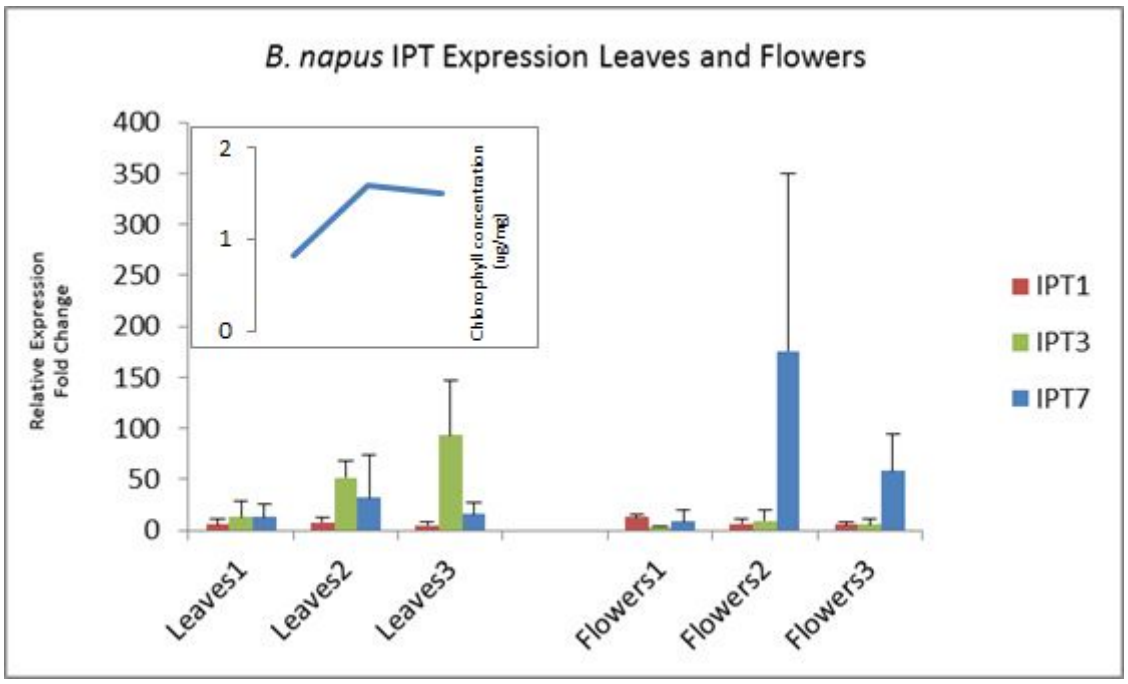


Figure 3.12 B.napus qRT-PCR gene expression IPT gene family members from leaves and flowers. Leaves (leaves 1, 2, and 3) (depicting youngest to oldest) and flowers (flowers 1, 2, and 3, depicting bud to fully open flower). Bars show SD of the mean of two biological and three technical replicates based on corrected CT number.

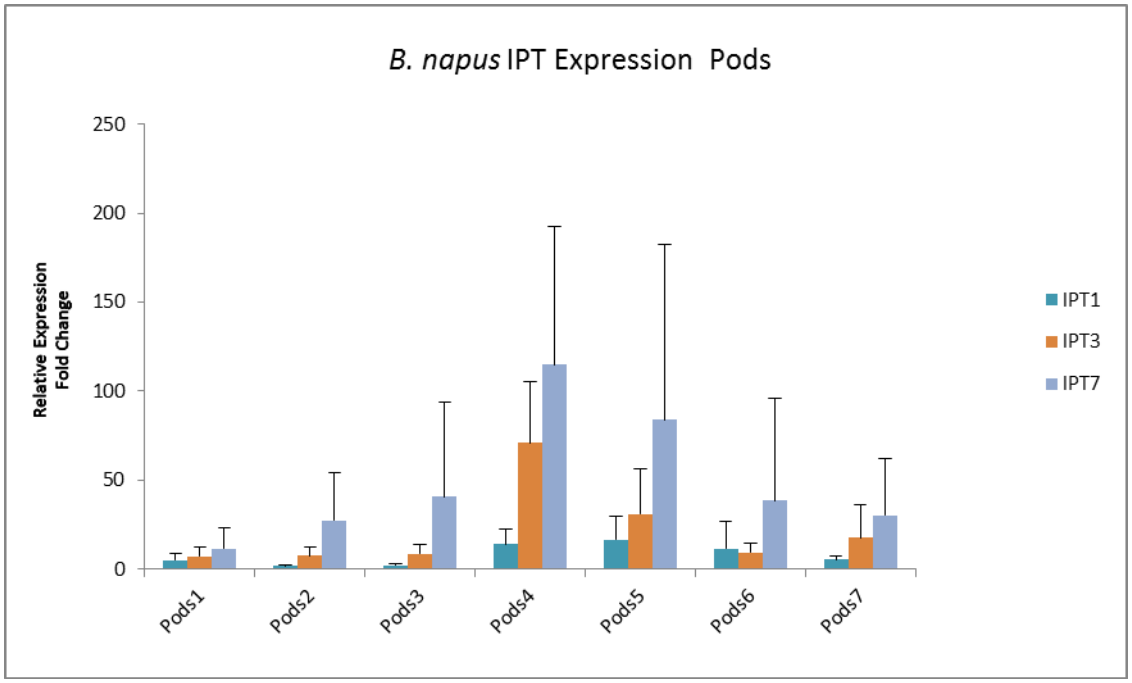


Figure 3.13 B.napus qRT-PCR gene expression IPT gene family members Pods (1, 2, 3, 4, 5, 6 and 7 depicting earliest pods through to earliest fertilised pods) Bars show SD of the mean of two biological and three technical replicates based on corrected CT number.

BrCKX1 expression was highest in young leaves. *BrCKX1*displayed a very low level of relative expression in flowers but expression started increasing at 2 DAP to peak at 7 DAP (Figure 3.14). This expression was at a similar level to expression older leaves. *BrCKX2* gene expression was highest in 7 and 14 DAP pods (Figure 3.14). There was also a high level of expression in young leaves and stems. Expression was relatively lower in flowers and stage 2 and 3 leaves.

BrCKX3 showed increased levels of relative expression in pods 7 DAP and early and late stage leaves (Figure 3.14). In all other tissues studied *BrCKX3* showed lower expression relative to these three tissue stages. *BrCKX5* was shown to express very highly in early and late stages of leaf development (Figure 3.14). In all other tissues studied *BrCKX5* showed low levels of expression relative to these tissue stages.

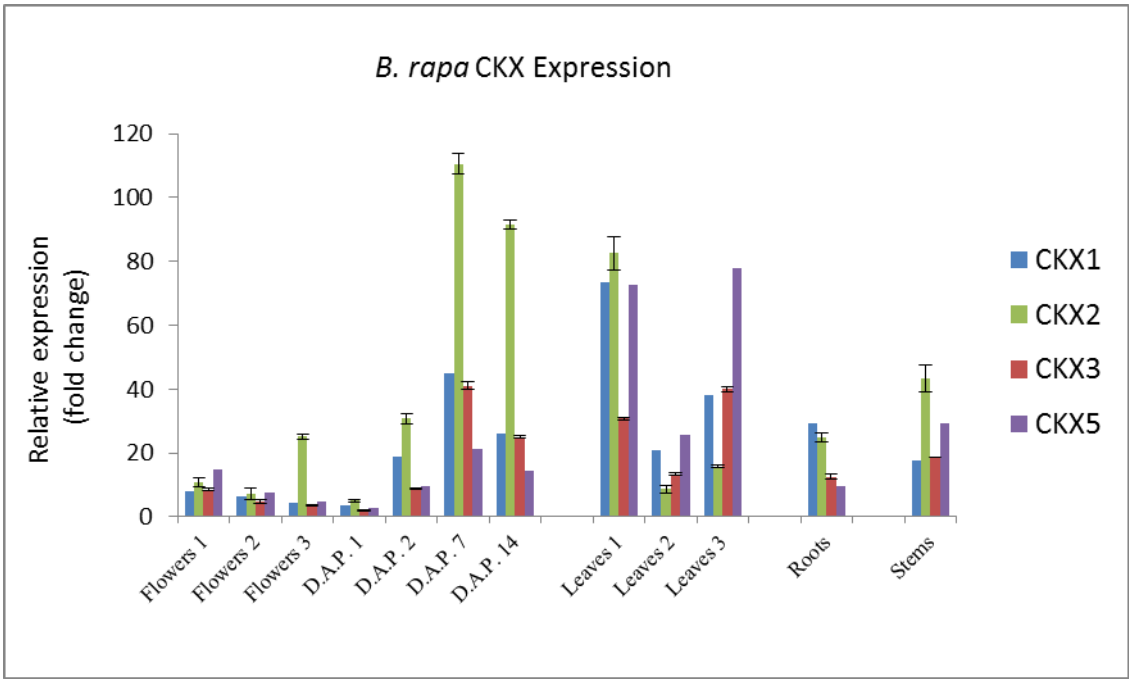


Figure 3.14 *B. rapa* qRT-PCR gene expression of CKX gene family members from buds and flowers (flowers 1, 2, and 3, depicting bud to fully open flower); pods from 1-, 2-, 7-, and 14-DAP; leaves (leaves 1, 2, and 3, depicting youngest to oldest); root tips and stems. Bars show ±SD of the mean of two biological and three technical replicates based on corrected CT number.

When seed was separated from pod cases and the tissues analysed separately, *BrCKX2* showed a more than 200 times increase in expression in seeds relative to pods by 14 DAP (Figure 3.15). In contrast, *BrCKX1, 3* and *5* all showed very little difference in relative expression throughout seed and pod development. There is a slight elevation in expression in seeds 14 DAP (Figure 3.15).

In *B. napus* plants, *BnCKX1* expression in leaves and flowers was highest in leaves 2 with expression in flowers being very low (Figure 3.16). *BnCKX1* expression increased slowly during pod development to show a peak at Pod 5 then expression dropped back after this stage (Figure 3.17). *BnCKX2* showed a marked increase in expression during flower development, this peaked at several hundred times the amount of gene product relative to the expression in leaves (Figure 3.16). A gradual increase was shown in expression of *CKX2* to Pod 5 which was then maintained throughout pod development (Figure 3.17). *BnCKX5* expression showed a rise during pod development but relative expression was low across all tissues (Figures 3.16 and 3.17). *BnCKX7* showed a slight increase in expression in Pod 5 relative to the other pod stages (Figure 3.17). Expression in leaves and flowers was very similar with all tissues showing a very low relative expression (Figure 3.16).

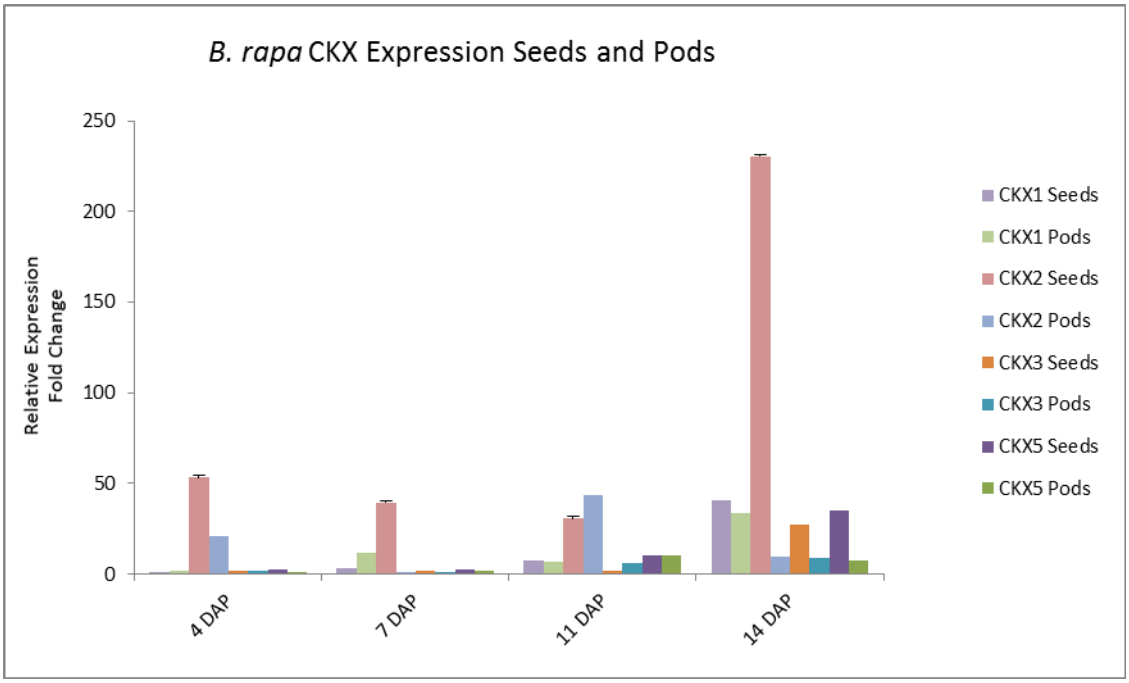


Figure 3.15 RT-qPCR gene expression of CKX gene family members extracted from pod cases and whole seeds. Pod cases and whole seeds 4, 7, 11, and 14 DAP. Bars show +SD of the mean of two biological and three technical replicates based on corrected CT number.

