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The Functional Characterization of the SnRK1 Protein Complex in Hybrid Poplar

by

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Dedication

To my family, especially Holly, for everything.

Abstract

The sucrose-nonfermenting-1 related kinase 1 (SnRK1) protein complex is a heterotrimeric serine/threonine protein kinase complex conserved in eukaryotes that acts as a regulator of carbon metabolism and energy homeostasis. The objective of this study was to determine if the SnRK1 protein complex has a role in the nitrogen response and during dormancy acquisition in poplar. Gene expression profiling of the *PtdSnRK1*, *PtdAKINβ*, and *PtdAKINγ* gene family members was carried out using a robust qRT-PCR assay. A subset of these genes showed modified expression patterns under differential nitrogen availability and during dormancy acquisition, suggesting that SnRK1 complexes comprised of specific subunits may be involved in the regulation of the response to nitrogen and during dormancy acquisition. The regulatory subunits *PtdAKINβ1.1*, *PtdAKINγ1.1* and *PtdAKINγ2.3* were often identified using principal component analysis as significantly responsible for distinguishing treatments from one another and therefore merit further study.

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List of Abbreviations

5PTase13 – myoinositol polyphosphate 5-phosphatase 13

ABA – abscisic acid

AGPase – ADP-glucose pyrophosphorylase

AREBP – ABA response element binding protein

AMPK – AMP-dependent kinase

AMPKβ - AMP-dependent kinase beta

AMPKγ - AMP-dependent kinase gamma

ASC domain – association with SNF1 complex

BSP – bark storage proteins

CBS motif – cystathionine β-synthase

EST – expressed sequence tag

F2KP – 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase

fru-2,6-P_2 – fructose 2,6-biphosphate

G6P – glucose-6-phosphate

GRIK1 – geminivirus Rep-interacting kinase-1

GRIK2 – geminivirus Rep-interacting kinase-1

HMG-CoA reductase – 3-hydroxy-3-methyl-glutaryl-CoA reductase

$InsP_3$ - inositol 1,4,5-trisphosphate ($InsP_3$)

KIS – kinase-interacting sequence

LPI – leaf plasticron index

MBSU - Molecular Biology Service Unit

MEP – methylerythritol 4-phosphate

MVA – mevalonate

NMT – N-myristoyltransferase

NR – nitrate reductase

PRKAB - protein kinase, AMP-activated, beta

PRKAG – protein-kinase, AMP-activated gamma

PRL1 – pleiotropic regulatory locus 1

qRT-PCR – quantitative reverse transcription polymerase chain reaction

SD – short days

SnAK1 – SnRK1-activating kinase-1

SnAK2 – SnRK1-activating kinase-2

SNF1 – sucrose-nonfermenting-1

SnRK – sucrose nonfermening 2 related kinase

SPS – sucrose phosphate synthase

T6P – trehalose-6-phosphate

TPS5 – trehalose-6-phosphate synthase 5

tZR – *trans*-zeatin riboside

VSP – vegetative storage proteins

1.0. Introduction

1.1. The SnRK1 protein complex

1.1.1. Overview of the SNF1/AMPK/SnRK1 protein kinases

SNF1/AMPK/SnRK1 complexes are evolutionarily conserved heterotrimeric serine/threonine protein kinases which have been studied in yeast, plants and mammals (Polge and Thomas, 2007; Halford and Hey, 2009). The three subunits making up the complex are generally termed the catalytic α subunit and the regulatory β and γ subunits (reviewed in Hardie, 2007). The specific names given to these subunits can vary depending on the species being studied. The α subunit contains a serine/threonine kinase domain at the N terminus; the C-terminal region is required for the formation of the complex with the β and γ subunits. The β subunit is sometimes called a scaffolding protein because its C-terminal appears to bind to both the α and γ subunits. The β subunit also contains a glycogen binding domain. The γ subunit contains regions which interact with the β subunit as well as four tandem cystathionine β-synthase (CBS) motifs which are involved in binding to AMP/ATP in mammals.

1.1.1.1. SNF1 in yeast

The SNF1 (sucrose non-fermenting 1) protein complex has a central role in energy homeostasis by responding to starvation stress caused by low glucose. The SNF1 protein complex regulates the transcription of genes involved in the metabolism of alternative carbon sources, gluconeogenesis, respiration, transport and meiosis, as well as directly regulating enzymes involved in fatty acid metabolism and carbohydrate storage (reviewed in Hedbacker and Carlson, 2008). SNF1 was first identified in a screen for yeast mutants that were unable to activate the invertase gene (*SUC2*) in response to glucose deprivation (Carlson *et al.*, 1981). The *snf1* mutations had pleiotropic effects, preventing the utilization of sucrose, galactose, maltose and other nonfermentable carbon sources. These carbon sources are alternate sources of energy and their utilization is glucose-repressible. Characterization of the *SNF1* gene showed that it encoded a 72 kD protein kinase (Celenza and Carlson, 1986). Later studies identified the β subunits SIP1, SIP2 (SNF1-interacting protein-1 and -2) and GAL83 (galactose metabolism-83) through a yeast two-hybrid assay and homology analysis (Yang *et al.*, 1992; Yang *et al.*, 1994) and the γ subunit SNF4 (Celenza and Carlson, 1989; Celenza *et al.*, 1989). The β subunits interact with both the α subunit at the KIS (kinase-interacting sequence) domain and the γ subunit at the ASC (association with SNF1 complex) domain (Yang *et al.*, 1994; Jiang and Carlson, 1997).

1.1.1.2. AMPK in mammals

The AMPK (AMP kinase) complex is found in mammals and has been characterized as a fuel gauge due to its ability to respond to increased AMP/ATP ratio. However, the AMPK complex has a role in a wide variety of physiological processes beyond energy sensing, such as the regulation of glucose uptake in muscle cells, regulation of cell growth and proliferation, establishment of cell polarity and response to oxidative stress (Hardie, 2007; Li and Keaney Jr., 2010). AMPK was named for its activation through allosteric interaction with AMP (Carling *et al.*, 1987; Carling et al., 1989) and was initially discovered through biochemical assays of protein phosphorylation (Beg e*t al.*, 1973; Carlson and Kim, 1973). Amino acid and nucleotide sequencing combined with phylogenetic and functional analyses showed that AMPK showed sequence similarity to the yeast SNF1, and AMPK was found to be functionally homologous to SNF1 (Carling *et al.*, 1994; Davies *et al.*, 1994; Mitchelhill *et al.*, 1994; Gao *et al.*, 1995; Woods *et al.*, 1996) and, like the SNF1 complex, is a heterotrimeric complex with an α catalytic subunit (AMPKα) and two regulatory subunits, β (AMPKβ or protein kinase, AMP-activated, beta (PRKAB)) and γ (AMPKγ, or protein-kinase, AMP-activated gamma (PRKAG)). Thus far, two isoforms of AMPKα, two isoforms of AMPKβ, and three isoforms of AMPKγ have been identified in mammals (Hardie, 2007)

1.1.1.3. SnRK1 in plants

For consistency, in plants the α subunit will be called SnRK1, the β subunit

will be called AKINβ and the γ subunit will be called AKINγ, except when referring to specific isoforms at which point the names used by the authors of the study will be used.

The SnRK1 (SNF1-related protein kinase) complex and its subunits have been largely identified by taking advantage of the homologous functions found between the SNF1, AMPK and SnRK1 complexes. *SnRK1*, the α subunit, was found in rye through complementation of the *snf1* mutation (Alderson *et al.*, 1991) and has also been studied in other plant species such as *Brassica oleracia, Hordeum vulgare, Arabidopsis thaliana, Nicotiana tabacum, Spinacia oleracea* and *Solanum tuberosum* (Halford *et al.*, 1992; Le Guen *et al.*, 1992; MacKintosh *et al.*, 1992; Muranaka *et al.*, 1994; Hannappel *et al.*, 1995; Man *et al.*, 1997; Sugden *et al.*, 1999*a,b*) . β subunits have been identified in *S. tuberosum* (Lokatos *et al.*, 1999) and *Arabidopsis* (Bouly *et al.*, 1999) using yeast two-hybrid assays, and γ subunits have been found in Arabidopsis using yeast-two hybrid assays and complementation of the yeast *snf4* mutant (Bouly *et al.*, 1999; Kleinow *et al.*, 2000).

Plants have unique gene families which encode members of the SnRK1 complex which are not found in fungi or mammals. The SnRK1 gene family is closely related to two other kinase families called SnRK2 and SnRK3 (Halford *et al.*, 2000; Hrabak *et al.*, 2003). Members of the SnRK2 family have been linked to response to abscisic acid (ABA) and some may be regulated by calcium while the SnRK3 family is involved in responses to salt stress and in sugar and ABA

signalling (reviewed in Hey *et al.*, 2010).

Plants also have a unique protein which contains an N-terminal KIS domain (usually seen in the β subunit) fused with a C-terminal usually found in the γ subunit (Lumbreras *et al.*, 2001). Given its structure, it is called AKINβγ and has been shown to both interact with SnRK1 and complement the yeast *snf4* mutation (Lumbreras *et al.*, 2001). Although it contains components of both the β and γ subunits, in *Arabidopsis* it appears that AKINβγ interacts in heterotrimeric complexes with SnRK1 and AKINβ and may be involved in pathogen resistance (Gissot *et al.*, 2006).

Other families related to AKINγ which appear to be unique to plants include the SnIP1 family and the PV42 family. The SnIP1 family was isolated in barley through two-hybrid screening with barley SnRK1 (Slocombe *et al.*, 2002). Sequence similarity search revealed sequences similar to SnIP1 in maize, *Arabidopsis*, and poplar. Homologues were not found outside of plants and, despite weak sequence similarity to yeast SNF4, SnIP1 was unable to complement a yeast *snf4* mutant (Slocombe *et al.*, 2002). The PV42 family, originally found in bean, also shows similarity to SNF4 but only shows similarity to the SnIP1 family at a short hydrophobic motif called the SnIP motif (Abe *et al.*, 1996; Slocombe *et al.*, 2002)

1.1.2. Characterized roles of the SnRK1 complex in plants

The SnRK1 complex appears to be a key modulator of carbon metabolism, acting as a central hub through which different signals are carried to different signalling networks through phosphorylation of specific targets and affecting the transcriptional activation of genes though it is not itself a transcription factor (Halford and Hey, 2009). Recent microarray results in transgenic *Arabidopsis KIN10* (a *SnRK1*) overexpression or *KIN10*-RNAi reduced expression lines demonstrate that misexpressing *KIN10* affects the transcriptional expression of hundreds of target genes (Baena-González *et al.*, 2007). Comparison of the target genes of *KIN10* to published data sets showed that gene expression profiles caused by sugar and energy starvation conditions positively correlated with *KIN10* target genes. Furthermore, the expression profiles of glucose- or sucrose-treated seedlings and adult leaves in intact plants grown in ambient CO_2 compared to low CO_2 negatively correlated with *KIN10* target genes. The data suggest that *SnRK1* plays a key role in mobilization of resources during energy deprivation (Baena-González *et al.*, 2007; Baena-González and Sheen, 2008).