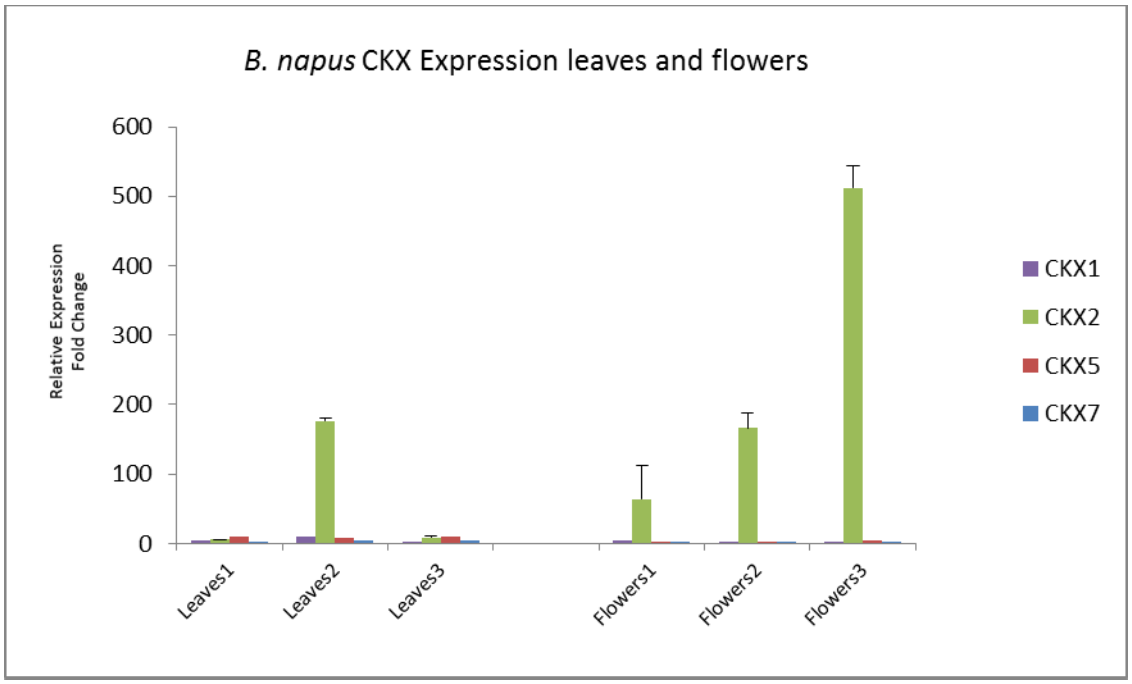


Figure 3.16 B.napus RT-qPCR gene expression CKX gene family members from leaves and flower. Leaves (leaves 1, 2, and 3, (depicting youngest to oldest) and flowers (flowers 1, 2, and 3, depicting bud to fully open flower). Bars show SD of the mean of two biological and three technical replicates based on corrected CT number.

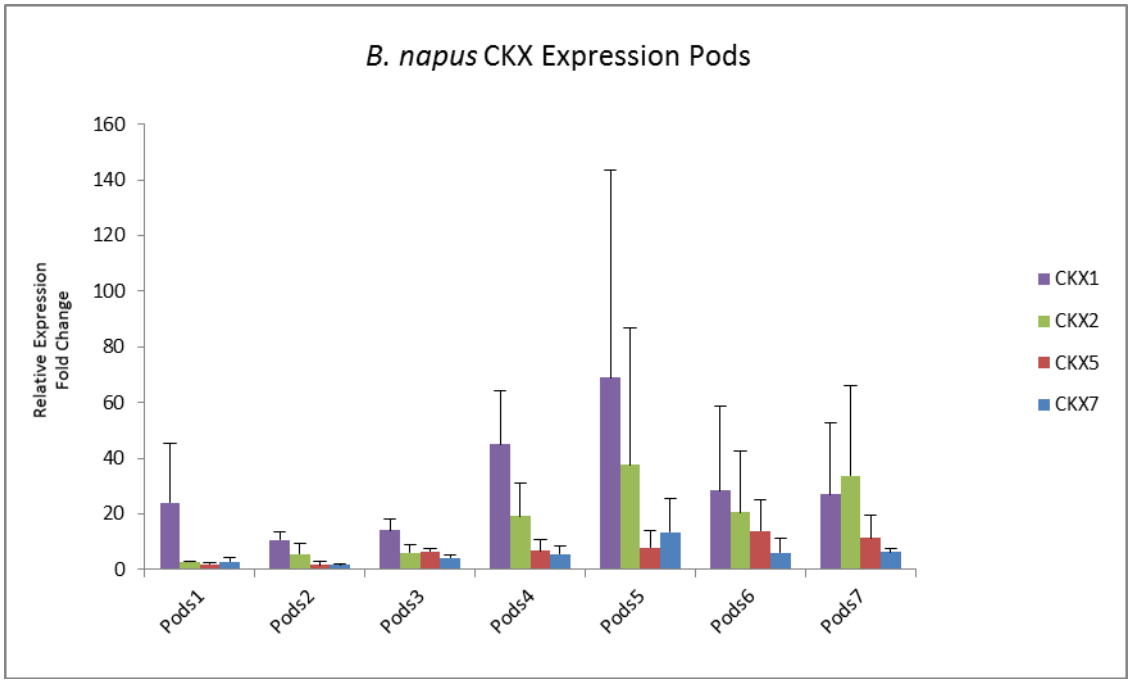


Figure 3.17 RT-qPCR gene expression of B. napus CKX gene family members from Pods (1, 2, 3, 4, 5, 6 and 7 depicting earliest pods through to earliest fertilised pods) Bars show SD of the mean of two biological and three technical replicates based on corrected CT number.

Chlorophyll readings (Figure 3.10 and 3.12) show that mid stage leaves contained the highest levels compared to growing young leaves and the start of senescence where nutrients are mobilised away from the older leaves (Buchanan-Woolaston).

Chapter 4

4.1 Discussion

The initial aim of this thesis was to isolate IPT and CKX gene family members from *B. rapa* and *B. napus.* The second aim was to determine if any of the putative *IPT* and /or *CKX* gene family members expressed specifically in developing seeds.

The phylogenetic tree (Figure 3.4) shows four major clades of *IPT* genes. 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, AtIPT5* and *AtIPT7.* 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 *IPT's, ZmIPT1* and *OsIPT6.*

The Brassica gene family members showed the expected close relatedness to *Arabidopsis*. BrIPT1 (Appendix 5.1.17) and BnIPT1 (Appendix 5.1.1) show close homology to *AtIPT1* (Figure 3.4) and a previously identified BrIPT1 (AB186132.1). In both sets of experiments, *BnIPT1* and *BrIPT1* expression occurred in most tissues, but with minimal expression in pods and only a low level in developing seeeds relative to other IPT family members. Using Rt-PCR, Miyawaki et al. (2004) reported *AtIPT1* to be abundant in flowers, siliques and roots, with the rest of the clade (AtIPT4, AtIPT6 and AtIPT8) expressed "almost exclusively" in floral buds, open flowers and siliques. As introduced in Section 1.8, AtIPT4 and AtIPT8::GUS transformants were expressed in immature seeds with highest expression in the chalazal endosperm (Miyawaki et al., 2004). These previous studies suggest that this clade should be the starting point of any study focusing on seed development. AtIPT4 and AtIPT8 show very close homology to AtIPT1 (Figure 3.4), which may have impeded actual discovery with the mixture of tissues used in the initial primer design stage. As discussed in Miyawaki et al. (2004) AtIPT8 had a slightly more extensive expression pattern in the endosperm and this persisted until late heart stage, whereas AtIPT4 was very specific to the chalazal cyst.The fact that these two gene family members had been shown to be so specific to seed development means that further efforts should be made to isolate them. Interestingly, Takei et al. (2004) show *AtIPT6* expressing in siliques, with only minimal expression of 4, or 8 detected using qRT-PCR.

BrIPT3 (Appendix 5.1.18) and *BnIPT3* (Appendix 5.1.2) show a close homology to *AtIPT3* and several other dicot IPTs (Figure 3.4). *AtIPT3* sits in a dicot clade with *AtIPT7* and *AtIPT5*. These gene family members had not been specifically implicated in seed development by Miyawaki et al., (2004) but low levels of *AtIPT3*, *5*, and *7* expression were shown by Takei et al. (2004) in siliques. However, it is clear that in both *B. rapa* and *B. napus* that *IPT3* was expressed during pod development. This was confirmed when pod walls and seed were extracted separately – *BrIPT3* showed strong expression in the developing seed.

AtIPT3 and *AtIPT7* have been reported to be present in "diverse tissues" and *AtIPT5* was found to be abundant in roots and rosette leaves (Miyawaki et al. 2004). Takei et al. (2004) showed *AtIPT3* to be strongly expressed in both rosette and cauline leaves. In this study, in both *B. rapa* 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.

BrIPT5 (Appendix 5.1.19) *and BnIPT5* (Appendix 5.1.3) also sit in the second clade. *BnIPT5* was identified and sequenced but due to an earthquake-related failure of the Stratagene qPCR machine the plates could not be run. *BrIPT5* was most highly expressed in roots and developing pods but showed very low relative expression in all stages of flowers, middle stages of leaves and stem tissue. This result is in accordance with other studies. Miyawaki et al., (2004) reported that *AtIPT5* was abundant in roots and rosette leaves of young plants. However, when pods and seeds were separated it is clear that *BrIPT5* was highly expressed in the developing seed. As it is also expressed in the roots, as shown also for *AtIPT5* by Takei et al. (2004), selection for increased expression of this gene family member may reveal plants with reduced root development.

BnIPT7 (Appendix 5.1.4) was also in the second clade. As *AtIPT7* had been shown to be expressed in many tissues (Miyawaki et al., 2004) this probably contributed to the relative ease which *BnIPT7* was isolated. *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 *IPT7* expression. Unfortunately pods and seeds were not separated and so the specificity of this expression has yet to be determined.

Overall, there was low expression of the identified *IPT* gene family members in developing pods, suggesting that maternally supplied cytokinin may be required during early pod development. None of *IPT1, 3, or 5* expressed specifically in developing pods and/or seeds, so may not be ideal targets for breeding. As mentioned above, further effort is needed to isolate *IPT4* and *8*, as well as further work with *IPT7*.

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 the focus of this study. The phylogenetic tree shows that *CKX* genes are more divergent than the *IPT* genes. Recent phylogenetic, molecular and comparative analyses show that local gene duplication and an ancient whole genome duplication event can be used to explain the groupings of the CKX gene family members (Mameaux et al 2012). Bae et al. (2008) compared the crystal structure of two unrelated CKX proteins and found that while ZmCKX1 and AtCKX7 shared only 39.4% amino acid identity, their structures and active sites were highly conserved. As with the IPT genes, the Brassica CKX genes show very close homology to their Arabidopsis counterparts.

BrCKX1 (Appendix 5.1.13) and *BnCKX1* (Appendix 5.1.5) are closely related to *AtCKX1* and two previously sequenced Brassica *BrCKX1* and *BoCKX1* (AB331924 and AB331918). Although a *BrCKX1* has already been sequenced it was slightly different to the sequence obtained in these results. There could be a number of reasons for the difference. The RCBr have been artificially selected for specific criteria and this may be a bi-product of that process. Irrespective of this variation, BrCKXI and BnCKX1 would be placed in Clade I of the six *CKX* clades (Gu et al. 2010). Werner et al. (2003) showed *AtCKX1*:*GUS* to be expressed in the shoot apex, in young floral tissues and in roots, but clearly*BrCKX1* is strongly expressed in young leaves and mature leaves and, at least at some level, in most tissues, including roots. Marmeaux et al. (2012) data ndicates *AtCKX1* expression is highest in roots and mature leaves.

BrCKX2 (Appendix 5.1.14) and *BnCKX2* (Appendix 5.1.6) show very close homology to *AtCKX2* and also *AtCKX4,* so are placed in Clade III (Gu et al. 2010). In the *B. rapa* study *BrCKX2* was shown to be highly expressed in developing seeds. In the *B. napus* study *BnCKX2* increased during pod development but independent seed analysis has yet to be done. Previously, *AtCKX2*:*GUS* expression

was observed in the shoot apex, stipules , and in the apical stem of flowering plants (Werner et al. 2003), while Gu et al. (2010) suggest *AtCKX2* is most highly expressed in male tissues.

BrCKX3 (Appendix 5.1.15) showed a very close homology to *BnCKX3* (Appendix 5.1.7) and *AtIPT3,* placing it in Clade III. *BrCKX3* showed increased relative expression in 7 DAP pods and early and late stage leaves (Figure 3.20). In all other tissues studied, *BrCKX3* showed very low levels of expression relative to these three tissue stages. Developmental expression of *BnCKX3* was not studied due to earthquake-induced machine failure. Heterologous expression of*AtCKX3::GUS* in tobacco (Galis et al 2005) showed GUS activity in leaf margins, in root primordial and strongly in pollen. Gu et al. (2010) also showed strong expression of *AtIPT3* in male tissues.

In the phylogenetic analysis, *BrCKX5* (Appendix 5.1.16) actually located close to *BnCKX7* (Appendix 5.1.9) and *AtCKX7* and may well have been misidentified. In the published paper (O'Keefe et al. 2011) it was annotated as *BrITP5/7. BnCKX5* (Appendix 5.1.8) was very similar to AtCKX5. *BrCKX5/7* was shown to express very highly in early and late stages of leaf development while *BnCKX5* showed a small rise during pod development but expression was relatively low across all tissues.Werner etal. (2003) showed *AtCKX5*:*GUS* expression to be localised at the very base of the youngest emerging leaves, marking the developing leaf petiole, and after bolting in the rib zone of the axillary meristems. In flowers, expression was detected in developing stamen and in ripening pollen grains. In roots, expression was confined to the vascular cylinder within the apical meristem. Expression was strongest in the vascular initials directly adjacent to the quiescent center. The earliest expression in lateral root primordia was detected at the start of vascular proliferation. Gu et al. (2010) showed high expresssion of *AtCKX5*in mature leaves and reproductive tissues.

BnCKX7 (5.1.9) showed closed homology to *AtCKX7* and locates to Clade VI. While it showed a marked increase in relative expression in Pod 5 compared to the other pod stages, its expression was insignificant compared to other gene family members. Expression in leaves and flowers was very similar, with all tissues showing a very low relative expression (Figure 3.22). Expression analysis of *AtCKX7* does not appear to have been carried out in other labs. However, according to Mameaux et al. (2010) other Clade VI members express preferentially in reproductive organs.

Overall, the *Brassic*a CKX genes expressed in a variety of tissues which is in line with other species. In maize, Vyroubalová et al. (2009), used qRT-PCR to show the expression of 12 *ZmCKX* family members in various tissues: *ZmCKX1*, 2, 3, 6 and 10 were the most widely expressed across tissue types, with *ZmCKX1* noticeably higher in the embryo, *ZmCKX2* particularly high in mature leaves, *ZmCKX2* in roots and *ZmCKX10* especially in silks and tassles. Brugière et al. (2003) had

earlier shown an increasing expression of *ZmCKX1* during kernel development. *OsCKX2 (*Ashikari et al. 2005), and *ZmCKX1* (Brugière et al. 2003), are both located in Clade IV and are gene family members closely associated with grain/kernel development. To date, the most active gene family member detected during *B. rapa* seed development is *BrCKX2*, which is probably located in Clade III. Mameaux et al. (2010) suggest that Clade III and VI family members preferentially expressed in reproductive tissues.

Plant breeders aim to develop plant varieties which display useful agronomic characteristics. But traditional or conventional methods of breeding are long, involved and reasonably hit and miss. This is where it is possible to combine the latest molecular techniques with the traditional plant breeders. When the genes of interest, such as *BrCKX2*, have been identified, the objective is to alter the expression of that gene family member to achieve the desired end-point – in this case increased yield and/or seed quality. For example, Ashikari (2005) located rice mutants that produced more seed, and showed that there were mutations in *OsCKX2,* whereas Zalewski et al. (2010) used RNAi to decrease expression of HvCKX1 in barley. There are several methods available to suppress expression of a gene of interest. Historically mutagenesis was one method used to enforce changes in genetic makeup and breeding lines were screened for mutants of interest. This work was very laborious and very costly in time and space. Today, with more advanced molecular genetic tools available, it is possible to target the specific gene at the mRNA level and either disable gene expression using RNAi, or utilise TILLING (Targeting Local Lesions in Genomes) to identify changes in the genome brought about through chemical or ionising mutagenesis.

4.1 Conclusions

The RT-qPCR data presented here involved simultaneous monitoring of three members of the *BrIPT* gene family and four members of the *BrCKX* gene family. The data presented support suggestions that CKX plays a major role in maintaining cytokinin homeostasis during seed development and that down regulation of *CKX*, specifically in developing seeds, would appear to be a logical next target for improvement of seed storage reserves in brassica species. The data also support early metabolism studies that point to the necessity for seed-based biosynthesis of cytokinins, but possibly some dependence of the maternal tissue on cytokinin supplied from elsewhere in the plant because there is minimal expression, at least of *BrIPT1, -3,* and *5*, at the very early stages of pod development.

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Appendix

5.1 Sequence Data

5.1.1 BnIPT1 aligned with PCR primers

```
IPT1
IPT1                -------------TACTCCGACAAAATCCAAGTCTACGAAGGACTAGAGATCACCACCAACCAGATTACCATCCCGGA
AtIPT1_NM_105517.1  -TCAGAGATCATAAACTCCGATAAAATCCAAGTCTACGAAGGATTAGAGATCACAACGAATCAGATTACGTTACAAGA
BrIPT1_AB186132.1   TTCAGAGATCATAAACTCCGACAAAATCCAAGTCTACGAAGGACTGGAGATCACCACCAACCAGATTACCATCCCCGA
FPrimer             --------------------------------------------------------------------------------
IPT1a               --------------------------------------------------------------------------------
RPrimer             --------------------------------------------------------------------------------

IPT1                CCGTCGCGGCGTTCCTCACCACCTCCTCGGTTATCTCCCCTCCGAAGACGGCGAACTCACCGCCGGAGATTTTCGTGT
AtIPT1_NM_105517.1  CCGTCGCGGCGTTCCTCACCATCTCCTCGGCGTCATCAACCCCGAACACGGCGAACTAACCGCCGGAGAGTTTCGCTC
BrIPT1_AB186132.1   CCGTCGCGGCGTTCCTCACCACCTCCTCGGTTACCTCCCCTCCGAAGACGGCGAACTCACCGCTGGAGATTTTCGTGC
FPrimer             ------------------------------------------------------------GAGATTTTCGTGT
IPT1a               ------------------------------------------------------------GAGATTTTCGTGT
RPrimer             --------------------------------------------------------------------------------

IPT1                AGACGCTTCAAACGCCGTTTCCGACATAATCTCCCGTAAAAAGCTTCCGATCATCGCCGGCGGATCCAACTCCTTCGT
AtIPT1_NM_105517.1  CGCCGCTTCAAACGTCGTCAAAGAGATAACTTCTCGTCAAAAGGTTCCGATTATCGCCGGTGGATCTAACTCTTTCGT
BrIPT1_AB186132.1   AGACGCTTCAAACGCCGTTTCCGACATAATCTCCCGTAAAAAGCTTCCGATCATCGCCGGCGGATCCAACTCCTTCGT
FPrimer             AGACGCTTC-------------------------------------------------------------------
IPT1a               AGACGCTTCAAACGCCGTTTCCGACATAATCTCCCGTAAAAAGCTTCCGATCATCGCCGGCGGATCCAACTCCTTCGT
RPrimer             --------------------------------------------------------------------------------

IPT1                CCACGCGCTCCTCGCCGAGAGATTCGACCCAAAGATCGATCCTTTCTC------GTCTTCTTCGATATGTCGGGATTT
AtIPT1_NM_105517.1  CCACGCACTCTTAGCTCAACGATTCGACCCAAAGTTCGATCCTTTTTCATCCGGGTCGTGTTTAATCAGCTCCGATTT
BrIPT1_AB186132.1   CCACGCGCTCCTCGCCGAGAGATTCGACCCAAAGATCGATCCTTTCGC------GTCTTCTTCGATATGTCGGGATTT
FPrimer             --------------------------------------------------------------------------------
IPT1a               CCACGCGCTCCTCGCCGAGAGATTCGACCCAAAGATCGATCCTTTCTC------GTCTTCTTCGATATGTCGGGATTT
RPrimer             --------------------------------------------------------------------------------

IPT1                GCGGTACGACAGCTGTTTCATCTGGGTAGATGTGTCGGAACCTGTGCTGTTCGAGTATCTTCTGAAACGGGTCGACGA
AtIPT1_NM_105517.1  GCGTTACGAGTGTTGTTTCATCTGGGTCGATGTATCGGAGACTGTTCTCTACGAGTATCTTCTCAGAAGAGTCGACGA
BrIPT1_AB186132.1   GCGTTACGACAGCTGTTTCATCTGGGTAGATGTGTCGGAACCTGTGCTGTTCGAGTATCTTCTGAAACGGGTCGACGA
FPrimer             --------------------------------------------------------------------------------
IPT1a               GCGATACGACAGCTGTTTCATCTGGGTAGATGTGTCGGAACCTGTGC------------------------------
RPrimer             ----------------------GGGTAGATGTGTCGGAACCTGTGC------------------------------
```

```
IPT1               GATGATGGGTTCGGGCATGTTCGAGGAGCTGTCCGGGTTTTACGACCCGGTGAAAGCGAGT---AGGGCCCGGTTTGG
AtIPT1_NM_105517.1 AATGATGGATTCAGGTATGTTCGAAGAGCTGTCTAGATTCTACGACCCGGTTAAATCCGGTTTAGAAACCCGGTTTGG
BrIPT1_AB186132.1  GATGATGGGTTCGGGCATGTTCGAGGAGCTGTCCGGGTTTTACGACCCGGTGAAAGCGAGT---AGGGCCCGGTTTGG
```

5.1.2 BnIPT3 aligned with PCR primers

```
IPT3
AtIPT3_AB062610.1 TCATGGGTGCTACCGGGACAGGCAAGTCACGACTCTCCGTGGATATAGCCACACGTTTTCGGGCTGAGATCATAAACTCA
FPrimer           --------------------------------------------------------------------------------
RPrimer           --------------------------------------------------------------------------------
BrIPT3_AB186133.1 TCATGGGCGCTACCGGAACAGGCAAGTCACGACTTTCCGTCGATCTTGCCACACGTTTTCAAGCAGAGATCATAAACTCC

IPT3              ------------------------------------------------------GAACAAAGAAGTTCGGTGAAACGTAGCA
AtIPT3_AB062610.1 GACAAGATACAAGTCCACCAAGGTCTAGACATTGTAACCAACAAGATCACGAGCGAGGAGAGCTGCGGGGTACCGCACCA
FPrimer           --------------------------------GTCACTAACAAGATCACGACCGA-----------------------
RPrimer           --------------------------------------------------------------------------------
BrIPT3_AB186133.1 GACAAGATCCAAGTTCACCAAGGTCTCGACATTGTCACTAACAAGATCACGACCGAGGAGAGATGCGGGGTACCGCACCA

IPT3              TCTCCTAGATGTCTTGCCGCCTGAGACTGACTTAACCGCTGTAAACTTCCGCCTT-TGGCGA-TCTCTCTGTTGAATCTG
AtIPT3_AB062610.1 TCTCCTCGGCGTCTTGCCGCCTGAAGCCGACTTAACCGCCGCGAATTACTGTCACATGGCGAATCTCTCCATTGAATCCG
FPrimer           --------------------------------------------------------------------------------
RPrimer           --------------------------------------------------------------------------------
BrIPT3_AB186133.1 TCTCCTCAGTGTTTTGCCGCCTCAAGCCGACTTAACCGCCGCAAACTTCTGCCACATGGCAAATCTCTCTGTTGAATCTG

IPT3              TTCTCAACCGTGGAAAGCTTCCAATCATCGTTGGAGGTTCCCACTCTTACGTTGAGGCTCTAGTCGACGACGACGACTAC
AtIPT3_AB062610.1 TCCTAAACCGTGGAAAGCTTCCAATCATCGTTGGAGGTTCCAACTCTTACGTGGAGGCTCTAGTGGATGACAAAGAAAAC
FPrimer           --------------------------------------------------------------------------------
RPrimer           --------------------------------------------------------------------------------
BrIPT3_AB186133.1 TTCTTAACCGTGGAAAGCTTCCAATCATCGTTGGAGGTTCCAACTCTTACGTCGAGGCTCTAGTCGACGACGACGACTAC

IPT3              AAGTTTAGGTCAAGATATGACTGTTGTTTTCTATGGGTTGACGTGGCACTTCCCGTTTTGAACGGGTTTGTGACTGAGAG
AtIPT3_AB062610.1 AAGTTCAGGTCGAGATACGACTGTTGTTTTCTATGGGTGGACGTGGCACTTCCCGTTTTGCACGGGTTCGTGTCTGAGAG
FPrimer           --------------------------------------------------------------------------------
RPrimer           --------------------------------------------------------------------------------
BrIPT3_AB186133.1 AAATTTAGGTCAAAGTACGACTGTTGCTTCCTATGGGTCGACGTGGCACTACCCGTTTTGAACCGGTTTGTGTCTGAGAG

IPT3              AGTTGATAAGATGGTGCAAAATGGAATGGTCGAAGAAGCTAGAGATTTTTTTGACTATTCGAATTCTGATTACTCGAGAG
AtIPT3_AB062610.1 AGTTGACAAGATGGTGGAGAGTGGAATGGTTGAGGAAGTCAGAGAATTTTTCGACTTTTCGAACTCTGATTACTCAAGAG
FPrimer           --------------------------------------------------------------------------------
RPrimer           ------------------------------------------------------------------------TACTCGAGAG
BrIPT3_AB186133.1 AGTTGATAAGATGGTGCAAAATGGAATGGTCGAAGAAGCTAGAGACTTTTTTGACTATTCGGATTCTGATTACTCGAGAG

IPT3              GGATCAAAAAAGCGAACCC-------------------------------------------------------------
AtIPT3_AB062610.1 GGATCAAGAAAGCAATCGGATTTCCGGAGTTTGACAGGTTTTTCAGGAACGAGCAGTTCTTGAATGTGGAAGACAGAGAA
FPrimer           --------------------------------------------------------------------------------
```

```
RPrimer           GAATCAAGAAAGCA------------------------------------------------------------
BrIPT3_AB186133.1 GAATAAAGAAAGCAATTGGAGTTCCAGAGTTCGACATATTTTTCAGGAACGAACCGTTCTTGAATTTGGGAGACAGAGAA
```

5.1.3 BnIPT5 aligned with PCR primers

```
IPT5
I5F               ----------------------------------------------------CCCTCGACGAGCTGTACTTCAAGAG
FPrimer           ----------------------------------------------------------------------------
RPrimer           ----------------------------------------------------------------------------
AtIPT5_NM_121909.2 CAGCAGGGGAAGTTAAAGGAGTTTTTATAAGAATGAAGCCATGCATGACGGCTCTAAGACAAGTGATTCAACCATTGTCG

I5F               ATATCGTGCGGCTTTGATATGGCCGACGTTCCCTATTTCCGGCGAAAGGACAAGGCTGTTTTCGACATGGGAGCAACCGG
FPrimer           ----------------------------------------------------------------------------
RPrimer           ----------------------------------------------------------------------------
AtIPT5_NM_121909.2 TTGAACTTCCAAGGAAACATGGTGGACGTTCCGTTTTTCCGGCGAAAAGACAAGGTTGTTTTCGTCATGGGAGCCACCGG

I5F               AACCGGCAAATCCCGTCTCGCCATCGACCTTGCCACTCGTTTTCCGGCGGAGATCGTAAATTCCGACAAGATCCAAGTCT
FPrimer           -----GCAAATCCCGTCTCGCCA--------------------------------------------------------
RPrimer           ----------------------------------------------------------------------------
AtIPT5_NM_121909.2 AACCGGCAAATCTCGTCTAGCCATTGACCTAGCCACTCGTTTTCCGGCGGAGATTGTAAACTCCGACAAGATCCAGGTCT

I5F               ACAAAGGTCTAGACATCGTGACCAACAAAGTCACTCCGGAGGAAAGCCTCGGCGTCCCTCACCACCTCCTCGGCACCGTG
FPrimer           ----------------------------------------------------------------------------
RPrimer           ----------------------------------------------------------------------------
AtIPT5_NM_121909.2 ATAAAGGTCTAGACATTGTGACTAACAAAGTCACTCCTGAGGAAAGCCTTGGCGTTCCTCACCACCTTCTCGGCACCGTC

I5F               GAAAACACTCACGAGGATTTCACCGCGGAGGATTTTCAGCGGGAAGCAATCAGAGCCGTTAAATCGATCGTGGAGAGAGA
FPrimer           ----------------------------------------------------------------------------
RPrimer           ----------------------------------TCAGCGGGAAGCAATCAGAG------------------------
AtIPT5_NM_121909.2 CACGACACTTACGAAGATTTCACGGCGGAGGATTTTCAGCGTGAAGCAATCAGGGCCGTCGAGTCAATCGTCCAGAGAGA

I5F               CCGTGTCCCGATCATAGA--------------------------------------------------------------
FPrimer           ----------------------------------------------------------------------------
RPrimer           ----------------------------------------------------------------------------
AtIPT5_NM_121909.2 CCGTGTCCCGATCATAGCCGGTGGTTCCAATTCTTACATCGAGGCTCTGGTCAACGATTGCGTTGACTTCCGGTTAAGGT
```