Although *SnRK1* clearly has a major effect on the transcriptome, the exact mechanism of how this protein kinase acts to affect gene transcription is not very well understood. While it has been shown that *SnRK1* regulation of the transcriptome is mediated in part by *bZIP* transcription factors, the signal transduction chain between these two regulators has yet to be fully elucidated (Baena-González *et al.*, 2007). The SnRK1 complex is known to directly regulate some processes, however, as several enzymes and other proteins that are direct

targets of SnRK1 phosphorylation have been discovered. These processes and identified SnRK1 targets are discussed below.

1.1.2.1. SnRK1 regulation of starch biosynthesis

Transgenic plants have been essential in showing the role of the SnRK1 complex in starch biosynthesis. Transgenic barley expressing *SnRK1* antisense RNA showed that pollen grains developed abnormally, arresting at the binucleate stage of development, and contained little to no starch (Zhang *et al.*, 2001). In rice, expression of reporter genes using *SnRK1* promoters showed that the expression of one *SnRK1* promoter correlated with starch accumulation in the pericarp, endosperm cells and basal part of the leaf sheath (Kanegae *et al.*, 2005). Consistent results were also found in maize and sorghum endosperm, and in maize microspores (Jain *et al.*, 2008). Transgenic potatoes overexpressing *SnRK1* under the control of a tuber-specific promoter were found to have increased starch levels in the tubers as well as decreased levels of glucose, although sucrose and fructose levels did not change (McKibbin *et al.*, 2006).

The SnRK1 protein complex appears to regulate starch biosynthesis, in part, through the transcriptional regulation of *sucrose synthase* and *α-amylase*. *Sucrose synthase* expression in potato tubers is reduced in transgenic potatoes expressing SnRK1 antisense RNA (Purcell *et al.*, 1998). *Sucrose synthase* expression was increased in transgenic potatoes overexpressing *SnRK1* under the control of a

tuber-specific promoter (McKibbin *et al.*, 2006). Sucrose synthase is responsible for the reversible conversion of sucrose and UDP to UDP-glucose and fructose. Sucrose synthase is a major determinant of tuber sink strength, and transgenic potatoes expressing *sucrose synthase* antisense RNA show reduced starch accumulation but increased glucose and fructose (Zrenner *et al.*, 1995).The α-amylase enzyme is responsible for starch hydrolysis and is therefore involved in the mobilization of stored carbohydrates. In wheat, it has been shown that the expression of a *SnRK1* antisense RNA leads to the repression of the α-amylase promoter (Laurie *et al.*, 2003).

Another avenue through which the SnRK1 protein complex acts is through redox regulation of ADP-glucose pyrophosphorylase (AGPase). Like sucrose synthase, AGPase expression is increased in transgenic potatoes overexpressing a *SnRK1* transcript (McKibbin *et al.*, 2006). AGPase catalyzes the first step of starch synthesis in the plastid and is subject to postranslational redox inactivation (Fu *et al.*, 1998; Ballicora *et al.*, 2000) which is prevented by glucose or sucrose in wildtype *Arabidopsis* (Tiessen *et al.*, 2003). Feeding of sucrose to tuber discs expressing antisense *SnRK1* was unable to prevent redox inactivation of AGPase, whereas feeding of glucose was able to do so, indicating that the SnRK1 protein kinase modulates the sucrose-dependent pathway (Tiessen *et al.*, 2003). Since *AGPase* is inducible by sucrose (Müller-Röber *et al.*, 1990), it is likely that the overexpression of *SnRK1* was a contributing factor of increased expression of *AGPase*, rather than induction caused by some other source (McKibbin *et al.*,

2006).

1.1.2.2. SnRK1 regulation of isoprenoid biosynthesis

Isoprenoids are a diverse class of chemicals. Isoprenoid compounds include phytosterols, which act as membrane components and hormones; carotenoids, which act as photosynthetic pigments and antioxidants; terpenoids that act as defense compounds; signalling compounds to other species which interact with or form symbiotic relationships with the plant; and other secondary metabolites (reviewed in, for example, Bouvier *et al.*, 2005; Gershenzon and Dudareva, 2007; Boutté and Grebe, 2009; Cazzonelli and Pogson, 2010). HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase) catalyzes the reduction of HMG-CoA to mevalonic acid, which is a key step in isoprenoid biosynthesis through the cytosolic mevalonate (MVA) pathway. Plants also have a second pathway of isoprenoid synthesis, through the plastidic methylerythritol 4-phosphate (MEP) pathway.

SnRK1 inhibits HMG-CoA reductase through phosphorylation of the serine-577 site, which is the same site inhibited by AMPK when phosphorylating mammalian HMG-CoA reductase (Ball *et al.*, 1994; Dale *et al.*, 1995; Ball *et al.*, 1995; Barker *et al.*, 1996; Sugden *et al.*, 1999b). Expression of modified HMG-CoA reductase lacking the SnRK1 phosphorylation site in tobacco showed increased expression of phytosterols in seeds, although not in leaves, suggesting a

seed-specific function of SnRK1 modulation of HMG-CoA reductase in phytosterol production in seeds (Hey *et al.*, 2006). The expression of the modified HMG-CoA reductase also led to aberrant flower phenotypes, such as shortened stamens and late anther development with little to no pollen production. Although the flower phenotypes were not correlated strongly with measured phytosterols known to have roles as hormones for growth and development, undetected and subtle changes may be the cause of the altered flower phenotypes (Hey *et al.*, 2006).

1.1.2.3. Roles for SnRK1 in ABA signalling

ABA is a phytohormone involved in plant growth, development and stress signalling (reviewed in, for example, Agarwal and Jha, 2010). As mentioned above, SnRK2 and SnRK3 appear to have a role in crosstalk with the ABA signalling pathway but evidence suggests that SnRK1 may also be involved. Antisense *SnRK1* in pea embryos leads to a phenotype similar to an ABA-insensitive phenotype (Radchuk *et al.*, 2006) while overexpression of *SnRK1* in *Arabidopsis* leads to an ABA hypersensitive response (Jossier *et al.*, 2009). In tomato and *Medicago truncatula*, *AKIN*γ is inducible by ABA (Bradford *et al.*, 2003; Bolingue *et al.*, 2010). SnRK1 may act through AREBPs (ABA response element binding proteins), which were found to have highly conserved SnRK1 target sites which, when expressed in peptides, are phosphorylated by SnRK1

(Zhang *et al.*, 2008). AREBPs are a family of bZIP transcription factors, which have been shown to mediate in part the role of the SnRK1 complex in the transcriptional regulation (Baena-González *et al.*, 2007).

1.1.2.4. Other SnRK1 targets

The SnRK1 complex has been shown to directly phosphorylate other targets involved in sucrose synthesis and nitrogen metabolism. In the case of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (F2KP), nitrate reductase (NR) and trehalose-6-phosphate synthase 5 (TPS5), the phosphorylated enzyme becomes associated with a 14-3-3 protein. 14-3-3 proteins bind to phosphorylated consensus motifs and act to aid conformational changes or by modulating interactions with other molecules (for review see Oeking and Jaspert, 2009)

F2KP catalyses the synthesis and degradation of fru-2,6-P_2 (fructose 2,6-biphosphate). Fru-2,6-P_2 is a signal metabolite which is believed to regulate sucrose synthesis (reviewed in Stitt, 1990). Transgenic plants with decreased fru-2,6-P_2 are found to have increased sucrose synthesis while increased fru-2,6-P_2 stimulates flux towards starch (Truesdale *et al.*, 1999; Theodorou and Kruger, 2001; Draborg *et al.*, 2001). F2PK can be phosphorylated by AMPK (at the time purified SnRK1 was unavailable) (Kulma *et al.*, 2004). Sucrose phosphate synthase (SPS), which catalyzes the reversible conversion of UDP-glucose and D-fructose 6-phosphate to UDP and sucrose 6-phosphate is also phosphorylated and

inactivated by SnRK1 (Sugden *et al.*, 1999b). Also, evidence suggests that at least one TPS5 can be phosphorylated by SnRK1 (Harthill *et al.*, 2006).

NR catalyzes the reduction of nitrate to nitrite, the first step in the assimilation of inorganic nitrogen. SnRK1 is able to phosphorylate and inactivate nitrate reductase (Douglas *et al.*, 1997; Sugden *et al.*, 1999b; Jossier *et al.*, 2009). Evidence suggests that the interaction of the SnRK1 complex and NR is mediated by the AKINβ1 subunit (Polge *et al.*, 2008). This activity is suggestive of a role for the SnRK1 complex in crosstalk between carbon and nitrogen metabolism. Indeed, transcription profiles in *Arabidopsis* indicate that the SnRK1 complex is involved in the transcriptional regulation of amino acid and protein synthesis and degradation (Baena-González *et al.*, 2007; Baena-González and Sheen, 2008).

1.1.3. Regulation of the SnRK1 complex in plants

1.1.3.1. Post-translational modification

The SnRK1 complex is regulated in part by reversible phosphorylation of its catalytic subunit on a threonine residue in the T-loop (Sugden *et al.*, 1999a; Jossier *et al.*, 2009). The *in vivo* identity of phosphatases which act on SnRK1 is unknown. It has been shown that phosphatase inactivation of SnRK1 in spinach is inhibited by 5'AMP (Sugden *et al.*, 1999a), although unlike AMPK, 5'AMP does not interact allosterically with the SnRK1 catalytic subunit.

Phosphorylation of SnRK1 is believed to be mediated by SnRK1-activating kinase-1 and -2 (SnAK1/2, also called GRIK1/2; Shen and Hanley-Bowdoin, 2006; Hey *et al.*, 2007). SnAK1/2 are able to functionally complement a yeast *elm1 sak1 tos3* triple mutant, which lacked upstream kinases for SNF1 (Hey *et al.*, 2007). SnAK1/2 are shown to interact with the geminivirus protein AL1 and are shown to have elevated protein levels in leaves during viral infection (Shen and Hanley-Bowdoin, 2006), hence the alternative name of GRIK1/2 (geminivirus Rep-interacting kinase-1 and -2). The interaction of AL1 with SnAK1/2 may be an early response to viral infection but it may be counteracted by the interaction of other geminivirus proteins AL2/L2 which have been shown to bind and inhibit SnRK1 (Hao *et al.*, 2003).

N-myristoyltransferase (NMT) catalyzes *N*-myristoylation, which involves the addition of the fatty acid myristate to proteins for a variety of reasons ranging from subcellular targeting to signaling (reviewed in Sorek *et al.*, 2009). NMT was shown to interact with two AKINβ subunits in *Arabidopsis*, inhibiting the SnRK1 complex and relocalizing the AKINβ subunits from the plasma membrane to either the nucleus or the cytosol (Pierre *et al.*, 2007).

1.1.3.2. Pleiotropic regulatory locus 1 (PRL1) and myoinositol polyphosphate 5-phosphatase 13 (5PTase13)

PRL1 is a regulatory protein containing a WD40 motif and is believed to act

as a negative regulator of glucose-responsive genes (Neméth *et al.*, 1998). *Arabidopsis prl1* mutants show hypersensitivity to growth arrest caused by feeding with glucose and sucrose. Mutant seedlings grown in the presence of sucrose showed inhibited stem and root growth and increased glucose, fructose, sucrose and starch content in leaves. Mutant seedlings were also sensitive to hormones cytokinin, ethylene, abscisic acid and auxin and showed transcriptional derepression of glucose-regulated genes (Neméth *et al.*, 1998). Furthermore, it was demonstrated that SnRK1 phosphorylation of sucrose phosphate synthase was enhanced in *prl1* mutants. A yeast two-hybrid assay confirmed the interaction of PRL1 and SnRK1. It was also demonstrated that PRL1 is able to inhibit the kinase activity of SnRK1 *in vitro* (Bhalerao *et al.*, 1999).