5.1.4 BnIPT7 aligned with PCR primers

```
IPT7
BrIPT7_AB062613.1 TCTTTTAAGTTTGTTACAACTCGTTGAGGTGCTTTTACAAAAGGACAAATCAATAAAACATATATTTTTTTCTCCCAC
FPrimer           --------------------------------------------------------------------------------
RPrimer           --------------------------------------------------------------------------------

I7F               ------------------------------------------------------------GGGAACCTAAC
I7R               ------------------------------------------------------------TTTCAAAAACAA
BrIPT7_AB062613.1 CCAAAAGTCAAAATACATCATGAAGTTCTCAATCTCAGCAATGAAGCAGGTACAACCAATCTTAAGCTTCAAGAACAA
FPrimer           --------------------------------------------------------------------------------
RPrimer           --------------------------------------------------------------------------------

I7F               ATCATCCATTGTCA-CGTCAACTCTTTCCTCCATCCTCACGAAAAAGTCGTCTTCGTAATGGGAGCCACTGGATCCGG
I7R               ACCATCCATTGTCAACGTCAACTCTTTCCTCCATCCTCACGAAAAAGTCGTCTTCGTAATGGGAGCCACTGGATCCGG
BrIPT7_AB062613.1 ACTCTCGATGGTCAACGTCAATTCTTTCCTCCGTCCCCAAGAAAAAGTCGTCTTCGTGATGGGAGCCACTGGTTCCGG
FPrimer           --------------------------------------------------------------------------------
RPrimer           --------------------------------------------------------------------------------

I7F               CAAGTCTCGTCTCGCCATCGACCTTGCAACACGGTTTCAAACAGATATCATAAACTCCGACAAGATCCAAGTTTACAA
I7R               CAAGTCTCGTCTCGCCATCGACCTAGCAACACGGTTTCAAACAGATATCATAAACTCCGACAAGATCCAAGTTTACAA
BrIPT7_AB062613.1 TAAGTCCCGGCTCGCCATCGACCTAGCAACTAGGTTTCAGACAGAGATCATAAACTCCGACAAGATCCAAGTTTACAA
FPrimer           --------------------------------------------------------------------------------
RPrimer           --------------------------------------------------------------------------------

I7F               GGGCCTCGACGTCCTTACAAATAAAGTGACACCTCAAGAATGTCGAGGCGTGCCTCACCACTTGCTCGGGGTATTTGA
I7R               GGGCCTCGACGTCCTTACAAATAAAGTGACACCTCAAGAATGTCGAGGCGTGCCTCACCACTTGCTCGGGGTATTTGA
BrIPT7_AB062613.1 GGGCCTCGACGTCCTTACAAATAAAGTAACACCTCAAGAATGCCGAGGCGTGCCTCACCACTTGCTCGGAGTGTTTGA
FPrimer           ------------------------------------------------------------CACTTGCTCGGGGTATTTGA
RPrimer           --------------------------------------------------------------------------------

I7F               CTCGGAAGATGGAAACCTAACGGCCACCGACTTCGCTCTCCTTGCGTCACAAGAGATCTCAACACTCTCAGCTAACAA
I7R               CTCGGAAGATGGAAACCTAACGGCCACCGACTTCGCTCTCCTTGCGTCACAAGAGATCTCAACACTCTCAGCTAACAA
BrIPT7_AB062613.1 CTCCGAAGCTGGCAACCTAACGGCCACCGACTTCTGCCGCCTTGCGTCACAAGAAATCTCAACACTCTCGGCTAACAA
FPrimer           CTCGGA--------------------------------------------------------------------------
RPrimer           --------------------------------------------------------------------------------

I7F               CAAGCTTCCTATAGTAGCCGGTGGATCAAACTCATACATCGAAGCACTTGCGAACCATTCGTCTGGACCATTGTTAAA
I7R               CAAGCTTCCTATAGTAGCCGGTGGATCAAACTCATACATCGAAGCACTTGCGAACCATTCGTCTGGACCATTGTTAAA
BrIPT7_AB062613.1 CAAGCTTCCTATCGTAGCCGGTGGATCAAACTCATACATCGAAGCACTTGCGAATCATTCGTCTGGATTCTTGTTAAA
FPrimer           --------------------------------------------------------------------------------
RPrimer           ------------------------------------------------TGCGAACCATTCGTCTGGACCATTGT----

I7F               CAACTACGAATGTTGTTTCATTTGGGTCGACGTTTCCTTACCAGTTCTTAACTCTTTCGTCTCAAAACGTGTCGATCG
I7R               CAACTACGAATGTTGTTTCATTTGGGTCGACGTTTCCTTACCAGT-CTTAACTC--TCGTCTCAAACTGCGG------
BrIPT7_AB062613.1 CAAGTACCAATGCTGTTTTATTTGGGTCGACGTTTCCTTACCGGTTCTTAACTCATTCGTCTCAAAACGTGTCGATCG
```

5.1.5 BnCKX1 aligned with PCR primers

```
CKX1
1F                ------------------------------------------------------------------------------
AtCKX1_NM_129714.3 CCCTCAGAGTAGTACTCTAAACCTCAAGTTTACCTCTACTTCTCTTATACCATTCCGCCTCTTATTCTTTGCAATTTCTC
1R                ------------------------------------------------------------------------------
FPrimer           ------------------------------------------------------------------------------
RPrimer           ------------------------------------------------------------------------------

1F                ------------------------------------------------------------------------------
AtCKX1_NM_129714.3 TCAACAAAGTAGAAATGGGATTGACCTCATCCTTACGGTTCCATAGACAAAACAACAAGACTTTCCTCGGAATCTTCATG
1R                ------------------------------------------------------------------------------
FPrimer           ------------------------------------------------------------------------------
RPrimer           ------------------------------------------------------------------------------

1F                ----------------------------------------AGGTGTCAGCTGCATGCCAGGTAGTATCAATCTTTT-G
AtCKX1_NM_129714.3 ATCTTGGTTCTAAGCTGTATACCAGGTAGAACCAATCTTTGTTCCAATCATTCTGTTAGTACCCCAAAAGAATTACCTTC
1R                ----------------------------------------------------------CATTTTAAAACCAATCTTTTTG
FPrimer           ------------------------------------------------------------------------------
RPrimer           ------------------------------------------------------------------------------

1F                ATTCAATCAATCAGAATATTAGTTCCTCA-ATAGAGTCACGAGATT-AGAGGGATACATAAC-CTTTGATGATGTCCACA
AtCKX1_NM_129714.3 TTCAAATCCTTCAGA-TATTCGTTCCTCA-TTAGTTTCACTAGATTTGGAGGGTTATATAAG-CTTCGACGATGTCCACA
1R                TTTCAATCATTCAGACTCTCGTCTCCTCATATAGATTCACTCGATTTAGAGGGTTACATACTTCTTTGATGATGTCCACA
FPrimer           ------------------------------------------------------------CTTTGATGATGTCCACA
RPrimer           ------------------------------------------------------------------------------

1F                ATGCGTCCAAGGACTTTGGCAACAGATACCAGTTCCCACCTTTGGCAATTCTCCATCCCAAGTCAGTTTCTGATAG-TCA
AtCKX1_NM_129714.3 ATGTGGCCAAGGACTTTGGCAACAGATACCAGTTACCACCTTTGGCAATTCTACATCCAAGGTCAGTTTTTGATATTTCA
1R                ATGCGTCCAAGGACTTTGGCAACAGATACCAGTTCCCACCTTTGGGGATTCACCATCCCAAGTCAGTTACTGAAATCTCA
FPrimer           ATGCT------------------------------------------------------------------------
RPrimer           ------------------------------------------------------------------------------

1F                ACAGTGATGAGACATATCTTACACGTGGGATCCACCTCAAATATTACAGTAGCAGCCAGAGGCCATGGTCACTCTCTTCA
AtCKX1_NM_129714.3 TCGATGATGAAGCATATAGTACATCTGGGCTCCACCTCAAATCTTACAGTAGCAGCTAGAGGCCATGGTCACTCGCTTCA
1R                ACAGTGCTGAGACATATCTTACACCTGGGATCCACCTCAAATATTACAGTACCTGCCAGAGGCCA---------------
FPrimer           ------------------------------------------------------------------------------
RPrimer           ------------------------------------------------------------------------------

1F                AGGCCAAGCAATAACTCATCAAGGTGTTGTCATCAACACGGAGTCACT-CGAAGTCATGATATCAAGATT-ACAAAAGGA
AtCKX1_NM_129714.3 AGGACAAGCTCTAGCTCATCAAGGTGTTGTCATCAAAATGGAGTCACTTCGAAGTCCTGATATCAGGATTTATAAGGGGA
1R                ------------------------------------------------------------------------------
FPrimer           ---------------TCATCAAGGTGTTGTCATCAACAC-------------------------------------
RPrimer           ------------------------------------------------------------------------------
```

```
1F                 AACAACCAAT-------------------------------------------------------------------------
AtCKX1_NM_129714.3 AGCAACCATATGTTGATGTCTCAGGTGGTGAAATATGGATAAACATTCTACGCGAGACTCTAAAATACGGTCTTTCACCA
1R                 --------------------------------------------------------------------------------
FPrimer            --------------------------------------------------------------------------------
RPrimer            --------------------------------------------------------------------------------

1F                 --------------------------------------------------------------------------------
AtCKX1_NM_129714.3 AAGTCCTGGACAGACTACCTTCATTTGACCGTTGGAGGTACACTATCTAATGCTGGAATCAGCGGTCAAGCATTCAAGCA
```

5.1.6 BnCKX2 aligned with PCR primers

```
CKX2
AtCKX2_NM_1275082 TTTAATCACGGTTTTAATGATCACCAAAT--CATCAAACGGTATTAAAATTGATTTACCTAAATCCCTTAACCTCACCCT
CKX2              AGTACGTTCTGATTTATTTACTTCCGAGCTGTGCTAA-TCGTCAGA-GATTATTCTACTTATTACGCTAAACCTCTCCCT
FPrimer           ----------------------------------------------------------------------------T
BnCKX2            -------TCTGATTTATTTACTTCCAAGCTGTATCAAATCGTCAGAAGATTATTCTACCCAAATCGTTAAACCTCACCCT
RPrimer               ------------------------------------------------------------------------------------

AtCKX2_NM_1275082 CTCTACCGATCCTTCCATCATCTCCGCAGCCTCTCATGACTTCGGAAACATAACCACCGTGACCCCCGGCGGCGTAATCT
CKX2              CCTAATCGATCCTTCCGCCATCTCCGTCGCCTCTCACGATTTTGGAAACATCAATACCGTGATACCCGGTGGCGTGATCT
FPrimer           CCTAAACGATCCTTCCG----------------------------------------------------------------
BnCKX2            CCTAACCGATCCTTCCGCCATCTCCGTCGCCTCTCACGATTTTGGAAACATCACTACCGTGATACCCGGTGGCGTGATCT
RPrimer           --------------------------------------------------------------------------------

AtCKX2_NM_1275082 GCCCCTCCTCCACCGCTGATATCTCTCGTCTCCTCCAATACGCCGCAAACGGAAAAAGTACATTCCAAGTAGCGGCTCGT
CKX2              GCCCTTCCTCTCCCGTTGAGATATCCCGTCTCCTCCCCTACCCCCTCCCCAACG-------------------------
FPrimer           --------------------------------------------------------------------------------
BnCKX2            GCCCTTCCTCTCCCGTTGAGATATCCCGTCTCCTCCCCTACGCATGACCGTACTATCACGGTAGTGATGTTTCCGAAATC
RPrimer           ----------------TGAGATATCCCGTCTCCTCCC--------------------------------------------

AtCKX2_NM_1275082 GGCCAAGGCCACTCCTTAAACGGCCAAGCCTCGGTCTCCGGCGGAGTAATCGTCAACATGACGTGTATCACTGACGTGGT
CKX2              --------------------------------------------------------------------------------
FPrimer           --------------------------------------------------------------------------------
BnCKX2            GTAAGAGGCGACAGACATGGCGG--AAGGATCGGTTAGGAGGGTGAGGTTTAACGATTTGGGTAGAATAATC--TCTGAC
RPrimer           --------------------------------------------------------------------------------

AtCKX2_NM_1275082 GGTTTCA-AAAGACAAGAAGTACGCTGA-CGTGGCGGCCGGGACGTTATGGGTGGATGTGCTTAAGAAGACGGCGGAGAA
CKX2              --------------------------------------------------------------------------------
FPrimer           --------------------------------------------------------------------------------
BnCKX2            GATTTGATACAGCTGGGAAGTAAATAAATCAGAACCACGTT?GAGTGATCATTAAACGAAGATAGGCATGTAA-------
RPrimer           --------------------------------------------------------------------------------
```

5.1.7 BnCKX3 aligned with PCR primers

```
CKX3
AtCKX3_AF303979.1 ATAAAACTTTGGAGTTAGGGTTAACGCCGGTTTCTTGGACGGATTATTTGTATTTAACAGTCGGTGGGACGTTATCAAAC
X3F               ----------------------------------------------------CGAGCAGTG--ACGTTATCCA-C
X3R               ------------------------------------------------TTGTATTTAACGGTCGGTGGAACGTTATCCAAC
RPrimer           ---------------------------------------------------------------------------
FPrimer           ---------------------------------------------------------------------------

AtCKX3_AF303979.1 GGCGGAATTAGTGGACAAACGTTTCGGTACGGTCCACAGATCACTAATGTTCTAGAGATGGATGTTATTACTGGAAAAGG
X3F               GGCGGA-TAAGCGGACAAACGTCTCGGTACGGTCCACAGATCAGTAATGTTCTTGAGCTGGATATTATTACTGGAAAAGG
X3R               GGCGGAATAAGCGGACAAACGTCTCGGTACGGTCCACAGATCAGTAATGTTCTTGAGCTGGATATTATTACTGGAAAAGG
RPrimer           ---------------------------------------------------------------------------
FPrimer           --------AAGCGGACAAACGTCTCGGTA-------------------------------------------------

AtCKX3_AF303979.1 AGAGATTGCAACTTGTTCCAAGGACATGAACTCGGATCTTTTCTTCGCGGTGTTAGGAGGTTTGGGTCAATTCGGCATTA
X3F               GGAGATTGCAACTTGTTCCAACGACATGAACTCAGATCTTTTCTACGCGGCGTTAGGAGGTTTGGGTCAATTCGGAATTA
X3R               GGAGATTGCAACTTGTTCCAACGACATGAACTCAGATCTTTTCTACGCGGCGTTAGGAGGTTTGGGTCAATTCGGAATTA
RPrimer           ---------------------------------------------------------------------------
FPrimer           ---------------------------------------------------------------------------

AtCKX3_AF303979.1 TAACAAGAGCCAGAATTAAACTTGAAGTAGCTCCGAAAAGGGCCAAGTGGTTAAGGTTTCTATACATAGATTTCTCCGAA
X3F               TAACAAGAGCCAGGATTAAACTCGAATTAGCTCCAAAAAGGGCTAAATGGTTAAGGTTTCTATACACTGATTTCTCTGAA
X3R               TAACAAGAGCCAGGATTAAACTCGAATTAGCTCCAAAAAGGGCTAAATGGTTAAGGTTTCTATACACTGATTTCTCTGAA
RPrimer           ---------------------------------------------------------------------------
FPrimer           ---------------------------------------------------------------------------

AtCKX3_AF303979.1 TTCACAAGAGATCAAGAACGAGTGATATCGAAAACGGACGGTGTAGATTTCTTAGAAGGTTCCATTATGGTGGACCATGG
X3F               TTCACAAGAGATCAAGAACGATTGATATCAGAAGCGGGCGGTTTACATTTCTTGGAAGGTTCCGTTATGCTTGACCATGG
X3R               TTCACAAGAGATCAAGAACGATTGATATCAGAAGCGGGCGGTTTACATTTCTTGGAAGGTTCCGTTATGCTTGACCATGG
RPrimer           ---------------------------------------------------------------------------
FPrimer           ---------------------------------------------------------------------------G

AtCKX3_AF303979.1 CCCACCGGATAACTGGAGATCCACGTATTATCCACCGTCCGATCACTTGAGGATCGCCTCAATGGTCAAACGACATCGTG
X3F               CCCACCTGATAACTGGAGATCTACTTACTATCCACCGTCCGATCACTTGAGGATCGCCTCAATGATCAAACGACACCAA-
X3R               CCCACCTGATAACTGGAGATCTACT-ACT-TCCAC-ATCCGTGCATGG-----------------------------
RPrimer           ---------------------------------------------------------------------------
FPrimer           CCCACCTGATAACTGGAGATC-----------------------------------------------------------

AtCKX3_AF303979.1 TCATCTACTGCCTTGAAGTCGTCAAGTATTACGACGAAACTTCTCAATACACAGTCAACGAGGAAATGGAGGAGTTAAGC
```

5.1.8 BnCKX5 aligned with PCR primers

```
CKX5
AtCKX5_NM_106199.5 ATAAACGCACAAACTCGTTAAATTTGTACGAATATAATTTTTTTTAAAACACTCGTTATAATATATTAAAGTTTCACCCA
RPrimer            --------------------------------------------------------------------------------
FPrimer            --------------------------------------------------------------------------------

CKX5               ----------------------------------------TTTACTAACAGTGTATCTGGGCTTACGTTAGCTCTC--GA
AtCKX5_NM_106199.5 AACCGAAAAAAGAGAGAATCTGTGCATGTTGCTCAGAAAATCTTCAAAGCGTA-ATCTGGGCTTACGTTAGCTCTCACGA
RPrimer            --------------------------------------------------------------------------------
FPrimer            ----------------------------------------------------ATCTGGGCTTACGTTAGCTCTC----

CKX5               ACTCCCCAGGATCTCCTATATATGTTTTTCCCTCTCCCCATAAAATCTCTCATCATCTAAAACATCTTTATTATCATTTC
AtCKX5_NM_106199.5 ACCCCCAAGGATCTTCTATATATGTTTTTTCATTTCCCCATAAAATCTTTCATTATCTAAAAAATATT-ATTATCGTATC
RPrimer            --------------------------------------------------------------------------------
FPrimer            --------------------------------------------------------------------------------

CKX5               CCCTTC--TATATATTCCTTTCCCCTTTAGTGTCCGGAGAGACACATATCTTGA---TTTCTTGATGACTCGTGAAATGA
AtCKX5_NM_106199.5 TTTTTTCTTCTATATATTCTTCCTCCTCAATCTT----GATTCTTGTTTCTTGAGTATTCTTTGATGAATCGTGAAATGA
RPrimer            --------------------------------------------------------------------------------
FPrimer            --------------------------------------------------------------------------------

CKX5               CGTCAAGCTTTCTTCTCGTGACATTCTCCATTTGTACACTGATCATAG-----------ACGTTGGTCCCAGCGAGCTC
AtCKX5_NM_106199.5 CGTCAAGCTTTCTTCTCCTGACGTTCGCCATATGTAAACTGATCATAGCCGTGGGTCTAAACGTGGGCCCCAGTGAGCTC
RPrimer            --------------------------------------------------------------------------------
FPrimer            --------------------------------------------------------------------------------

CKX5               CTCCGCATCGGAGCCATAGACGTCAACGGCCACTTCACCTTCAAGCCTTCCGACTTAGCCTCCGTCTCGTCGGACTTCGG
AtCKX5_NM_106199.5 CTCCGCATCGGAGCCATAGATGTCGACGGCCACTTCACCGTCCACCCTTCCGACTTAGCCTCCGTCTCCTCAGACTTCGG
RPrimer            --------------------------------------------------------------------------------
FPrimer            ------------------------------------------------CTTCCGACTTAGCCTCCGTCT-------------

CKX5               TATGCTAAAGTCGCCGGAGGAGCCATTAGCCGTACTTCATCCATCCTCAGCTGAAGACGTGGCACGGCTCATCAGAACAG
AtCKX5_NM_106199.5 TATGCTGAAGTCACCTGAAGAGCCATTGGCCGTGCTTCATCCATCATCGGCCGAAGACGTGGCACGACTCGTCAGAACAG
RPrimer            --------------------------------------------------------------------------------
FPrimer            --------------------------------------------------------------------------------

CKX5               CTTACGGCTCAAACTT---------------------------------------------------------------
AtCKX5_NM_106199.5 CTTACGGTTCAGCCACGGCGTTTCCGGTCTCAGCCCGAGGCCACGGCCATTCCATAAACGGACAAGCCGCGGCGGGGAGG
```

5.1.9 BnCKX7 aligned with PCR primers

```
CKX7
AtCKX7_NM_180532.2 ACAGACAAACAAAAAAAGGTTTGGTTCCAAAAGCTAAAAAGCTTCCATCTACATTAGAGTCTCTCTATTTAGCATTTA
CKX7F_Seq          --------------------------------------------------------------------------------
FPrimer            --------------------------------------------------------------------------------
RPrimer            --------------------------------------------------------------------------------

CKX7R_Seq          -------------------------------------CATTGCTTACATAGAGCCATACTTCTTGGATAACGACGG
AtCKX7_NM_180532.2 CACACAATCACACACACACACACACACACACACCAAAATGATAGCTTACATAGAACCATACTTCTTGGAAAACGACGC
CKX7F_Seq          ------------------------------------------------------------------------TA
FPrimer            --------------------------------------------------------------------------
RPrimer            --------------------------------------------------------------------------

CKX7R_Seq          TGAAGCCGCCTC---------CGTCACCGGACAATCTTTTGATGGCGTCTCCGAGTCACTCCACATCCAGGGAGAAAT
AtCKX7_NM_180532.2 CGAGGCTGCCTCTGCCGCCACCGCCGCCGGAAAATCTACGGATGGTGTTTCTGAGTCACTTAACATCCAAGGAGAAAT
CKX7F_Seq          GGCGTAGCGCCT---------CGTCACCGGACA-TCTTTTGATGGCGTCTCCGAGTCACTCGACATCCAGGGAGAAAT
FPrimer            --------------------------------------------------------------------------
RPrimer            --------------------------------------------------------------------------

CKX7R_Seq          CTTATGCGGCGGTGCTGCGGCGGATATCGCCGGGAAAGACTTCGGTGGCATGAACTGCGTGAAGCCTCTTGCGGTGGT
AtCKX7_NM_180532.2 CTTATGTGGTGGAGCTGCGGCGGATATCGCCGGGAGAGATTTTGGCGGCATGAACTGTGTGAAGCCTCTTGCTGTGGT
CKX7F_Seq          CTTATGCGGCGGTGCTGCGGCGGATATCGCCGGAAAAGACTTCGGTGGCATGAACTGCGTGAAGCCTCTTGCGGTGGT
FPrimer            -------------------------------GGGAAAGACTTCGGTGGCA---------------------------
RPrimer            --------------------------------------------------------------------------

CKX7R_Seq          GAGACCCGTGGGACCGGAGGATATCGCCGGAGCGGTGAGAGCAGCTCTGAGGTCCGATAAACTCACGGTGGCGGCGCG
AtCKX7_NM_180532.2 GAGACCAGTGGGACCGGAAGATATCGCCGGAGCGGTGAAAGCGGCTCTGAGGTCAGATAAACTAACGGTGGCGGCGCG
CKX7F_Seq          GAGACCCGTGGGACCGGAGGATATCGCCGGAGCGGTGAGAGCAGCTCTGAGGTCCGATAAACTCACCGTGGCGGCGCG
FPrimer            --------------------------------------------------------------------------
RPrimer            --------------------------------------------------------------------------

CKX7R_Seq          TGGAAACGGCCATTCTATCAACGGCCAGGCCATGGCGGAAGGAGGACTTGTCGTAGATATGCGTTCCACGGCGGAGAA
AtCKX7_NM_180532.2 TGGAAACGGCCATTCTATCAACGGTCAAGCCATGGCGGAAGGAGGACTCGTTGTCGATATGAGTACCACGGCGGAGAA
CKX7F_Seq          TGGAAACGGCCATTCTATCAACGGCCAGGCCATGGCGGAAGGAGGACTCGTCGTCGATATGCGTTCCACGGCGGAGAA
FPrimer            --------------------------------------------------------------------------
RPrimer            --------------------------------------------------------------------------

CKX7R_Seq          TCATTTCGAGGTTGGTTTTTTACCCGGCGG---------GGCGTTTGTCGACGTCTCCGGAGGGGCATTATGGGAAAA
AtCKX7_NM_180532.2 TCATTTCGAGGTTGGTTATTTATCCGGCGGTGATGCCACGGCGTTTGTTGATGTCTCCGGAGGGGCATTATGGGAAGA
CKX7F_Seq          TCATTTCGAGGTTGGTTTTTTACCCGGCGG---------GGCGTTTGTCGACGTCTCCGGAGGGGCATTATGGGAAAA
FPrimer            --------------------------------------------------------------------------
RPrimer            --------------------------------------------------------------------------

CKX7R_Seq          CGTGTTGAAACGGTGCGTTTCGGAGTATGGCTTGGCGCCTCGGTCGTGGACTGATTACCTCGGGT-GACGGTAGGCGG
AtCKX7_NM_180532.2 TGTATTGAAACGGTGCGTTTCGGAGTACGGTTTGGCTCCGAGGTCTTGGACTGATTATCTTGGGTTAACGGTGGGAGG
```

```
CKX7F_Seq          CGTGTTGAAACGGTGCGTTTCGGAGTATGGCTTGGCGCCTCGGTCGTGGACTGATTACCTCGGGTTGACGGTAGGCGG
FPrimer            ----------------------------------------------------------------------------
RPrimer            -------------------TCGGAGTATGGCTTGGCG-----------------------------------------

CKX7R_Seq          ACGTGCATGCC---------------------------------------------------------------------
AtCKX7_NM_180532.2 TACGTTGTCAAATGCCGGCGTTAGTGGTCAAGCGTTCCGTTACGGACCAC---------------------------
CKX7F_Seq          GACGTTGTCGAATGCCGGCGTTAGTGGACAAGCGTTTCCATTACCCCG---------------------------
```

5.1.10 BnACT aligned with PCR primers

```
Sequence           --------------------------------TGTGACAATGGAACTGGAATGGTGAAGGCTGGGTTTGCTGGTGACGAT
AtACT_NM_125328.3 TGGCGGACGGTGAAGACATTCAACCGCTTGTTTGTGACAATGGAACTGGAATGGTTAAGGCCGGATTCGCGGGTGACGAT
Rev_Seq            ------------------------------------------------------------CGTAATTGCTGCTGA-GAT
FPrimer            --------------------------------TGTGACAATGGAACTGGAATGGT-------------------------

Sequence           GCTCCCAGGGCTGTGTTCCCGAGTATTGTTGGTCGTCCTAGG-CACACTGGTGTCATGGTTGGGATGGGTCAGAAAGACG
AtACT_NM_125328.3 GCACCAAGAGCGGTTTTTCCGAGCATTGTAGGCCGTCCTCGC-CACACGGGTGTGATGGTAGGGATGGGACAAAAGGATG
Rev_Seq            GCTCC-AGGGCTGTGTTCCCGAGTATTGTTGGTCGTCCTAGGACACACTGGTGTCATGGTTGGGATGGGTCAGAAAGACG
FPrimer            ----------------------------------------------------------------------------
RPrimer            ----------------------------------------------------------------------------

Sequence           CTTACGTTGGTGACGAAGCTCAGTCCAAAAGAGGTATTCTTACCCTCAAGTATCCGATTGAGCATGGTATTGTGAGCAAC
AtACT_NM_125328.3 CTTATGTTGGAGACGAGGCTCAATCAAAACGTGGTATCTTGACTCTGAAGTACCCAATTGAGCATGGAATTGTTAATAAT
Rev_Seq            CTTACGTTGGTGACGAAGCTCAGTCCAAAAGAGGTATTCTTACCCTCAAGTATCCTATTGTGCATGGTATTGTGAGCAAC
FPrimer            ----------------------------------------------------------------------------
RPrimer            ----------------------------------------------------------------------------

Sequence           TGGGATGACATGGAGAAGATCTGGCATCACACTTTCTACAACGAGCTCCGTGTTGCACCTGAAGAGCACCCGGTTCTTCT
AtACT_NM_125328.3 TGGGATGACATGGAGAAGATTTGGCATCACACTTTCTACAATGAGCTTCGTGTTGCCCCTGAAGAACATCCGGTTCTCTT
Rev_Seq            TGGGATGACATGGAGAAGATCTGGCATCACACTTTCTACAACGAGCTCCGTGTTGCCCCTGAAGAGCACCCGGTTCTTCT
FPrimer            ----------------------------------------------------------------------------
RPrimer            ----------------------------------------------------------------------------

Sequence           CACAGAGGCGCCTCTTAACCCTAAAGCCAACAGGGAGAAGATGACTCAGATCATGTTCGAGACGTTCAATGTCCCTGCCA
AtACT_NM_125328.3 GACCGAAGCTCCTCTCAATCCGAAAGCTAACCGTGAGAAGATGACTCAGATCATGTTTGAGACATTCAATACTCCTGCTA
Rev_Seq            CACAGAGGCGCCTCTTAACCCTAAGGCTAACAGGGAGAAGATGACTCAGATCATGTTCGAGACTTTCAATGTCCCTGCCA
FPrimer            ----------------------------------------------------------------------------
RPrimer            ----------------------------------------------------------------------------

Sequence           TGTATGTTGCTATCCAGGCTGTTCTTTCCCTCTACGCTAGTGGTCGTACCTACCGGTATTGTGCT-GACTCTGGTGATGG
AtACT_NM_125328.3 TGTATGTTGCCATTCAAGCTGTTCTCTCACTCTATGCCAGTGGCCGTAC-AACTGGTATTGTTTTGGACTCTGGAGATGG
Rev_Seq            TGTATGTTGCTATCCAGGCTGTTCTTTCTCTCTACGCTAGTGGGCGTAC-TACCGGTATTGTGCTCGACTCTGGTGATGG
FPrimer            ----------------------------------------------------------------------------
RPrimer            ----------------------------------------------------------------------------
```

```
Sequence          TGTGTCTCACACCG-GCCATCACATTGC--------------------------------------------------
AtACT_NM_125328.3 TGTGAGCCACACGGTACCAATCTACGAGGGTTACGCTCTCCCACACGCAATCCTACGTCTTGACCTAGCAGGTCGTGACC
Rev_Seq           TGTGTCTCACACTGTGCCAATCTACGAGGGTTATGCTCTTCCTCACGCTATCCTCCGTA--------------------
FPrimer           ------------------------------------------------------------------------------
RPrimer           ----------------------------------CTTCCTCACGCTATCCTCCGT----------------------
```