The inhibition of SnRK1 may involve proteosomal degradation, as PRL1 is believed to be a substrate receptor for a CUL4-ROC1-DDB1-PRL1 E3 ligase (Lee *et al.*, 2008). PRL1 binding appears to be antagonistic or competitive with SnRK1 binding to SKP1/ASK1, part of the SCF ubiquitin ligase (Farras *et al.*, 2001), where SnRK1 may be involved in phosphorylation of proteasomal subunits (Farras *et al.*, 2001).

Inositol 1,4,5-triphosphate ($InsP_3$) is a driver of the inositol signalling pathway and is modulated by 5PTases. The WD40 repeat region of the *5PTase13* gene has been shown to interact with SnRK1. *5ptase13* mutants were shown to have decreased SnRK1 activity in conditions of low-nutrient or sugar conditions due to proteasomal degradation of SnRK1 (Ananieva *et al.*, 2008). This particular

phenotype contrasts with the phenotype of *prl1* mutants. Furthermore, while the *prl1* is sugar and ABA hypersensitive, *5ptase13* mutants displayed the opposite. This suggests that 5PTase13 and PRL1 may have opposing roles in regulating SnRK1 degradation.

1.1.3.3. Sugar signalling

1.1.3.3.1. Glucose-6-phosphate (G6P)

Early evidence in spinach showed inconsistent inactivation of SnRK1 by G6P *in vitro* (Sugden *et al.*, 1999b). It was initially believed that the inactivation was caused by contaminants in commercial preparations of G6P, as subsequent purification of G6P showed that there was no inactivation of SnRK1 (Sugden *et al.*, 1999b). Later studies suggested that SnRK1 inhibition by G6P can be lost upon storage of the enzyme at 0^{o}C and that, under Mg^{2+} concentrations and pH levels which are closer to physiological levels, G6P can act as an inhibitor of SnRK1 kinase activity (Toroser *et al.*, 2000). Zhang *et al.* (2009) also confirmed inhibition of SnRK1 by G6P in desalted *Arabidopsis* tissue extracts.

1.1.3.3.2. Trehalose-6-phosphate (T6P)

T6P is a precursor of trehalose and acts as a signalling molecule in plants

(reviewed in Paul *et al.*, 2008; Smeekens *et al.*, 2010). In *Arabidopsis*, increased T6P correlates with increased expression of *SnRK1* (Schluepmann *et al.*, 2004). Furthermore, feeding of trehalose (which leads to increased levels of T6P) leads to the redox activation of AGPase and stimulates starch biosynthesis (Kolbe *et al.*, 2005). T6P acts through an unknown intermediate to inhibit SnRK1 in *Arabidopsis* seedling and young tissue (Zhang *et al.*, 2009). Interestingly, the unknown intermediate appears to be missing from mature leaves. The transcriptome profile of seedlings overexpressing *T6P synthase* (which would elevate levels of T6P) appears to be the opposite of the transcriptome profile of *Arabidopsis* overexpressing *SnRK1* (Baena-González *et al.*, 2007; Zhang *et al.*, 2009). The profile was not as significantly correlated between seedlings overexpressing *T6P phosphatase* (which would lower levels of T6P) and *Arabidopsis* overexpressing *SnRK1*, but this may be due to a higher level of G6P (Schluepmann *et al.*, 2003), which could result in inhibition of *SnRK1*.

1.1.4. Using poplar to explore potential roles for SnRK1 in woody perennials

All studies conducted to date on the SnRK1 complex in plants have been conducted in herbaceous (annual) model organisms such as *Arabidopsis*. While this knowledge can be used to make inferences about the roles that SnRK1 may play in woody perennials such as forest trees, nothing is known about how SnRK1 may function in processes that are different in or unique to woody perennials.

Poplar is an ideal model for investigating SnRK1 roles in such processes because of its rapid growth, relative ease of experimental manipulation and growing range of genetic tools, including the genomic sequence of *Populus trichocarpa* (Tuskan *et al.*, 2006; Jansson and Douglas, 2007). In this thesis, I have focused on two processes that are either different in or unique to woody perennials compared to herbaceous annuals: responses to nitrogen availability and dormancy acquisition (Jansson and Douglas, 2007).

1.2. Genus *Populus*

Poplars (genus *Populus*) are fast-growing, dioecious, deciduous or semievergreen trees (Eckenwalder, 1996). The genus is traditionally divided into morphologically and ecologically similar groups called sections. Barriers to hybridization typically exist between sections, although not always, and this makes a consensus of the number of *Populus* species difficult to achieve (Echkenwalder, 1996). Species native to Canada include aspen (*Populus tremuloides* Michx.), cottonwood (*Populus deltoides* Bartr. ex Marsh.), black cottonwood (*Populus trichocarpa* Torr. & A. Gray), balsam poplar (*Populus balsamifera* L.), narrowleaf cottonwood (*Populus angustifolia* James), and largetooth aspen (*Populus grandidentata* Michx.) (Richardson *et al.*, 2007). Some of these species readily hybridize with each other, such as *P. deltoides* and *P. angustifolia*. Many of these species also readily hybridize with poplars which are

not native to North America that are used in agricultural shelterbelts and urban settings, such as *P. nigra*.

Since poplars are fast-growing, diverse, grow in a variety of environmental conditions and readily hybridize, they have been cultivated since historical times (Richardson *et al.*, 2007). While they are cultivated for a variety of wood products, they are also used for stream bank protection, windbreaks and shelterbelts, in remediation, and have been studied as a potential carbon sink to offset climate change. For instance, financial analysis in Alberta of hybrid poplar stands suggests that hybrid poplars are potentially financially viable candidates for intensive forest management which would increase the value of the boreal forest resources without making use of additional land which may have competitive uses, such as for agriculture (Anderson and Luckert, 2007). As another example, the Forest 2020 Plantation Demonstration and Assessment Initiative, which was created to ascertain the viability of mitigating greenhouse gas emissions through afforestation, made extensive use of hybrid poplar plantations (Dominy *et al.*, 2010).

The tools for molecular and genomic studies in poplar have increased significantly in recent years, most notably with the sequencing of the poplar genome (Tuskan *et al.*, 2006). A variety of tools are now freely available online to facilitate poplar research (reviewed in Yang *et al.*, 2009), including PopulusDB (http://www.populus.db.umu.se/; Sterky *et al.*, 2004), an EST database built from 19 cDNA libraries; the poplar eFP browser

(http://bbc.botany.utoronto.ca/efppop/cgi-bin/efpWeb.cgi; Wilkins *et al.*, 2009), which is a user-friendly graphical representation of microarray transcript levels; and PopGenIE (http://www.popgenie.org/; Sjödin *et al.*, 2009), a central resource of tools for the exploration of the poplar genome and expression data.

1.2.1. The gene families encoding putative α, β and γ subunits of the SnRK1 protein kinase complex in *P. trichocarpa*

The publication of the *Populus* genome (Tuskan *et al.*, 2006) has made it possible to use an *in silico* approach to discover homologues of the α, β and γ subunits of the SNF1 complex in poplar. This study was undertaken by Fedosejevs (2008) who identified homologues of the SnRK1 complex by taking advantage of *SnRK* homologue sequences which have already been elucidated. Briefly, Fedosejevs (2008) used sequences from *Arabidopsis* and other species as seed sequences to query the *Populus trichocarpa* genome (release 1.1, http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html; Tuskan *et al.*, 2006) using TBLASTN and/or BLASTN (NCBI BLAST 2.2.14; Altschul *et al.*, 1997) to identify gene models corresponding to putative SnRK, AKINβ and AKINγ subunits. As a further check, *P. trichocarpa* sequences which appeared to be *bona fide SnRK1* complex family member gene models were used to iteratively search the *Populus* genome for additional *SnRK1* complex family members. ESTs were then used as a check of the gene models and to provide evidence for

combining redundant gene models together. This yielded 43 *SnRK*s (of which 3 are part of the SnRK1 family), 7 *AKINβ*s, 6 *AKINβγ*s and 10 *AKINγ*s. Fedosejevs (2008) also constructed phylogenetic trees using all *P. trichocarpa*, *Oryza sativa* and *Arabidopsis* sequences found, as well as representative characterized sequences from *Homo sapiens, Hordeum vulgare*, *Lycopersicon esculentum* and *Saccharomyces cerevisiae* (Figure 1.1; used with permission).

1.2.2. Effect of nitrogen availability on whole plant physiology and molecular biology in poplar

Regulation of nitrogen uptake in woody perennials is more complex than in herbaceous plants as there is a need to meet not only the current demands of tissues but also to deal with seasonal changes which demand nitrogen cycling (Geβler *et al.*, 2004). Poplar species have different preferences for NO_3^- and NH_4^+. The uptake of inorganic nitrogen is also influenced by a variety of environmental factors including abundance of NO_3^- and NH_4^+, heat, drought, soil pH and soil temperature (reviewed in Rennenberg *et al.*, 2010). NO_3^- acts as both a nutrient and a signalling molecule for its own uptake (Crawford, 1995). The 5' proximal region of NR was found to be nitrate inducible in transgenic tobacco (Lin *et al.*, 1994) and levels of NR were increased with increasing nitrate concentrations in nutrient solutions provided to legumes (Fan *et al.*, 2002). In trees, NO_3^- is largely reduced in the roots to the transportable amino compounds

(usually glutamine) and transported through the xylem to tissues which require it (reviewed in Geβler *et al.*, 2004). Girdling experiments in poplar show that phloem has a role in organic nitrogen transport, as there is a significant accumulation of glutamine, asparagine and arginine below the girdle in both xylem and phloem (Cooke *et al.*, 2003).

In poplars, increased nitrogen availability leads to diverse changes to plant architecture, resource allocation, and gene expression (Cooke *et al.*, 2003; Cooke *et al.*, 2005). It has been shown that feeding with glutamine reduces NO_3^- uptake and *glutamine synthase 1* transcript levels in roots, as well as shifting the distribution of nitrogen reduction to shoots rather than roots (Dluzniewska *et al.*, 2006). Phytohormones such as cytokinins are also involved in nitrogen assimilation. For instance, treatment of poplar seedlings with tZR (*trans*-zeatin riboside) led to an increase in NR transcripts in roots (Dluzniewska *et al.*, 2006). Poplars treated with higher levels of NH_4NO_3 were significantly taller, produced sylleptic branches and had more leaves and more leaf area (Cooke *et al.*, 2005). At the molecular level, transcripts for vegetative storage proteins were higher in shoot tips, stems and roots of poplars treated with higher levels of nitrogen. In leaves, transcripts of *AGPase* and *starch synthase* were found to be more abundant in poplars in limiting nitrogen conditions (Cooke et al., 2003). The ratio of C:N in roots, stems and leaves decreases as nitrogen availability increases (Cooke *et al.*, 2003).

Increased nitrogen abundance has a direct effect on wood morphology. In

high nitrogen conditions, xylem fibres are wider and thicker, with a significant thickening of the cell walls. The internal cell wall layer is enriched in cellulose (Pitre *et al.*, 2007a). High nitrogen availability also leads to reduced lignin content and an altered S:G lignin subunit ratio, which is characteristic of the early stages of cell wall development, and suggests either an increase in the number of cells in the early stages of proliferation or a delay in completion of maturation (Pitre *et al.*, 2007b).