5.1.11 BnGAP aligned with PCR primers

```
FPrimer             ------------------------------------------------------------------------------
ATGAPDH_NM_101214.3 ACAAGAAGATCAGAATCGGAATCAACGGTTTCGGAAGAATCGGTCGTTTGGTTGCTAGAGTTGTTCTTCAGAGGGATGAT
Seq1                ----------------------AACGGTTTTCGGAAGAATCGGTCGTTTGGTGGCCAGAGTTATCCTTCAGAGGAACGAT
RPrimer             ------------------------------------------------------------------------------
Seq2                -------------------------------------------------CGGTGGCAGAA--TATCTTC---AAGGACGAT

FPrimer             --------------------GATCCCTTCATCACCACCGAGTA-----------------------------------
ATGAPDH_NM_101214.3 GTTGAGCTCGTCGCTGTTAACGATCCTTTCATCACCACCGAGTACATGACATACATGTTTAAGTATGACAGTGTTCACGG
Seq1                GTTGAGCTCGTCGCTGTTAACGATCCCTTCATCACCACCGAGTACATGACGTACATGTTTAAGTATGACAGTGTTCACGG
RPrimer             ------------------------------------------------------------------------------
Seq2                GTTGAGCTCGTCGCTGTTAACGATCCCTTCATCACCACCGAGTACATGACGTACATGTTTAAGTATGACAGTGTTCACGG

FPrimer             ------------------------------------------------------------------------------
ATGAPDH_NM_101214.3 TCAGTGGAAGCACCATGAGCTTAAGGTGAAGGATGACAAAACTCTTCTCTTCGGTGAGAAGCCAGTCACTGTTTTCGGCA
Seq1                TCAGTGGAAGCACAATGAGCTCAAGGTTAAGGATGAGAAGACACTTCTCTTCGGTGAGAAGCCAGTCACTGTTTTCGGCA
RPrimer             ------------------------------------------------------------------------------
Seq2                TCAGTGGAAGCACAATGAGCTCAAGGTTAAGGATGAGAAGACACTTCTCTTCGGTGAGAAGCCAGTCACTGTTTTCGGCA

FPrimer             ------------------------------------------------------------------------------
ATGAPDH_NM_101214.3 TCAGGAACCCTGAGGACATCCCATGGGGTGAGGCTGGAGCTGACTTTGTTGTTGAGTCTACTGGTGTCTTCACTGACAAA
Seq1                TCAGGAACCCTGAGGATATCCCATGGGGTGAGGCCGGAGCTGACTTTGTTGTTGAGTCTACTGGTGTCTTCACTGACAAG
RPrimer             ------------------------------------------------------------------------------
Seq2                TCAGGAACCCTGAGGATATCCCATGGGGTGAGGCTGGAGCTGACTTTGTTGTTGAGTCTACTGGTGTCTTCACTGACAAG

FPrimer             ------------------------------------------------------------------------------
ATGAPDH_NM_101214.3 GACAAGGCTGCTGCTCACTTGAAGGGTGGTGCTAAAAAGGTTGTCATCTCTGCCCCAAGCAAAGATGCGCCCATGTTCGT
Seq1                GACAAGGCCGCTGCTCACTTGAAGGGTGGTGCCAAGAAAGTTGTCATCTCTGCACCAAGCAAAGATGCTCCCATGTTCGT
RPrimer             ------------------------------------------------------------------------------
Seq2                GACAAGGCTGCTGCTCACTTGAAGGGTGGTGCCAAGAAAGTTGTCATCTCTGCCCCAAGCAAAGATGCTCCCATGTTCGT

FPrimer             ------------------------------------------------------------------------------
ATGAPDH_NM_101214.3 TGTTGGTGTCAACGAGCACGAGTACAAGTCTGACCTTGACATTGTTTCCAACGCTAGTTGCACCACTAACTGCCTTGCTC
Seq1                TGTTGGTGTCAATGAGCATGAGTACAAGTCTGATCTCAACAGACATCT-----------------------------
RPrimer             ------------------------------------------------------------CACTAACTGCCTTGCTC
Seq2                TGTTGGTGTCAATGAGCATGAATACAAGTCTGATCTTAACATTGTTTCCAACGCTAGTTGCACCACTAACTGCCTTGCTC
```

```
FPrimer             ------------------------------------------------------------------------------
ATGAPDH_NM_101214.3 CTCTTGCCAAGGTTATTAATGACAGGTTTGGCATTGTTGAGGGACTCATGACCACTGTCCACTCTATCACTGCTACTCAG
Seq1                ------------------------------------------------------------------------------
RPrimer             CCC---------------------------------------------------------------------------
Seq2                CC----------------------------------------------------------------------------
```

5.1.12 BnElf aligned with PCR primers

```
Sequence            ----------------------TGGAGGCTGGCTGAGATGAACAAGAGGTCCTTCAAGTACGCGTGGGTGTTGGACAAA
AtElf1_NM_100667.3 GTGTGATCGAGAGGTTCGAGAAGGAGGCTG-CTGAGATGAACAAGAGGTCCTTCAAGTACGCATGGGTGTTGGACAAA
FPrimer             ----------------------AGGAGGCTG-CTGAGATGAACAA---------------------------------
RPrimer             ------------------------------------------------------------------------------

Sequence            CTTAAGGCCGAGCGTGAGCGTGGTATCACCATTGATATTGCTCTCTGGAAGTTCGAGACCACCAAGTACTACTGCACG
AtElf1_NM_100667.3 CTTAAGGCTGAGCGTGAGCGTGGTATCACCATTGACATTGCTCTCTGGAAGTTCGAGACCACCAAGTACTACTGCACT
FPrimer             ------------------------------------------------------------------------------
RPrimer             ------------------------------------------------------------------------------

Sequence            GTCATTGATGCTCCTGGACATCGTGATTTCATCAAGAACATGATTACTGGTACCTCCCAGGCTGATTGTGCTGTTCTG
AtElf1_NM_100667.3 GTCATTGATGCTCCTGGTCATCGTGATTTCATCAAGAACATGATCACTGGTACCTCCCAGGCTGATTGTGCTGTCCTT
FPrimer             ------------------------------------------------------------------------------
RPrimer             ------------------------------------------------------------------------------

Sequence            ATCATTGACTCCACCACTGGTGGTTTTGAAGCTGGTATCTCCAAGGATGGTCAGACCCGTGAGCATGCTCTC--TGCT
AtElf1_NM_100667.3 ATCATTGACTCCACCACTGGTGGTTTTGAGGCTGGTATCTCCAAGGATGGTCAGACCCGTGAGCACGCTCTCCTTGCT
FPrimer             ------------------------------------------------------------------------------
RPrimer             ------------------------------------------------------------------------------

Sequence            T-CACGCT--GTACAA------------------------------------------------------------
AtElf1_NM_100667.3 TTCACCCTTGGTGTCAAGCAGATGATCTGCTGTTGTAACAAGATGGATGCCACTACCCCCAAGTACTCCAAGGCCAGG
FPrimer             ------------------------------------------------------------------------------
RPrimer             ----------------------TGATTTGCTGCTGTAACAAGATGG-----------------------------
```

5.1.13 BrCKX1 submitted to Genebank

```
LOCUS       HM587033                599 bp    mRNA    linear   PLN 27-OCT-2010
DEFINITION  Brassica rapa cytokinin oxidase (CKX1) mRNA, partial cds.
ACCESSION   HM587033
VERSION     HM587033
KEYWORDS    .
SOURCE      Brassica rapa
 ORGANISM  Brassica rapa
           Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
           Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
           rosids; malvids; Brassicales; Brassicaceae; Brassica.
REFERENCE   1  (bases 1 to 599)
 AUTHORS   O'Keefe,D., Song,J. and Jameson,P.E.
 TITLE     Spatial and temporal expression of cytokinin regulatory genes in
           Rapid Cycling Brassica
 JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 599)
 AUTHORS   O'Keefe,D., Song,J. and Jameson,P.E.
 TITLE     Direct Submission
 JOURNAL   Submitted (23-JUN-2010) School of Biological Sciences, University
           of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand
FEATURES             Location/Qualifiers
    source          1..599
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                    /db_xref="taxon:3711"
    gene            <1..>599
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                    /codon_start=1
                    /product="cytokinin oxidase"
                    /translation="VGGTLSNAGISGQAFKYGPQINNVYQLEIVTGKGEVMTCSEKQN
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                    DYVEGFVTVNRTDLLDNWRSSFSPHDSIGASQFKSEGKTLYCLEVVKYFNLEEANSTN
                    LEVEKLLSELSYIPSTLFSSEVTYIEFLDRVHIAEIKRRA"
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       61 atcaacaacg tttaccagct agaaatcgtc acagggaaag gagaagtcat gacatgttct
      121 gagaagcaaa actctgaact tttctacagt gttcttggag ggcttggaca gtttggcata
      181 atcaccaggg caaggatagc tcttggacca gcaccacata tggtgaaatg gatcagggta
      241 ctctactcag acttctctgc gttttcaagg gaccaagaac atttgattac caagaagaat
      301 ggtttcgact atgttgaagg gtttgtgaca gtgaatcgaa ctgacctcct cgataactgg
      361 aggtcatcat ttagtcccca tgattccata ggagcaagcc agttcaagtc agaagggaaa
      421 actctgtact gtctagaagt tgtgaaatat ttcaacttag aagaagctaa ctctactaat
      481 ctggaagtgg agaagttact ttcagagctg agttatattc catctacttt gttctcatct
      541 gaagtgacat acattgaatt tctggaccgt gtgcatatcg ccgagataaa acgtagagc
```

5.1.14 BrCKX2 submitted to Genebank

```
LOCUS       HM587034                536 bp    mRNA    linear   PLN 27-OCT-2010
DEFINITION  Brassica rapa cytokinin oxidase (CKX2) mRNA, partial cds.
ACCESSION   HM587034
VERSION     HM587034
SOURCE      Brassica rapa
 ORGANISM  Brassica rapa
           Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
           Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
           rosids; malvids; Brassicales; Brassicaceae; Brassica.
REFERENCE   1  (bases 1 to 536)
 AUTHORS   O'Keefe,D.J., Song,J. and Jameson,P.E.
 TITLE     Spatial and temporal expression of cytokinin regulatory genes in
           Rapid Cycling Brassica
 JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 536)
 AUTHORS   O'Keefe,D.J., Song,J. and Jameson,P.E.
 TITLE     Direct Submission
 JOURNAL   Submitted (23-JUN-2010) School of Biological Sciences, University
```

```
           of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand
FEATURES             Location/Qualifiers
    source          1..536
                    /organism="Brassica rapa"
                    /mol_type="mRNA"
                    /db_xref="taxon:3711"
    gene            <1..>536
                    /gene="CKX2"
    CDS             <1..>536
                    /gene="CKX2"
                    /codon_start=2
                    /product="cytokinin oxidase"
                    /translation="AGVPDGVIINMTCLAGVVVSDDMKYADVAGGTLWVDVLKNTAEK
                    GVSPVSWTDYLHVSVGGTLSNAGIGGEVFRNGPQISNVLELDVITGKGEMLACSPQLN
                    SELFYGVLGGLGQFGIITRARIVLNHAPKRAKWFRMLYSDFTAFTKDQERLISMANDT
                    GVDYLEGQLFMSNGVVDT"
ORIGIN
        1 agcaggcgtc cccgacggag tcattatcaa catgacgtgt ctcgctggcg tagtggtttc
       61 cgatgacatg aagtatgctg acgtggccgg tgggacgtta tgggttgatg tgctgaaaaa
      121 tacggcggag aaaggcgttt cgccggtttc ttggacggat tatttgcatg ttagcgtygg
      181 aggaacactg tcaaatgctg gaatcggcgg tgaagtcttt agaaacggtc ctcagattag
      241 taacgtyctt gagttggacg ttataactgg gaaaggtgaa atgttagcat gctcgccaca
      301 gttaaactca gagttgttct atggagtttt aggaggtttg ggacaatttg gaataatcac
      361 gagagccaga attgttttga accatgcacc taaaagggcg aaatggtttc gaatgctgta
      421 cagtgacttc acagctttta caaaggacca agaacgtttg atatcaatgg ccaatgatac
      481 tggagttgac tatttagaag gtcaactatt catgtcaaac ggcgtggtag acacct
//
```