1.2.3. Dormancy acquisition in poplar

Seasonal dormancy is a necessary survival characteristic for many perennials in order to deal with seasonal unfavourable conditions in the environment (Rohde and Bhalerao, 2007). It can be described as the cessation of growth until the return of favourable conditions. Dormancy can be divided into three types. Ecodormancy is caused by unfavourable or limiting environmental factors, such as a short photoperiod. Paradormancy is imposed when one part of the plant induces dormancy in another part, such as in the case of lateral buds. Endodormancy, sometimes called true dormancy, is imposed from within the dormant tissue itself (Lang, 1987).

Carbohydrates are stored in xylem ray cells, building up during the growing season and then depleting during the winter (Fege and Brown, 1984). Starch accumulation is stimulated by a short day photoperiod (Nelson and Dickson,

1981) but is hydrolyzed and converted to sucrose, raffinose and stachyose in response to lower temperatures (Sauter and van Cleve, 1991). Transcript profiling using microarrays show an increase in expression of genes encoding galactinol synthase, raffinose synthase and inositol monophosphatase, enzymes involved in raffinose synthesis, during dormancy acquisition in stem and cambial meristem (Druart *et al.*, 2007; Park *et al.*, 2008), as well as an increase in genes involved in starch degradation (Schrader *et al.*, 2004). Stems also show extensive changes in the expression profiles of many other genes. For instance, during the early winter development of cold hardiness and dormancy, there is an overrepresentation of cell defense genes and an underrepresentation of signal transduction and protein synthesis genes. Many genes involved in cell wall modification, such as pectinesterases, pectin methylesterases, pectin-glucuronyltransferases and beta-1,3-glucanases, were also upregulated in bark (Park *et al.*, 2007). Dormant cambium showed upregulation of stress response genes, nitrogen recycling genes, and starch degradation genes (Druart *et al.*, 2007; Schrader *et al.*, 2004).

In shoot tips, carbohydrate metabolism shows two separate phases of response to a short photoperiod. The first is an early response to the short day photoperiod. Starch is mobilized and disappears, along with a transient decrease in other sugars such as glucose, G6P and sucrose. After approximately two weeks, there is a shift towards the accumulation of storage carbohydrates (Ruttink *et al.*, 2007). Galactinol synthase is also expressed after one week (Rohde *et al.*, 2007).

Vegetative storage proteins (VSPs) are a major form of stored nitrogen in

vegetative tissue (Staswick, 1994; Stepien *et al.*, 1994). Important VSPs in poplars are the bark storage proteins (BSPs), of which one of the subfamilies is also named the BSP gene family. Increasing *BSP* subfamily mRNA levels have been shown to positively correlate with NH_4NO_3 availability in both short and long photoperiods, suggesting that the BSP subfamily plays a role in nitrogen storage in both actively growing and dormant tissue (Coleman *et al.*, 1994; Cooke *et al.*, 2003; Cooke *et al.*, 2005). Glutamine and NH_4NO_3 are able to induce *P. deltoides BSP* expression, as tested by a *BSP* promoter:reporter construct (Zhu and Coleman, 2001). BSP levels after a dormant period were also found to be higher in transgenic poplar expressing glutamine synthase (Jing *et al.*, 2004). *BSP* transcript abundance increased during dormancy acquisition in the cambial meristem, as well as the accumulation of amino acids for storage protein synthesis (Druart *et al.*, 2007). *BSP* has also been shown to be induced within the first two weeks of short day photoperiod in shoot tips (Ruttink *et al.*, 2007).

1.3. The present study

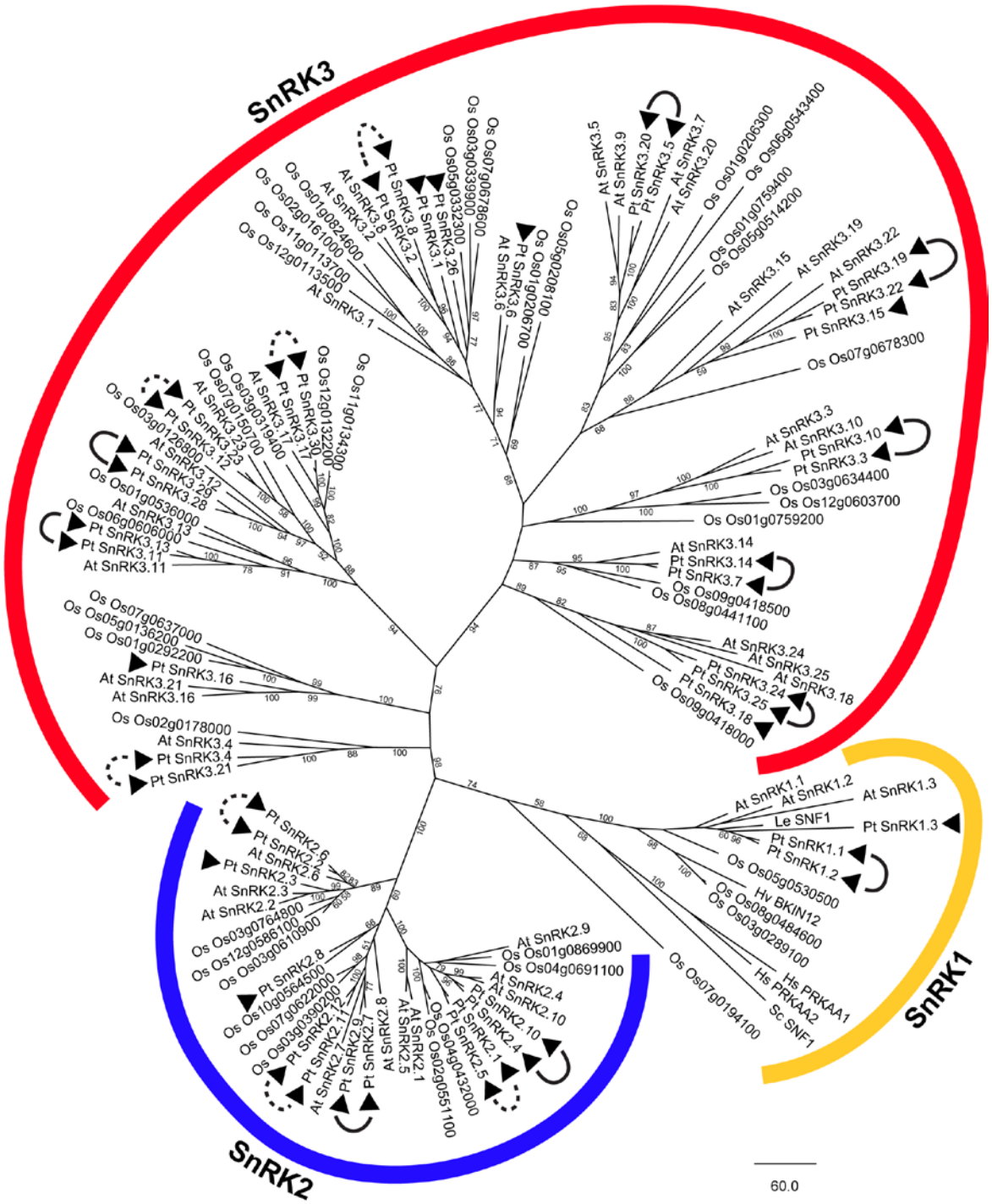
The present study aims to begin the characterization of the SnRK1 complex in poplars in order to facilitate future study of the role of the SnRK1 complex in processes such as the nitrogen response and seasonal dormancy. The overall goals are to identify if the SnRK1 protein complex potentially plays a role during differential nitrogen availability and dormancy acquisition, and if a subset of the

genes encoding members of the SnRK1 protein complex in poplar should be targeted for future in-depth study.

To achieve these goals, genes encoding members of the α, β and γ gene families of the SnRK1 protein complex were cloned, and qRT-PCR assays were developed to determine the expression profiles of these genes in different poplar tissues, as well as in different tissues under conditions of differential nitrogen availability and during dormancy acquisition. It is hypothesized that genes encoding certain subunits of the SnRK1 protein complex will be differentially expressed under different levels of nitrogen availability or under dormancy-inducing short day conditions. Furthermore, it is hypothesized that some genes will be expressed minimally while others will be expressed at high levels in different tissues under different environmental conditions. These data will allow me to infer whether the SnRK1 protein complex plays a role in processes associated with the nitrogen response and during dormancy acquisition, and which genes may be encoding subunits which form specific SnRK1 protein complexes that function in these roles. This information will then serve as the basis for future studies.

1.4. Figures

A



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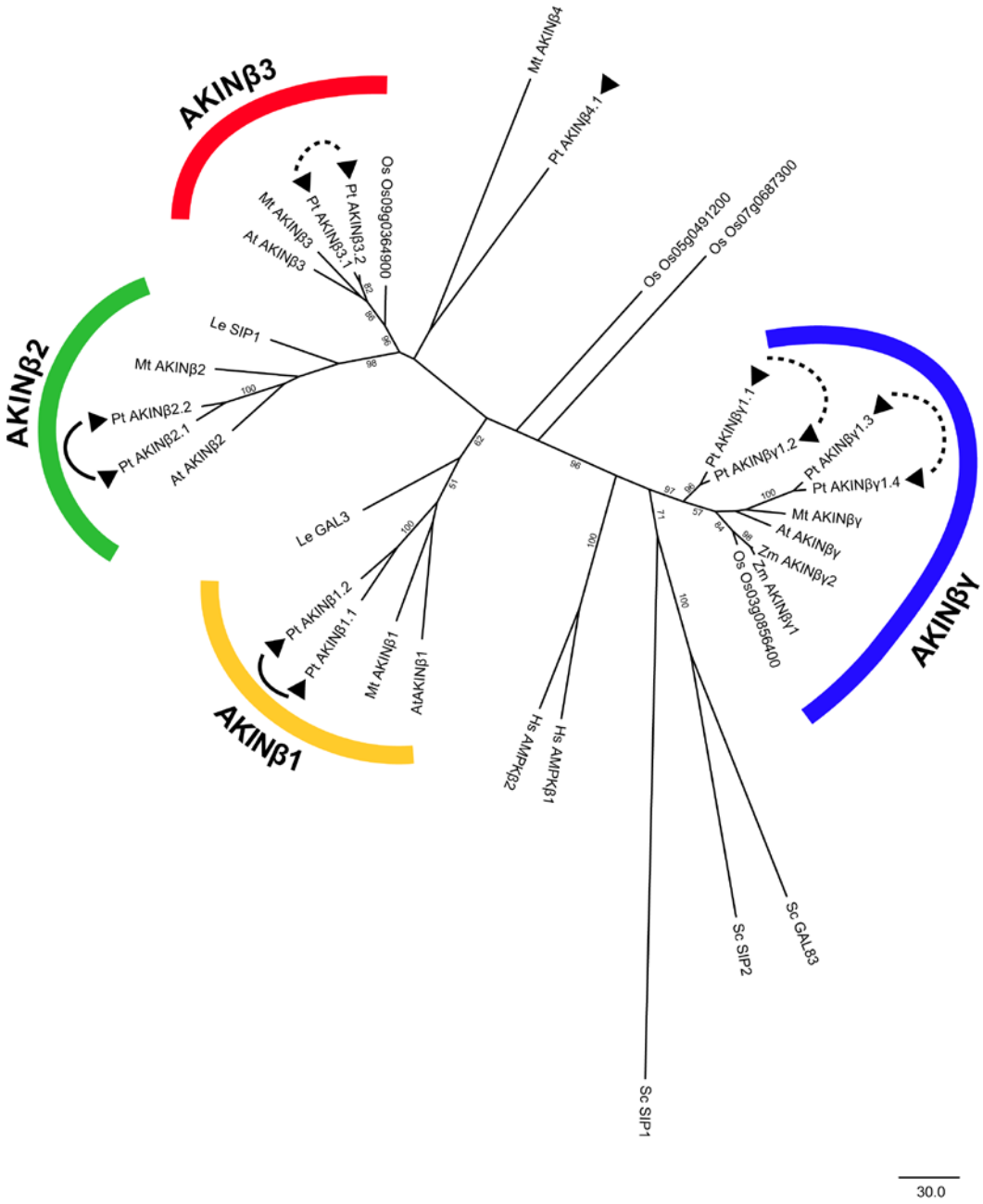
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Os Os07g0687300
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Mt AKINβ2
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Pt AKINβ2.1
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Zm AKINβγ1
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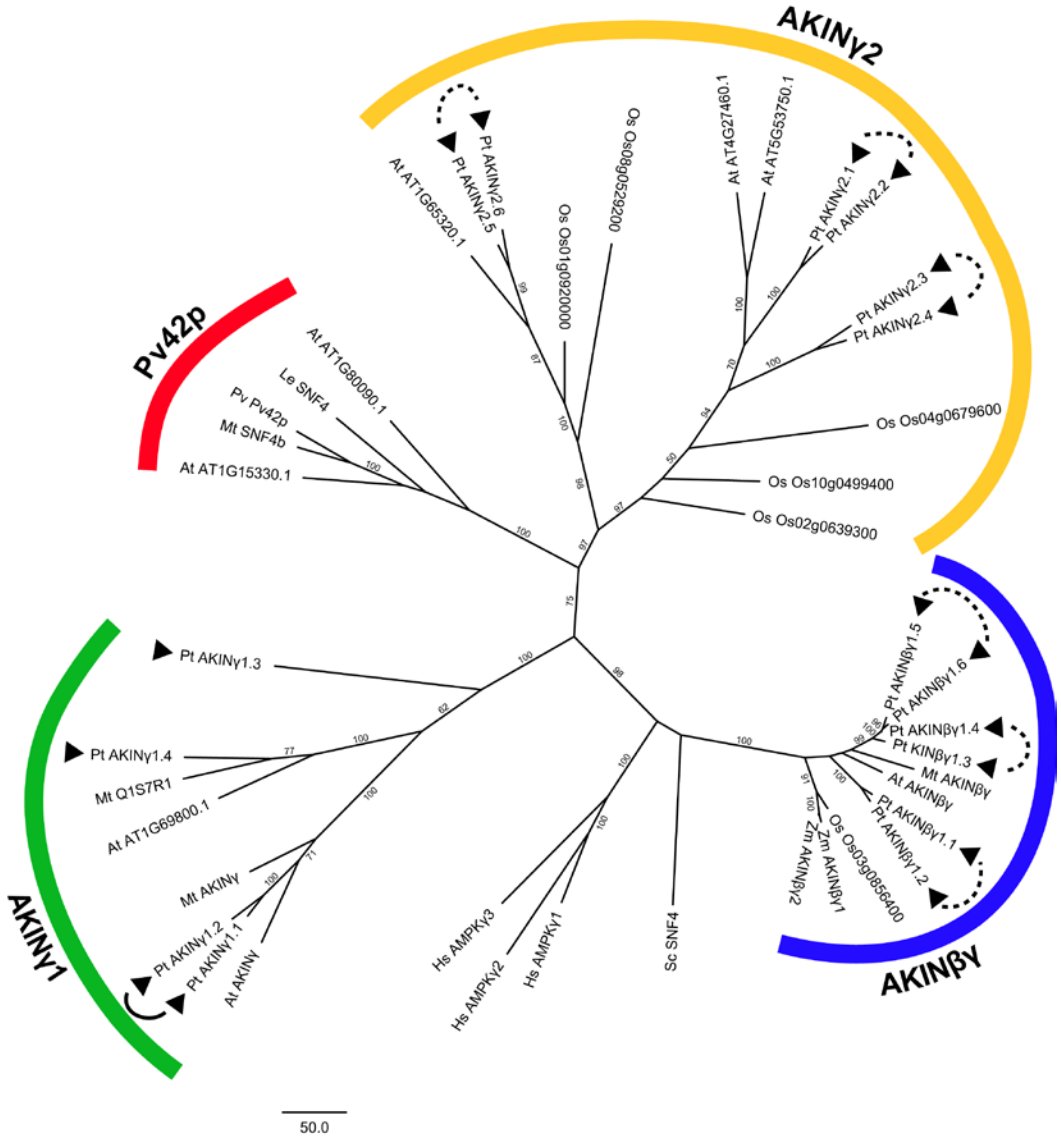
Sc SIP2
Sc GAL83
30.0

C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At AT5G53750.1
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Pt AKINγ2.2
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Pt AKINγ2.4
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Pv Pv42p
Mt SNF4b
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Pt AKINγ1.4
Mt Q1S7R1
At AT1G69800.1
Mt AKINγ
Pt AKINγ1.2
Pt AKINγ1.1
At AKINγ
Hs AMPKγ3
Hs AMPKγ2
Hs AMPKγ1
Sc SNF4
Pt AKINβγ1.5
Pt AKINβγ1.6
Pt AKINβγ1.4
Pt KINβγ1.3
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Zm AKINβγ1
Zm AKINβγ2
50.0

Figure 1.1. Maximum parsimony trees of *PtdSnRK*, *PtdAKINβ*, and *PtdAKINγ* gene families containing all revised non-redundant *P. trichocarpa*, *A. thaliana*, and *O. sativa* deduced amino acid sequences (Fedosejevs, 2008; used with permission). In each case, one most parsimonious tree from PAUP 4.0b10 is shown. Bootstrap support values from 100 bootstrap replicates are displayed on branches. Species abbreviations are as follows: At, *Arabidopsis thaliana*; Hs, *Homo sapiens*; Hv, *Hordeum vulgare*; Le, *Lycopersicon esculentum*; Mt, Medicago trunculata; Pt, *Populus trichocarpa*, Pv, *Phaseolus vulgaris*; Sc, *Saccharomyces cerevisiae*; Zm, *Zea mays*. *P. trichocarpa* gene models are indicated by arrows. Gene models whose linkage group positions suggest duplication during the recent Salicoid duplication event (Tuskan et al., 2006) are joined by solid lines while gene models suspected of such duplication but in which one or both gene models are located on scaffolds unintegrated into linkage groups are joined by dashed lines.
(A) Maximum parsimony tree of the SnRK family. One of nine most parsimonious trees is shown.
(B) Maximum parsimony tree of the AKINβ family. One of three most parsimonious trees is shown. The AKINβ-homologous region of AKINβγ sequences was used for their inclusion in the tree; the gene models for AKINβ1.5 and 1.6 lack this region and were omitted.
(C) Maximum parsimony tree of the AKINγ family. The single most parsimonious tree is shown. The AKINγ-homologous region of AKINβγ sequences was used for their inclusion in the tree. The gene models for AKINβγ1.6, AKINγ1.1, AKINγ1.4, and AKINγ2.1 each consist of two original JGI-predicted gene models. In the case of AKINβγ1.6 (JGI protein ID 733606 and 674428) and AKINγ2.1 (JGI protein ID 810738 and 793630), both original gene models were located on unintegrated scaffolds, while in the case of AKINγ1.1 and AKINγ1.4, one original gene model was located on a linkage group (JGI protein ID 733606 and 577408, respectively) and one original gene model was located on an unintegrated scaffold (JGI protein ID 585804 and 674145, respectively).

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2.0. Development of qRT-PCR assays for members of the SnRK1 protein complex

2.1. Introduction

SnRK1 is a serine/threonine protein kinase composed of three different subunits: an α catalytic subunit (SnRK1), a β regulatory subunit (AKINβ) and a γ regulatory subunit (AKINγ). In recent years, investigations of SnRK1 in *Arabidopsis* and other herbaceous plant species have revealed diverse roles for this protein kinase, including the regulation of starch biosynthesis (Purcell *et al.*, 1998; Zhang *et al.*, 2001; Laurie *et al.*, 2003; Tiessen *et al.*, 2003; Kanegae *et al.*, 2005; McKibbin *et al.*, 2006; Jain *et al.*, 2008), isoprenoid biosynthesis (Ball *et al.*, 1994; Dale *et al.*, 1995; Ball *et al.*, 1995; Barker *et al.*, 1996; Sugden *et al.*, 1999b, Hey *et al.*, 2006), sucrose synthesis (Sugden *et al.*, 1999b; Kulma *et al.*, 2004), nitrogen metabolism (Douglas *et al.*, 1997; Sugden *et al.*, 1999b; Baena-González *et al.*, 2007; Baena-González and Sheen, 2008; Polge *et al.*, 2008; Jossier *et al.*, 2009) and crosstalk with the ABA signalling pathway (Bradford *et al.*, 2003; Radchuk *et al.*, 2006; Zhang *et al.*, 2008; Jossier *et al.*, 2009; Bolingue *et al.*, 2010). Taking advantage of the publication of the *Populus* genome (Tuskan *et al.*, 2006), Fedosejevs (2008) used an *in silico* approach to discover homologues of the α, β and γ subunits of the SNF1 complex in poplar by using homologous sequences from other species which had already been elucidated.

ESTs were used to verify gene models and combine redundant gene models.

We are interested in discovering roles for SnRK1 in poplar. The SnRK1, AKINβ and AKINγ subunits are each encoded by multi-member gene families in poplar. A logical first step in understanding the role that the SnRK1 protein complex plays in biological processes in poplar is to examine the expression patterns of the genes from each of these three gene families which potentially assemble to form SnRK1 complexes. Expression profiles for the *SnRK1*, *AKINβ* and *AKINγ* gene family members in different tissues during the course of response to internal or external cues should serve to reveal whether the composition of SnRK1 complexes is altered - e.g. by modulating abundance of certain subunits - not only implying a role for SnRK1 in the biological process being investigated, but also potential roles for specific SnRK1 subunits in modulating SnRK1 action. Expression profiles for each of the members of these gene families may also allow us to deduce which members of one subunit family could be associating with other members in specific tissues to form SnRK1 complexes, or at least rule out certain members of these gene families. Interesting candidates found in such a manner will serve as the basis for future studies.