5.1.15 BrCKX3 submitted to Genebank

```
LOCUS       HM587035                403 bp    mRNA    linear   PLN 27-OCT-2010
DEFINITION  Brassica rapa cytokinin oxidase (CKX3) mRNA, partial cds.
ACCESSION   HM587035
VERSION     HM587035
SOURCE      Brassica rapa
 ORGANISM  Brassica rapa
           Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
           Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
           rosids; malvids; Brassicales; Brassicaceae; Brassica.
REFERENCE   1  (bases 1 to 403)
 AUTHORS   O'Keefe,D.J., Song,J. and Jameson,P.E.
 TITLE     Spatial and temporal expression of cytokinin regulatory genes in
           Rapid Cycling Brassica
 JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 403)
 AUTHORS   O'Keefe,D.J., Song,J. and Jameson,P.E.
 TITLE     Direct Submission
 JOURNAL   Submitted (23-JUN-2010) School of Biological Sciences, University
           of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand
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      241 aaatggttaa ggtttctata cactgatttc tctgaattca caagagatca agaacgattg
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5.1.16 BrCKX5 submitted to Genebank

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LOCUS       HM587036                224 bp    mRNA    linear   PLN 27-OCT-2010
DEFINITION  Brassica rapa cytokinin oxidase (CKX5) mRNA, partial cds.
ACCESSION   HM587036
VERSION     HM587036
KEYWORDS    .
SOURCE      Brassica rapa
 ORGANISM  Brassica rapa
           Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
           Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
           rosids; malvids; Brassicales; Brassicaceae; Brassica.
REFERENCE   1  (bases 1 to 224)
 AUTHORS   O'Keefe,D.J., Song,J. and Jameson,P.E.
 TITLE     Spatial and temporal expression of cytokinin regulatory genes in
           Rapid Cycling Brassica
 JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 224)
 AUTHORS   O'Keefe,D.J., Song,J. and Jameson,P.E.
 TITLE     Direct Submission
 JOURNAL   Submitted (23-JUN-2010) School of Biological Sciences, University
           of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand
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     121 tggaaacggc cattctatca acggccaggc catggcggaa ggaggactcg tcgtcgatat
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5.1.17 BrIPT1 submitted to Genebank

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LOCUS       HM587037                 82 bp    mRNA    linear   PLN 27-OCT-2010
DEFINITION  Brassica rapa cytokinin oxidase (IPT1) mRNA, partial cds.
ACCESSION   HM587037
VERSION     HM587037
KEYWORDS    .
SOURCE      Brassica rapa
 ORGANISM  Brassica rapa
           Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
           Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
           rosids; malvids; Brassicales; Brassicaceae; Brassica.
REFERENCE   1  (bases 1 to 82)
 AUTHORS   O'Keefe,D.J., Song,J. and Jameson,P.E.
 TITLE     Spatial and temporal expression of cytokinin regulatory genes in
           Rapid Cycling Brassica
 JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 82)
 AUTHORS   O'Keefe,D.J., Song,J. and Jameson,P.E.
 TITLE     Direct Submission
 JOURNAL   Submitted (23-JUN-2010) School of Biological Sciences, University
           of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand
FEATURES             Location/Qualifiers
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5.1.18 BrIPT3 submitted to Genebank

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DEFINITION  Brassica rapa cytokinin oxidase (IPT3) mRNA, partial cds.
ACCESSION   HM587038
VERSION     HM587038
KEYWORDS    .
SOURCE      Brassica rapa
 ORGANISM  Brassica rapa
           Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
           Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
           rosids; malvids; Brassicales; Brassicaceae; Brassica.
REFERENCE   1  (bases 1 to 416)
 AUTHORS   O'Keefe,D.J., Song,J. and Jameson,P.E.
 TITLE     Spatial and temporal expression of cytokinin regulatory genes in
           Rapid Cycling Brassica
 JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 416)
 AUTHORS   O'Keefe,D.J., Song,J. and Jameson,P.E.
 TITLE     Direct Submission
 JOURNAL   Submitted (23-JUN-2010) School of Biological Sciences, University
           of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand
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5.1.19 BrIPT5 submitted to Genebank

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ACCESSION   HM587039
VERSION     HM587039
KEYWORDS    .
SOURCE      Brassica rapa
 ORGANISM  Brassica rapa
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           Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
           rosids; malvids; Brassicales; Brassicaceae; Brassica.
REFERENCE   1  (bases 1 to 247)
 AUTHORS   O'Keefe,D.J., Song,J. and Jameson,P.E.
 TITLE     Spatial and temporal expression of cytokinin regulatory genes in
           Rapid Cycling Brassica
 JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 247)
 AUTHORS   O'Keefe,D.J., Song,J. and Jameson,P.E.
 TITLE     Direct Submission
 JOURNAL   Submitted (23-JUN-2010) School of Biological Sciences, University
           of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand
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     241 cggttta
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5.2 Recipes

5.2.1 25 x TAE Buffer

121g Tris base

28.5ml glacial acetic acid

9.3g EDTA

Made up to 1L with distilled H_2O

5.2.2 1 M Tris Stock

121 g Tris bace

Made up to 1 L with nanopure water, Adjust pH to 8.0 with HCL

5.2.3 0.5 M EDTA stock

146 g EDTA

Made up to 1 L with nanopure water, Adjust pH to 8.0 with HCL

5.2.4 TE Buffer

10 ml 1 M Tris (pH 8)

2 ml 0.5 M EDTA (pH 8)

Made up to 1 L with nanopure water

5.2.5 6 x agarose gel loading dye

60mM EDTA

10mM Tris-HCl (pH 7.6)

0.03% Xylene cyanol FF

0.03% Bromophenol blue

60% glycerol

5.2.6 RT master mix

4 µl 5x RT buffer (Roche)

0.8 µl RNAsecure™ Reagent (Ambion)

1 µl 20 mM dNTPs

1 µl Expand Reverse Transcriptase, (50 U/µl) (Roche)

3.2 µl DEPC water

5.2.7 PCR

For 20 µl PCR reaction, the recipe was scaled for different reaction volumes.

2.5 µl dNTPs (2mM)

2 µl 10*Taq buffer

1 to 2 µl 25mM $MgCl_2$ (adjusted to optimise reaction)