Several methods can be used to measure transcript abundance. In 1977, the Northern blot was introduced as a method of quantifying mRNA levels (Alwine *et al.*, 1977). RNA is electrophoretically separated under denaturing conditions, transferred to a membrane and hybridized with a specific probe. Northern blotting has several advantages, including the possibility of detecting different mRNAs

using the same membrane. There are also several drawbacks, including the need for relatively high amounts of RNA, the length of time required to perform the procedure, and its low sensitivity (VanGuilder *et al.*, 2008; Böhm-Hofstätter *et al.*, 2010). End point RT-PCR took advantage of PCR to quantify expression levels by amplifying the target for a discrete number of cycles and analyzing the results through visualization on an agarose gel. This technique suffers from certain flaws, most notable of which is that the quantification may not be accurate. When reagents are not limiting, the PCR reaction shows exponential growth; however, limiting reagents leads to a plateau of amplified product. The plateau can be reached by different samples for a variety of reasons, including the amount of starting template and primer efficiency. Furthermore, quantification using an agarose gel lacks sensitivity. In recent years, quantitative reverse transcription polymerase chain reaction (qRT-PCR) has become a gold standard in measuring expression levels (Ginginzer, 2002; VanGuilder *et al.*, 2008). The introduction of real-time qRT-PCR overcomes this issue by quantifying a fluorescent signal at each PCR cycle, which means that the sample is being quantified during the exponential growth stage of PCR. Fluoresence detection during the PCR reactions also eliminates post-PCR handling of the sample, which helps to minimize experimental error (VanGuilder *et al.*, 2008). Comparison of end point RT-PCR and real time qRT-PCR shows that real-time qRT-PCR has greater sensitivity to smaller differences and is able to accurately detect a larger range of quantities (Schmittgen *et al.*, 2000).

Although the premise is simple, there are a variety of protocols and options which may be employed to tailor qRT-PCR to the needs of a particular study. Nolan *et al.* (2006) categorized the qRT-PCR experimental workflow into four major themes: sample validation and data collection, assay validation and optimization, the qRT-PCR assay itself, and data analysis. Sample validation and data collection involves not only the design of an experiment and the collection of biological samples, but also the extraction and validation of RNA prior to cDNA synthesis. Unsurprisingly, variability during the sample collection and processing stage can lead to differences in expression that reflects methodological variability rather than true differences in biological processes. Assay validation and optimization involves determining how to generate the fluorescent signal, primer and/or probe design, and optimization of standards. The exact choices made need to take into account the goals of the study. The qRT-PCR assay consists of both the production of cDNA and the qPCR assay itself. Data analysis includes any manipulation of data which occurs after the qRT-PCR assay, including data transformation and statistical analyses.

Within each theme are multiple options which require careful consideration with regard to the objectives of the experiment. Assay validation and optimization may be of the most importance as it ultimately informs how data analysis will occur and provides grounds for confidence in the results of the study. It typically involves choosing the fluorescence method to be used and the method of quantification.

In general, there are three common methods of fluorescent labeling used in qRT-PCR: probe sequences, fluorescent hairpins and intercalating dyes (reviewed in VanGuilder *et al.*, 2008). Probe sequences and fluorescent hairpins are similar in that there is a fluorescent reporter molecule and quencher attached to a sequence designed to bind to the gene of interest. In the case of probe-based fluorescence, the probe is designed to bind to the target sequence between the forward and reverse primers. Extension of the primers leads to the degradation of the probe through the 5'-3' exonuclease activity of Taq DNA polymerase, releasing the fluorophore and leading to fluoresence. In the case of fluorescent hairpins, degradation by Taq DNA polymerase is not necessary. When not hybridized to a target sequence, the primer forms a hairpin which quenches the reporter molecule. Binding to the target sequence leads to fluorescence. Intercalating dyes fluoresce upon binding to double-stranded DNA. They are easily detectable and inexpensive, but because they bind indiscriminately to any double-stranded DNA, it is necessary to ensure that the primers designed for amplification of the target sequence are specific and that primer-dimers are not being formed. This can be accomplished by examining the melting curves of the amplification products, as different sequences will produce different melting curves. A single melting curve denotes a single product. Furthermore, it is possible to test the efficiency of primers for a particular sequence through the use of a standard curve, allowing researchers to test the efficiency of amplification of sequences which are closely related to the target sequence.

The method of quantification used informs how the data of the qRT-PCR assay can be analyzed. Data can be analyzed in either absolute levels or relative levels (reviewed in VanGuilder *et al.*, 2008). Absolute quantification requires the use of a standard curve which consists of a serial dilution of a known quantity of the gene of interest so that it can be compared to samples. Relative quantification involves the comparison of the expression of the gene of interest to another gene, the comparison of the expression of the gene of interest between different samples, or both. The most common method for relative quantification is the ΔΔCT method. The expression level of the gene of interest is normalized to a reference gene and then the expression levels between different experimental conditions can be compared. The reference gene, also known as an endogenous control, is a gene whose expression levels are constant between samples and so can be used as a normalizing factor to account for variability in experimental conditions, such as during cDNA synthesis (Bustin *et al.*, 2005). For example, it has been shown that when using SuperScript II (Invitrogen) the standard deviation of C_t when conducting quantification of known samples can vary from 0.04 to as high as 0.98 depending on whether the target is present in low copy number or high, and on the amount of background RNA present (Levesque-Sergerie *et al.*, 2007). Normalizing to a reference gene helps to minimize erroneous conclusions caused by differential expression patterns that are actually caused by the technical methods rather than biological differences. Relative quantification can also involve a standard curve. Rather than directly comparing the C_t's of the gene of

interest and the reference gene, the gene of interest and the reference gene are first quantified using a standard curve. This has the advantage of accounting for different primer efficiencies which can make $\Delta\Delta C_t$ problematic, as $\Delta\Delta C_t$ assumes nearly 100% primer efficiency.

In general, it is desirable to have a reference gene which is expressed invariantly or constitutively in all the experimental conditions of interest. In the past, so-called “housekeeping genes” were used under the belief that because they were involved in processes which occur in all cells they must be constitutively and invariantly expressed. Some common examples include GAPDH, ubiquitins, actins, and rRNA (Czechowski *et al.*, 2005; Gutierrez *et al.*, 2008). Studies testing the veracity of this belief have shown that the concept of “housekeeping genes” is outdated since their expression profiles in different tissues and under different experimental conditions are often not invariant (Volkov *et al.*, 2003; Brunner *et al.*, 2004; Czechowski *et al.*, 2005; Nicot *et al.*, 2005; Gutierrez *et al.*, 2008). The choice of reference gene must therefore be based on evidence collected through validation of invariant expression patterns rather than through inference based on function. There has also been a shift towards using more than one reference gene and combining the expression levels of the reference genes mathematically to produce an invariant expression pattern for normalization of the gene of interest (Vandesompele *et al.*, 2002).

Consistency of method and care in validation are paramount in ensuring accurate results and reproducibility. In order to lend consistency and reliability to

published literature, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) was developed to provide guidelines and standardized nomenclature for qRT-PCR experiments in order to allow other researchers to judge the validity of a qRT-PCR assay (see Table 2.1, obtained from Bustin *et al.*, 2009). The MIQE is organized with a variety of other guidelines by the Minimum Information for Biological and Biomedical Investigations (MIBBI; http://www.mibbi.org).

The overall goal of my thesis is to develop transcript abundance profiles for members of the *SnRK1*, *AKINβ* and *AKINγ* gene families in three different experiments: a survey of different tissue types in plants grown under standard conditions, an experiment comparing responses to high and low nitrogen availability in different tissues over time, and an experiment in which different tissues were examined during dormancy acquisition under short days (SD). These data will be used to infer whether SnRK1 may be playing roles in these biological processes, and if so, which genes may be contributing to SnRK1 complexes that are involved in these biological processes. qRT-PCR was the method of choice to develop profiles of transcript abundance for members of the *SnRK1*, *AKINβ* and *AKINγ* gene families in these three experiments. In order to accomplish this goal, it was necessary to develop robust, reliable, and sensitive qRT-PCR assays for each of these genes. Thus, the objective of this component of my study was to develop a robust qRT-PCR assay for the *SnRK1*, *AKINβ* and *AKINγ* members of the SnRK1 complex in *Populus trichocarpa* Torr. & Gray × *Populus deltoides*

Bartr. ex Marsh. Gene specificity and proper reference gene selection are imperative in order to ensure the reliability of the assay and confidence in the results when using qRT-PCR to test gene expression in several poplar experiments. To accomplish this objective, cDNAs representing members of the *SnRK1* and *AKINγ* gene families were cloned from *P. trichocarpa* × *deltoides*, gene specific qRT-PCR primers were designed and validated for *SnRK1*, *AKINβ* and *AKINγ* genes, and suitable reference genes were identified for the tissue survey, nitrogen availability and dormancy acquisition experiments.

2.2. Materials and methods

All chemicals were obtained from Bioshop (Burlington, ON, Canada), Sigma (St. Louis, MS, USA) or Fisher (Hampton, NH, USA) unless otherwise noted.

2.2.1. Standard poplar growth conditions

Populus trichocarpa Torr. & Gray × *Populus deltoides* Bartr. ex Marsh. (clone H11-11) plants were propagated from rooted cuttings in Sunshine Mix #4 and grown in growth chambers at the University of Alberta. The plants were grown individually in 1.5 gallon pots. The plants were fertilized weekly with 0.5 g/L of 20-20-20 (N, P, K), alternated with 0.5g/L each of 10-52-10 and 15.5-0-0 19% Ca. They were treated biweekly with Thrips Eliminator (Applied

BioNomics, Sidney, BC, Canada), a biological control for thrips. Plants were grown in long day conditions (16h light, 8h dark) at a temperature of 25^{o}C (day)/18^{o}C (night) and a humidity of 70%. Three growth chambers were used and the ranges of photosynthetic active radiation for each chamber as measured with an LI-205A light meter (LI-COR Biosciences, Lincoln, NE, USA) were 232.8 – 258.3 μmol/s m^2, 230.3 – 259.2 μmol/s m^2 and 245.9 – 268.0 μmol/s m^2.

2.2.2. Experimental design

Three experiments were conducted for qRT-PCR analysis of *SnRK*, *AKINβ*, and *AKINγ* transcript abundance: a tissue survey, a nitrogen availability experiment, and a dormancy experiment. Each of these experiments was conducted with *P. trichocarpa* × *deltoides* rooted cuttings.

2.2.2.1. Tissue survey experiment

Poplar plants grown under the standard conditions described above were grown to a height of 60-80 cm. Shoot tips, young (still expanding) leaves, mature (fully expanded) leaves, old (showing first signs of senescence) leaves, bark (constituting mainly secondary phloem), secondary xylem and roots were harvested from three plants. Shoot tips, young leaves and mature leaves were determined using the leaf plastichron index (LPI; Larson and Isebrands, 1971).

Shoot tip contained tissue above LPI 0 (inclusive). LPI 0 is defined as a developing leaf approximately 2 cm long with a one-half expanded lamina. Young leaves were denoted as leaves with LPI 1-3 and mature leaves had LPI 6-9. Old leaves consisted of four leaves taken above senescing leaves which were determined by looking for yellowing. Stems were collected from midway down the tree to approximately 6-8 inches above the soil. The stem was separated into the secondary xylem and the bark. Because the bark consists primarily of secondary phloem, bark tissue will be referred to as secondary phloem. Roots were washed in water to remove the Sunshine Mix and harvested whole. Upon harvesting, the tissue was immediately frozen in liquid nitrogen and stored at -80^{o}C.

2.2.2.2. Nitrogen availability experiment

Poplar plants were divided into three randomized complete blocks. Plants were fertilized with modified Hocking's Complete Fertilizer (Hocking, 1971) supplemented with either a limiting level of nitrogen (0 mM of NH_4NO_3) or luxuriant level of nitrogen (10 mM of NH_4NO_3). The modified Hocking's Complete Fertilizer consisted of 2 mM magnesium sulfate heptahydrate, 3.75 mM potassium chloride, 0.03 mM potassium phosphate monobasic, 0.37 mM potassium phosphate dibasic, 2 mM calcium chloride dihydrate, 0.4 μM sodium molybdate dihydrate, 13.5 μM boric acid, 1.1 μM cupric chloride, 2.8 μM zinc

chloride, 47.6 μM manganous chloride, and 39 μM Plant Products chelating micronutrient mix (Plant Products, Brampton, ON, Canada). The same tissues harvested in the tissue survey experiment were harvested as described above from three independent plants (one from each block) per experiment on day 0, 1, 3, 7 and 14 of nitrogen treatment. Young leaves, secondary phloem, secondary xylem and roots were used in the subsequent qRT-PCR assay. The experiment was repeated once for a total of six biological replicates.

2.2.2.3. Dormancy acquisition experiment

Poplar plants were divided into three randomized complete blocks and grown in short day conditions (8 h light, 16 h dark) over the course of 8 weeks in order to ensure dormancy was acquired. The same tissues harvested from the tissue survey experiment were harvested as described above on week 0, 2, 4, 6 and 8 of short day conditions from three independent plants per experiment. Shoot tip, mature leaves, secondary phloem, secondary xylem and roots were used in the subsequent qRT-PCR assay. The experiment was repeated twice, though for one experiment only shoot tips were harvested. Six biological replicates were chosen from these experiments.

2.2.3. Preparation of cDNA

2.2.3.1. RNA extraction

Harvested tissue was ground to a fine powder by hand with a mortar and pestle using liquid nitrogen. Root tissue samples were ground using 60 mL metal jars on the MM301 MixerMill (Retsch, Hann, Germany). RNA was extracted from the tissue samples using the protocol described by Chang *et al.* (1993) with some modifications. The hexadecyltrimethylammonium bromide (CTAB) extraction buffer consists of 2% CTAB, 2% polyvinylpyrrolidinone K 30 (PVP), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 0.5 g/L spermidine and 2% beta-mercaptoethanol (added just before use). For large scale extractions, 20 mL of CTAB buffer was placed in Oakridge centrifuge tubes and preheated in a water bath to 65^{o}C. Each ground tissue sample (approximately 1 g) was added to the hot buffer in separate tubes and shaken vigorously for at least three minutes in total. This mixture was extracted with 15 mL of chloroform: isoamyl alcohol (24:1) and samples were spun for 10 minutes at 12000g in a Beckman JA-20 rotor (Beckman Coulter, Brea, CA, USA). The aqueous phase was retrieved and extracted again with 15mL of chloroform: isoamyl alcohol (24:1). A quarter volume of 10 M LiCl was added to the aqueous phase and the samples were incubated at 4^{o}C in an ice and water slush bath for 11.5 hours in order to precipitate the RNA. The samples were spun at 12000g in a Beckman JA-20 rotor for 20 minutes to pellet the RNA and the supernatent discarded. Pellets were resuspended in SSTE (1.0 M NaCl, 0.5% SDS, 10 mM Tris-HCl with a pH of 8.0,

and 1 mM EDTA with a pH of 8.0) and extracted with 500 μL of chloroform:isoamyl alcohol (24:1). Samples were spun for 7 minutes at 14000 rpm on a table top centrifuge and the aqueous phase retrieved. Two volumes of 95% ethanol were added to the sample and RNA was precipated for one hour at -80C. The sample was spun at 14000 rpm for 20 minutes to pellet the RNA, and the sample was washed with 70% ethanol. The RNA was then resuspended in RNase free water (Baxter). Quantification of the RNA from the tissue survey experiments occurred using an Ultraspec 3000 spectrophotometer (Pharmacia Biotech, Stockholm, Sweden) while quantification of the nitrogen availability experiments and dormancy acquisition experiments occurred using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). The quality of the RNA was ascertained by running the RNA on a 1% agarose gel and visualized using ethidium bromide. RNA extraction for the tissue survey experiment and shoot tip tissue from the nitrogen availability and dormancy acquisition experiments was done as large scale extractions.

RNA from leaves, secondary phloem, secondary xylem and roots from the nitrogen availability and dormancy acquisition experiments was extracted using a small scale CTAB protocol to increase throughput (Pavy et al., 2008). A small amount of tissue was added to 750 μL of pre-heated CTAB buffer in a microcentrifuge tube. Samples were extracted twice with 500 μL of chloroform:isoamly alcohol (24:1) and spun on a table top centrifuge at 14000 RPM. After the addition of ¼ volume of 10M LiCl, samples were incubated at -

20°C for 1 hour. Samples were centrifuged at 14000 RPM for 15 minutes and the pellet washed with 80% ethanol. The RNA was then resuspended in RNAse free water (Baxter).

2.2.3.2. cDNA synthesis

1.5 μg of RNA was treated with 1U of RNAse-free DNaseI (New England BioLabs, Ipswich, MA, USA) in a 10 μL volume including 10X reaction buffer and autoclaved RNase-free water (Baxter). The mixture was incubated at 37°C for 15 minutes and then treated with 1 μL 25 mM EDTA for 10 minutes at 65°C.

SuperScript™ II Reverse Transcriptase (SSII RT; Invitrogen, Carlsbad, CA, USA) was used to synthesize DNA using the manufacturer's instructions with some modifications. 1 μL of 50 μM oligo-dT_{23}VN (Integrated DNA Technologies, Coralville, IA, USA) and 1 μL of 10 mM dNTP (Fermentas, Burlington, ON, Canada) was added to the DNAse treated RNA and the mixture heated at 65°C for 5 minutes and quick chilled on ice. 4 μL of 5X First-Strand Buffer and 0.1 M DTT was added. No RnaseOUT™ was added since more than 50 ng of starting RNA was being used. The contents were mixed gently and incubated at 42°C for 2 minutes. 1 μL of SSII RT was added to the mixture. The mixture was incubated at 42°C for 50 minutes, and then the reaction was inactivated by heating at 70°C for 15 minutes. 1 μL (2 units) of RNase H (New England Biolabs) was added and the mixture was incubated at 37°C for 20

minutes.

For cloning, cDNA was used undiluted. For the qRT-PCR assays, cDNA was diluted ten-fold prior to use.

2.2.4. Cloning *PtdSnRK1* and *PtdAKINγ* gene family members

2.2.4.1. Cloning

Gene models of the three *P. trichocarpa SnRK1* and ten *AKINγ* subunits were provided (Fedosejevs, 2008). Primers for cloning were designed using Primer3/Primer3Plus (Rozen and Skaletsky, 2000; Untergasser *et al.*, 2007), using the default settings (Table 2.2). In order to design gene specific primers, closely related sequences were aligned using MEGA3.0 (Kumar *et al.*, 2008) and primers were designed in areas where there were at least three nucleotide differences between the sequences. When possible, primers were designed in the putative 3' and 5' UTR.

Putative genes were cloned from a mixture of cDNA from mature leaves, xylem and phloem of *P. trichocarpa* × *deltoides* using a standard PCR reaction mix and touchdown PCR where necessary into either the pGEM-T or pGEM-T Easy vectors (Promega, Madison, WI, USA; see Table 2.3). Plasmids were used to transform *Escherichia coli* strain DH5α. Colonies were grown on LB plates supplemented with 100μg/mL ampicillin 100 μL of 100mM isopropyl β-D-1-

thiogalactopyranoside (IPTG) and 20μL of 50mg/mL bromo-chloro-indolyl-galactopyranoside (X-Gal). Clone identities of the three *SnRK1* genes and nine *AKINγ* were confirmed by sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). 2 μL of BigDye premix and a buffer of 200 mM Tris (pH 9.0) and 5 mM $MgCl_2$ was added to 1 μL of template. 1 μL of either SP6 (5'-TAC GAT TTA GGT GAC ACT ATA G-3') or T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') was used as a primer. The mixture was incubated in 25 cycles of 96^{o}C for 30 seconds, 50^{o}C for 15 seconds and 60^{o}C for 1 minute. The product was precipitated using a mixture of ethanol, NaOAc, and EDTA and washed with 70% ethanol. The product was then sequenced by MBSU.

Members of the poplar *AKINβ* gene family were cloned by Fedosejevs (2008). These are *PtdAKINβ1.1*, *PtdAKINβ1.2*, *PtdAKINβ2.1*, *PtdAKINβ2.2*, *PtdAKINβ3.1*, *PtdAKINβ3.2*, and *PtdAKINβ4.1*.

2.2.4.2. *In silico* analysis of cloned sequences

Cloned sequences were aligned in MEGA3 (Kumar et al, 2004) with gene models from release 1.1. Once release 2.0 of the *Populus* genome was available (Phytozome; http://www.phytozome.net/poplar), gene models corresponding to the original gene models found by Fedosejevs (2008) were obtained and subsequent alignments to determine if there were major differences between the

gene models or the clone sequences were done using MEGA4 (Tamura *et al.*, 2007). The cloned sequences were interrogated with InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) to confirm domain structures described by Fedosejevs (2008) in the gene models.

2.2.5. Candidate reference gene identification

A literature search and personal communication served as the basis for the identification of candidate reference genes. Candidate reference genes already used in *Populus* were identified by searching the Web of Science database for poplar experiments with similar variables as those described above. The qRT-PCR primer sequences were used as reported (see Table 2.5).

Furthermore, Czechowski *et al.* (2005) was used as a starting point for the identification of other potential reference genes for the tissue survey experiment and the nitrogen availability experiments. For the dormancy experiment, a list of putative reference genes was derived using published microarray data from poplar. In total, 50 candidate reference genes were screened.

2.2.6. qRT-PCR assays

2.2.6.1. Designing qRT-PCR primers

qRT-PCR primers were designed manually in Primer Express 3.0 (Applied Biosystems) using the default settings for testing the suitability of the primers. Because gene specific primers were desired for SnRK1 protein complex subunits, closely related sequences were aligned using MEGA3.0 (Kumar *et al.*, 2008) and primers were chosen in regions that exhibited differences of at least three nucleotides between two sequences.

2.2.6.2. Preparation of standard curves

cDNAs encoding the SnRK1 complex subunits and the appropriate reference genes were amplified from pGEM-T vectors using M13F (5'-CGC CAG GGT TTT CCC AGT CAC GAC-3') and M13R (5'-AGC GGA TAA CAA TTT CAC ACA GG-3') universal primers. The amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Germantown, MD, USA), using the manufacturer's instructions. The amplicons were quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and the concentration was converted from ng/μL to molecules/μL. Standard curves were prepared by serial dilution of the originally quantified amplicon, and ranged in concentration from 4×10^1 molecules/μL to 4×10^8 molecules/μL. The final quantity of amplicon cDNA in each well ranged from 1×10^2 to 1×10^9 molecules.

2.2.6.3. qRT-PCR assay

The qRT-PCR mix consisted of 5 μL of 2X qRT-PCR Master mix, 2.5 μL of a mixture of the forward and reverse gene-specific primer (1.6 μM each) and 2.5 μL of cDNA which had been diluted ten-fold following synthesis and RNase treatment. The 2X qRT-PCR Master mix (*Dynamite*) used is a proprietary mix developed, and distributed by the Molecular Biology Service Unit (MBSU) in the department of Biological Science at the University of Alberta (Edmonton, AB, Canada). It contains Tris (pH 8.3), KCl, MgCl2, glycerol, Tween 20, DMSO, dNTPs, ROX (Invitrogen) as a normalizing dye, SYBR Green (Invitrogen) as the detection dye, and the antibody inhibited Taq polymerase-Platinum Taq (Invitrogen).