1 µl Primers*2 (10pmol/µl)

2 µl DNA template

0.2 µl Taq (5U/µl)

Water to make up to 20 µl

5.2.8 DEPC water

DEPC is added to Nanopure water (0.1% (v/v)) and vigorously shaken to mix, then incubated on a shaker for 12 hours, then autoclaved

for 45min to break down DEPC.

5.2.9 Applied Biosystems BigDye® Terminator v3.1

For 10 µl reaction volume.

0.5 µl BDT

1.75 µl 5x sequencing buffer

1 µl (3.2 pmole) primer

3 to 10 ng purified template

Water to make up to 10 µl

Sequencing Program: 96°C for 10 s, 50°C*[1] for 10 s, 60°C*[1] variable*[2]

*[1] adjusted to match primer. *2 adjusted for sequence length

5.2.10 Sephadex

25 g Sephadex

500 ml Nanopure water

Mix Sephadex and water and stand for 10 min. Suck out water and refill, repeat until water is clear, store in the fridge. To use, suck

water to 1cm above the gel and mix.

5.2.11 2×SYBR green qPCR reaction buffer

15 µl 200×SYBR green (Appendix 0)

930 µl qPCR buffer

40 µl 20 mM dNTP

15 µl Bioline taq plomerase

5.2.12 200×SYBR green

2 µl 10,000×SYBR Green 1 (Invitrogen)

998 µl Dimethyl sulfoxide

5.3 IPT genes used for bioinformatics

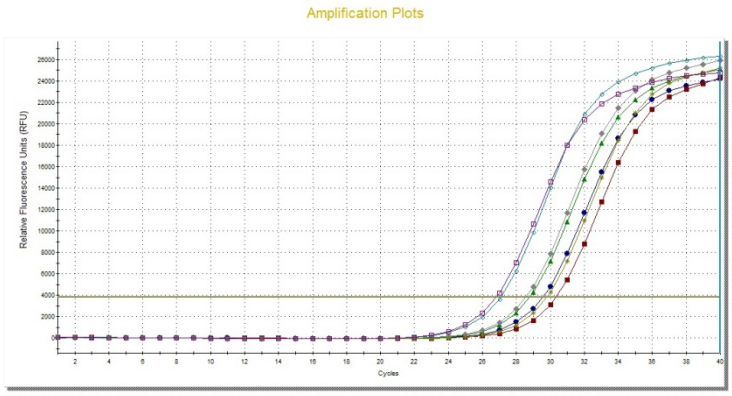
Gene name	Species	Accession No
AtIPT1	***Arabidopsis thaliana***	**AB062607.1**
AtIPT2	***Arabidopsis thaliana***	**AB062609.1**
AtIPT3	***Arabidopsis thaliana***	**AB062610.1**
AtIPT4	***Arabidopsis thaliana***	**AB061402.1**
AtIPT5	***Arabidopsis thaliana***	**AB062608.1**
AtIPT6	***Arabidopsis thaliana***	**AB062612.1**
AtIPT7	***Arabidopsis thaliana***	**AB062613.1**
AtIPT8	***Arabidopsis thaliana***	**AB061406.1**
AtIPT9	***Arabidopsis thaliana***	**NM_180712.1**
BrIPT1	***Brassica rapa***	**AB186132.1**
BrIPT3	***Brassica rapa***	**AB186133.1**
BrIPT5	***Brassica rapa***	**AB186134.1**
VvIPT1	***Vitis Vinifera***	**GQ981408.1**
MaIPT	***Morus alba***	**AY781335.1**
MdIPT1	***Malus x domestica***	**HQ606061.1**
MdIPT3	***Malus x domestica***	**HQ585948.1**
PnIPT1	***Ipomoea nil***	**AB371300.1**
PnIPT2	***Ipomoea nil***	**AB371301.1**
LjIPT1	***Lotus japonicus***	**DQ436462**
LjIPT2	***Lotus japonicus***	**DQ436463**
LjIPT3	***Lotus japonicus***	**DQ436464**
LjIPT4	***Lotus japonicus***	**DQ436465**
PsIPT1	***Pisum sativum***	**AB194606**
PsIPT2	***Pisum sativum***	**AB194607**
GmIPT1	***Glycine max***	**AY550884**
ZmIPT1	***Zea mays***	**EU263125**
ZmIPT2	***Zea mays***	**EU263126**
ZmIPT4	***Zea mays***	**EU263127**
ZmIPT5	***Zea mays***	**EU263128**
ZmIPT6	***Zea mays***	**EU263129**
ZmIPT7	***Zea mays***	**EU263130**
ZmIPT8	***Zea mays***	**EU263131**
OsIPT1	***Oryza sativa***	**AB239797**
OsIPT2	***Oryza sativa***	**AB239798**

OsIPT3	***Oryza sativa***	**AB239799**
OsIPT4	***Oryza sativa***	**AB239800**
OsIPT5	***Oryza sativa***	**AB239801**
OsIPT6	***Oryza sativa***	**AB239803**
OsIPT7	***Oryza sativa***	**AB239804**
OsIPT8	***Oryza sativa***	**AB239805**
RfIPT	***Rhodococcus fascians***	**X62428**

5.4 CKX genes used for bioinformatics

Gene name	Species	Accession No
BrCKX1	***Brassica rapa***	**AB331924**
BoCKX1	***Brassica oleracea***	**AB331918**
DsCKX1	***Dendrobium sonia***	**AJ294542.2**
OsCKX2	***Oryza sativa***	**AB205193.1**
OsCKX11	***Oryza sativa***	**gi|42409493**
ZvCKX	***Zinnia violacea***	**AB439582.1**
ZmCKX1	***Zea mays***	**NM_001112121.1**
ZmCKX2	***Zea mays***	**NM_001112056.1**
ZmCKX3	***Zea mays***	**NM_001111693.1**
ZmCKX4	***Zea mays***	**GU160398.1**
ZmCKX5	***Zea mays***	**GU160400.1**
ZmCKX6	***Zea mays***	**GU160401.1**
GhCKX	***Gossypium hirsutum***	**FJ644277.1**
StCKX	***Solanum tuberosum***	**DQ822452.1**
MtCKX	***Medicargo trun catula***	**XM_003625052.1**
TrCKX2	***Trifolium repens***	**JF968418.1**
PhCKX1	***Petunia x hybrida***	**AB588039.1**
PsCKX1	***Pisum sativum***	**EF030477**
PtCKX	***Populus triocarpa***	**XM_002307645.1**

5.5 Amplification plots



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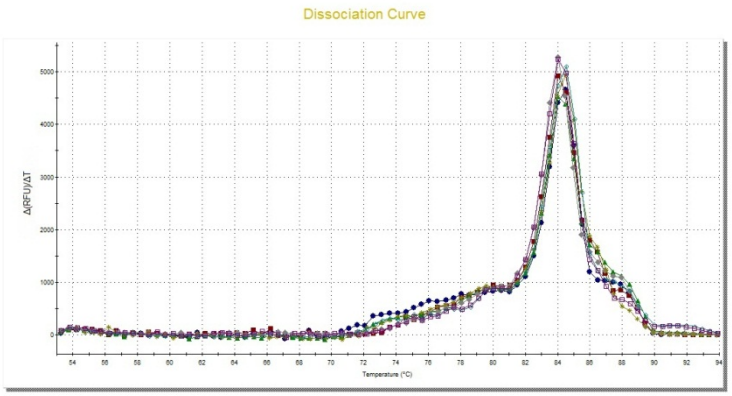
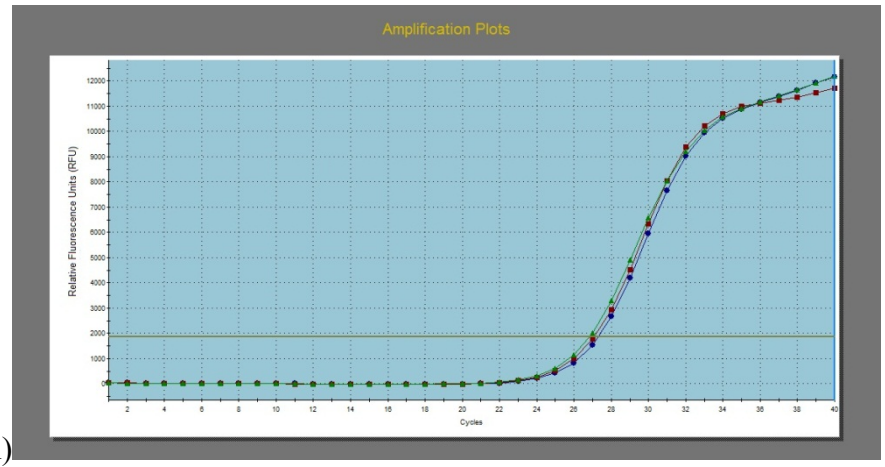


(B)

Figure 5.5.1 BnIPT3 amplification plot

BnIPT3 amplification plot (A) and melting curve (B), qPCR performed with leaf and flower cDNAs and the BnIPT3 primers BnIPT3F/ BnIPT3R.

(A)



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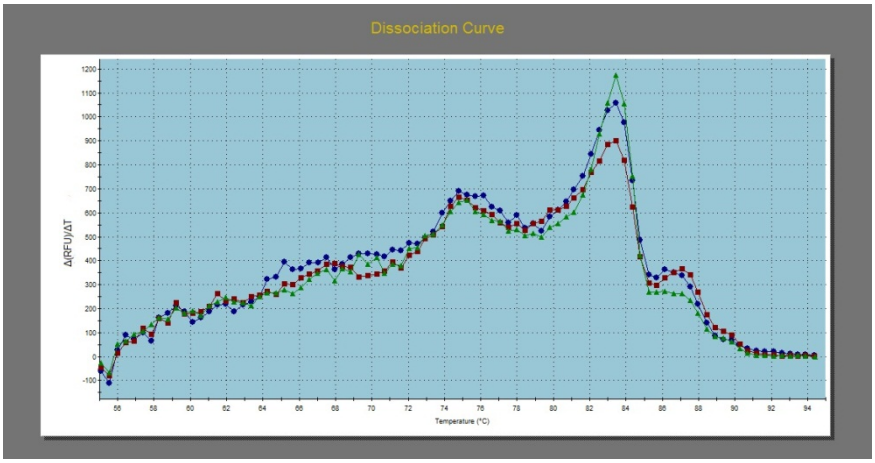
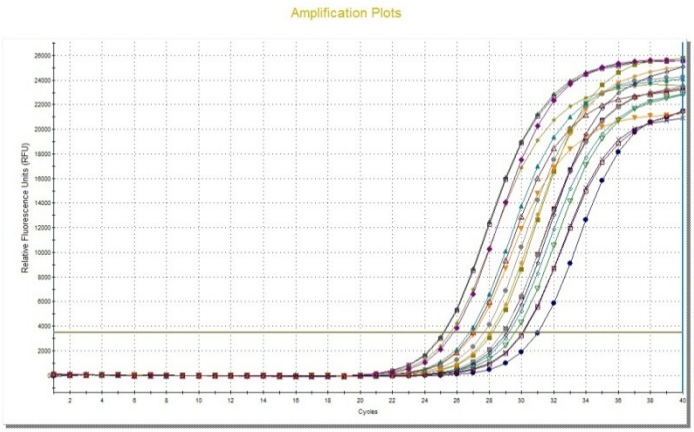


Figure 5.5.2 *BnIPT5* amplification plot

BnIPT5 amplification plot (A) and melting curve (B), qPCR performed with leaf and flower cDNAs and the *BnIPT5* primers *BnIPT5F*/ *BnIPT5R*.

(A)



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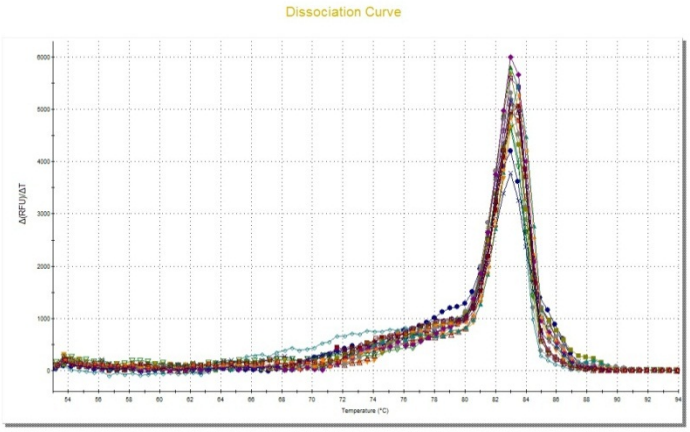
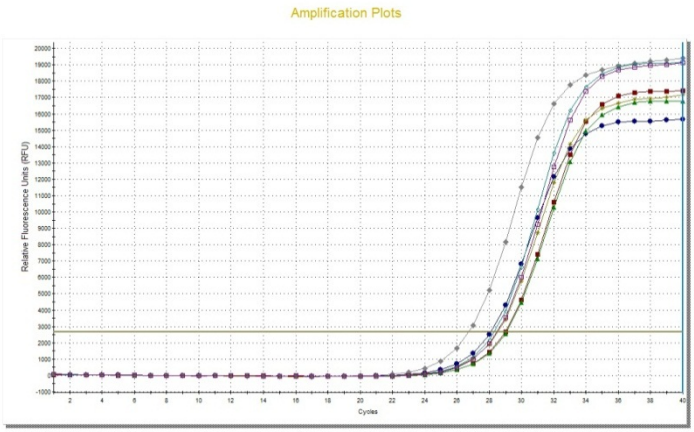


Figure 5.5.3 *BnIPT7* amplification plot

BnIPT7 amplification plot (A) and melting curve (B), qPCR performed with leaf and flower cDNAs and the *BnIPT7* primers *BnIPT7F*/ *BnIPT7R*.

(A)



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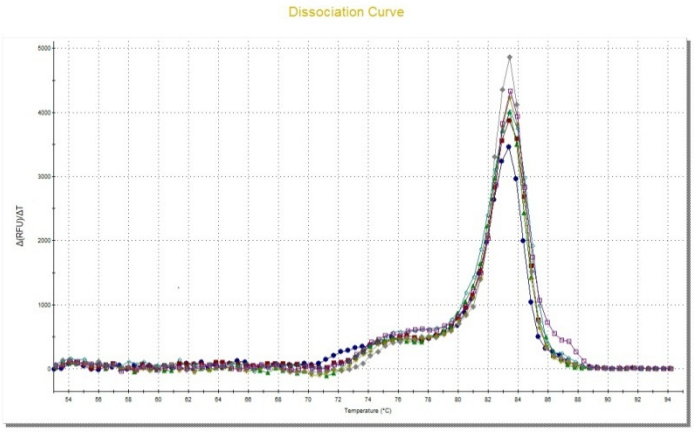
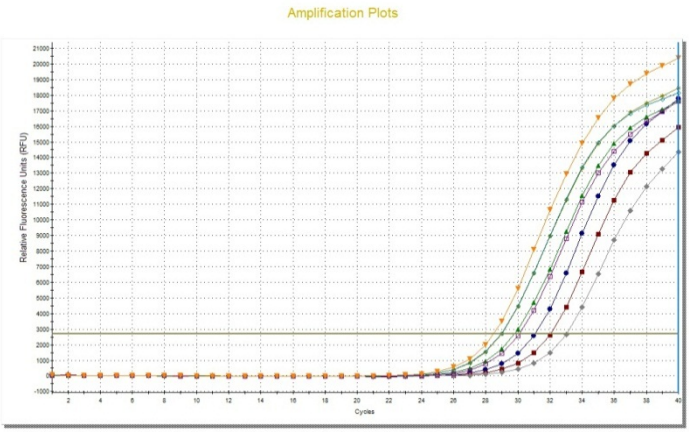


Figure 5.5.4 *BnCKX1* amplification plot

BnCKX1 amplification plot (A) and melting curve (B), qPCR performed with leaf and flower cDNAs and the *BnCKX1* primers *BnCKX1F*/ *BnCKX1R*.

(A)



(B)

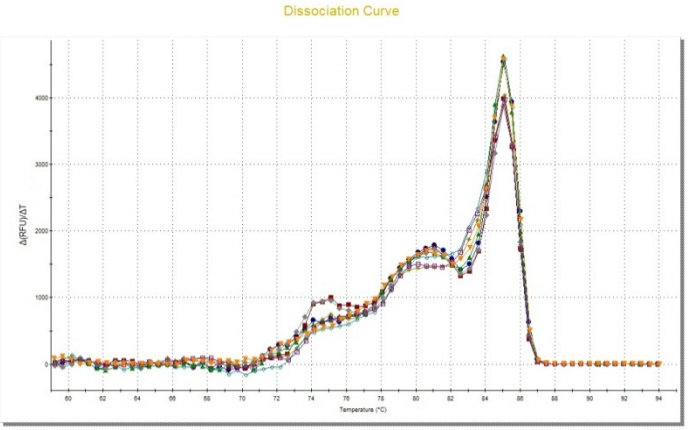
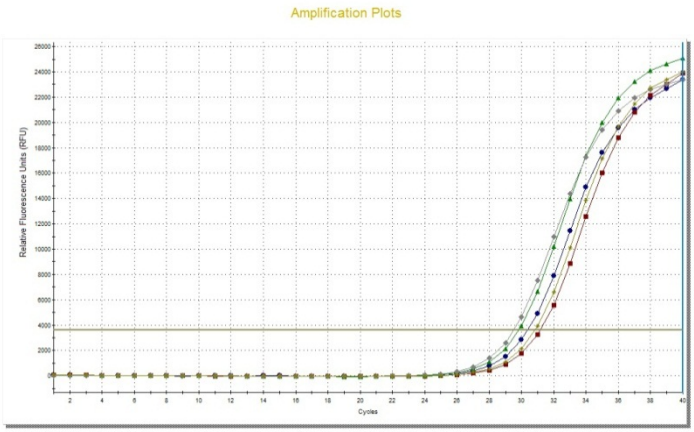


Figure 5.5.5 *BnCKX2* amplification plot

BnCKX2 amplification plot (A) and melting curve (B), qPCR performed with leaf and flower cDNAs and the *BnCKX2* primers *BnCKX2F*/ *BnCKX2R*.

(A)



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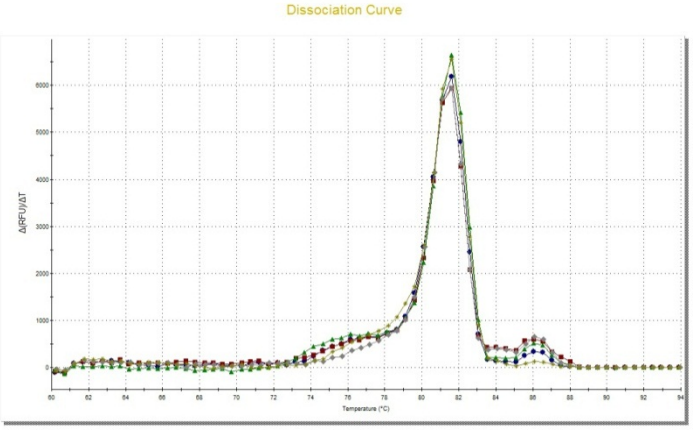
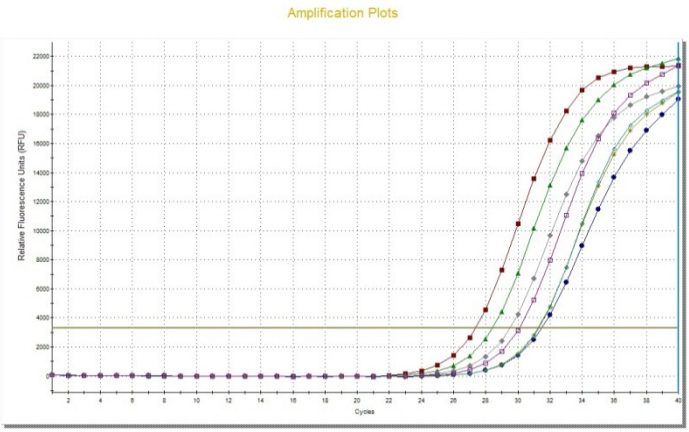


Figure 5.5.6 *BnCKX3* amplification plot

BnCKX3 amplification plot (A) and melting curve (B), qPCR performed with leaf and flower cDNAs and the *BnCKX3* primers *BnCKX3F*/ *BnCKX3R*.

(A)



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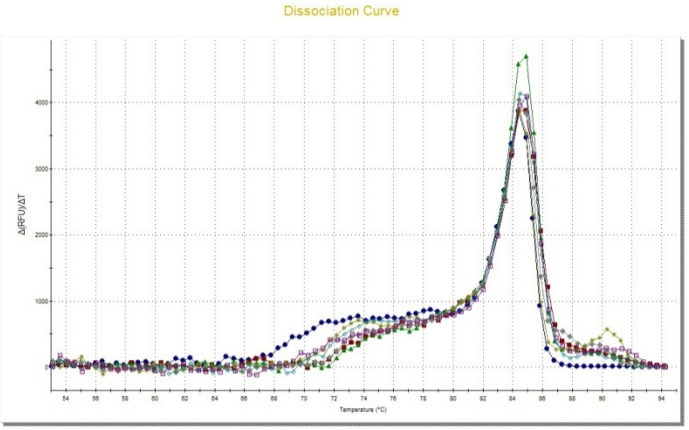
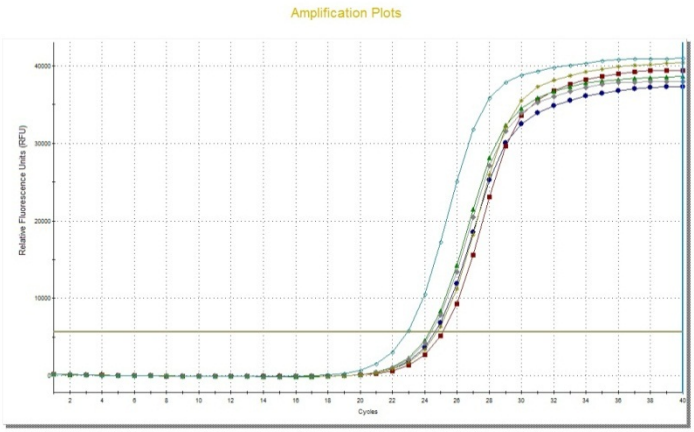


Figure 5.5.7 *BnCKX5* amplification plot

BnCKX5 amplification plot (A) and melting curve (B), qPCR performed with leaf and flower cDNAs and the *BnCKX5* primers *BnCKX5F*/ *BnCKX5R*.

(A)



(B)

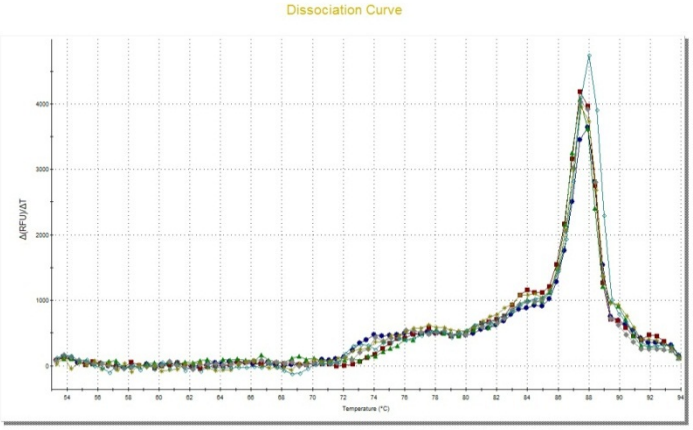


Figure 5.5.8 BnCKX7 amplification plot

BnCKX7 amplification plot (A) and melting curve (B), qPCR performed with leaf and flower cDNAs and the *BnCKX7* primers *BnCKX7F*/ *BnCKX7R*.