The qRT-PCR assay was performed either on the 7500 Fast Real-Time PCR system (Applied Biosystems) in MicroAMP Fast Optical 96-well Reaction Plate with Barcode or the 7900 HT Fast Real-Time PCR System (Applied Biosystems) in 384-Well Clear Optical Reaction Plate with Barcode. Reagents and cDNA were pipetted either manually or by using the Biomek 3000 (Beckman Coulter), respectively.

The thermal profile consisted of three stages. The first stage was one cycle of 95^{o}C for 2 minutes. The second stage was 40 cycles of 95^{o}C for 15 seconds and 60^{o}C for 1 minute. Data was collected at the end of each cycle during stage 2. The third stage was a dissociation cycle of 95^{o}C for 15 seconds, 60^{o}C for 1 minute, 95^{o}C for 15 seconds and 60^{o}C for 15 seconds. The dissociation cycle generates a

dissociation curve using the first derivative of the rate of change in fluorescence as a function of temperature, which in turn is used to detect nonspecific amplification. Three technical replicates were used of each sample. Results were analyzed using the 7500 Fast System SDS Software (96-well plates) or SDS2.3 (384-well plates; Applied Biosystems).

2.2.6.4. qRT-PCR assay for gene specificity

Gene specificity of SnRK1 complex subunit qRT-PCR primers was verified using qRT-PCR, with standard curves. Members of the *PtdSnRK1* gene family, the *PtdAKINγ1* gene subfamily, and the *PtdAKINγ2* gene subfamily were tested against dilution series composed of a single member of the respective family or subfamily, as well as a mix of all members of that family. The dilution series concentrations ranged from 4×10^1 molecules/μL to 4×10^8 molecules/μL.

Members of the *PtdAKINβ* gene family were verified in a similar fashion by Fedosejevs (2008).

2.2.6.5.1. qRT-PCR assay of candidate reference genes

Reference genes for the tissue survey experiment were tested on the 7500 Fast Real-Time PCR system (Applied Biosystems). Reference genes for the nitrogen experiment and the dormancy acquisition experiment were tested on the

7900 HT Fast Real-Time PCR System (Applied Biosystems).

Expression levels of candidate reference genes for the tissue survey experiment were compared between all seven tissues harvested. Expression levels of candidate reference genes for the nitrogen experiment were compared within a single tissue between tissue treated with high nitrogen and tissue treated with low nitrogen and across the five timepoints. Expression levels of candidate reference genes for the dormancy acquisition experiment were compared within a single tissue across the five timepoints. Three biological replicates were used when testing candidate reference genes.

2.2.6.5.2. Statistical analysis of reference genes

Expression levels of the reference genes were averaged across plates and analyzed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Normality was tested using the Shapiro-Wilk test, and homogeneity of variance was tested using Bartlett's test. When necessary to meet the assumptions of normality and homogeneity of variance, the data was transformed, either by log transformation or square root transformation. A one-way ANOVA test was performed for the reference gene used in the tissue survey experiment and the dormancy acquisition experiment. A two-way ANOVA was used in the nitrogen availability experiment. If the p-value derived from the ANOVA was less than 0.05, Tukey's Studentized range test was used to determine if there were any significant differences at a p-

value of 0.05.

2.3. Results

2.3.1. Cloning *PtdSnRK1* and *PtdAKINγ* gene family members

cDNAs that were cloned to enable gene expression profiling are listed in Table 2.3. In order to denote that the cDNAs were cloned from *P. trichocarpa* × *deltoides*, the prefix “Ptd” was affixed to cDNA names. Table 2.3 also includes the corresponding gene models for the SnRK, AKINβ, and AKINγ gene families from both v1.1 and v2.0 of the *P. trichocarpa* genome. The twelve cloned cDNAs of the members of the *PtdSnRK1* and *PtdAKINγ* gene families aligned with the transcripts inferred from the *P. trichocarpa* gene models can be seen in Figures 2.1-2.12. In general, models from v1.1 and v2.0 were very similar, and in cases where there were differences, they generally differed based on the length of the UTRs. The exceptions are *PtdSnRK1.2* (Figure 2.2), *PtdAKINγ2.6* (Figure 2.6) and *PtdAKINβ2.2* (Figure 2.13). In the case of *PtdSnRK1.2*, the v1.1 gene models estExt_fgenesh4_pg.C_986004 and eugene3.00170430 correspond with the splice variants POPTR_0017s12380.1 and POPTR_0017s12380.2, respectively. The cloned sequence for *PtdSnRK1.2* corresponds with the POPTR_0017s12380.1. Furthermore, POPTR_0017s12380.1 predicts 285 bases of coding sequence at the 5' end which is not predicted by estExt_fgenesh4_pg.C_9860004. The gene model

of *PtdAKINγ2.6* for the v1.1 assembly (fgenesh4_pg.C_LG_XIX000546) predicts an exon with 363 bases which is not predicted by the gene model for the v2.0 assembly (POPTR_0019s07980.1). The cloned sequence includes the sequence predicted by fgenesh4_pg.C_LG_XIX000546. The gene model for *PtdAKINβ2.2* from the v1.1 assembly (gw1.XVI.605.1) has been split into two gene models in the v2.0 assembly (POPTR_0016s00810.1 and POPTR_0016s00820.1). The cloned sequence (obtained from Fedosejevs, 2008) aligns with both v2.0 gene models, indicating that the split may be inaccurate (Figure 2.13).

cDNAs tended to align closely to the gene models with only occasional base substitutions. Exceptions include *PtdAKINγ2.2* (Figure 2.8) and *PtdAKINγ2.4* (Figure 2.10). *PtdAKINγ2.2* includes a sequence of 42 bases in the middle of the sequence (887-928) which do not correspond with either the gene models from either release 1.1 or 2. *PtdAKINγ2.4* includes 6 bases in exon 1 which are not found in the predicted gene models in either release, nor in the genomic sequence.

PtdAKINγ1.3 was not successfully cloned, although efforts were made by varying the reaction mix and conducting touchdown PCR. Search for EST evidence of *PtdAKINγ1.3*'s presence showed that most ESTs which were originally believed to be *PtdAKINγ1.3* were actually representative of upstream or downstream genes (Fedosejevs, 2008). Furthermore, the poplar eFP browser (Wilkins *et al*., 2009) was used in order to determine if *PtdAKINγ1.3* may be highly tissue specific. *PtdAKINγ1.3* was found to be minimally expressed in all tissues except for female catkins. As tissue from female catkins was unavailable

for use, efforts to clone *PtdAKINγ1.3* were ended.

Fedosejevs' (2008) InterProScan results for the v1.1 *P. trichocarpa PtdSnRK1* and *PtdAKINγ* gene models agree with InterProScan results obtained for the *P. trichocarpa* × *deltoides* cDNAs. The *PtdAKINγ* subunits were found to have a cystathionine beta-synthase core sequence (IPR000644), with the exceptions of *PtdAKINγ2.2*, *PtdAKINγ2.3* and *PtdAKINγ2.4*. *PtdSnRK1.1* and *PtdSnRK1.3* contain a serine/threonine-protein kinase domain (IPR002290) and the serine/threonine-protein kinase active site (IPR008271). Although the cloned sequence for *PtdSnRK1.2* was not found to have either of these motifs, it shares in common with the other *PtdSnRK1* genes a protein kinase catalytic domain (IPR000719).

2.3.2. Primer validation

Table 2.4 shows the primer sequences for gene specific primers for the *PtdSnRK1* and *PtdAKINγ* gene family members. The specificity of these primers was tested by conducting qRT-PCR on standard curves of the target cDNA, closely related cDNAs, and mixtures of these cDNAs. Primers were considered to be gene specific and suitable for qRT-PCR if they produced a standard curve with an r^2 value greater than 0.99 and relatively high efficiency against the target cDNA but not closely related cDNAs. Further proof of specificity was provided if similar slopes and r^2 values were obtained against the target when mixed with

other closely related cDNAs. Efficiency is calculated by using the formula

$$E=10^{(-1/slope)}-1$$

where E is the efficiency of the reaction and the slope is the slope of the regression line generated from a plot of the C_t and the log quantities of the standard curve (Ginginzer, 2002). While a higher primer efficiency is desirable, the need for gene specific primers led to a criterion of an efficiency between 0.7 to 1.1 rather than a more stringent efficiency. This corresponds to a slope range of approximately -3.1 to -4.4.

Figures 2.14-2.25 show standard curves generated by each set of primers against various target cDNAs and the calculated linear regression. Figure 2.24 indicates that either the *PtdAKINγ2.5* primer sequences are not particularly efficient or that not enough points are present to demonstrate the linear range of the standard curve. Figure 2.26 shows that it is the latter case rather than the former.

Melting curves were checked to determine if there were multiple peaks, indicating that there are multiple products being produced during amplification. None were found except at very low quantities of the template (see Appendix for melting curves). In the end, gene specific primers lacking interfering secondary structure were successfully designed against all cloned cDNAs.

2.3.3. Reference genes

Table 2.5 lists the candidate reference genes that were tested and Table 2.6 indicates which genes were tested in the nitrogen availability and dormancy acquisition experiments. Czechowski *et al.* (2005) was used as a starting point for potential reference genes. Czechowski *et al.* (2005) identified a number of putative reference genes in *Arabidopsis* by analyzing data from Affymetrix ATH1 GeneChips, deriving a list of genes with stable expression over a variety of different conditions. They confirmed the expression levels with qRT-PCR using diverse cDNA samples. *Arabidopsis* genes which showed stable expression across tissues and during differential nitrogen availability were chosen as candidate reference genes to test in my poplar experiments. The coding sequences for the *Arabidopsis* genes were used to BLAST the *Populus trichocarpa* genome Jamboree Models (v1.1) to find the most closely related *Populus* gene model.

Candidate reference genes were considered potentially suitable if the difference in the level of expression between two treatments was less than two-fold, i.e. within one PCR cycle, assuming 100% efficient amplification.

2.3.3.1. Selection of candidate reference gene for tissue survey

A member of the elongation factor alpha family (named EF1α-3) was tested across all tissues collected using qRT-PCR with three biological replicates. The qRT-PCR assay revealed an average C_t range of 18.64 to 19.58, with standard deviations of 0.36 and 0.53, respectively. Statistical analysis was performed to

confirm that expression of this gene was not statistically different across tissues. The data across all experimental plates were $\log_{10}$ transformed to meet the assumption of normality. Post-hoc statistical tests of the expression level of EF1α-3 across all experimental plates showed that EF1α-3 was not statistically differentially expressed ($p = 0.8406$; Figure 2.27).

2.3.3.2. Selection of candidate reference gene for nitrogen availability experiments

Reference genes chosen to be tested for the nitrogen experiment were tested in three biological replicates. In order to streamline the process, candidate reference genes were initially tested only between high and low nitrogen treatments at day 14. Potential reference genes that performed suitably were then tested across all time points. The reference gene chosen was a different member of the elongation factor alpha family (named EF1α-1; Figure 2.28). A two-way ANOVA of the expression level of EF1α−1 across all experimental plates within each tissue were performed. The data from young leaves were $\log_{10}$ transformed to meet the assumption of normality and an overall p-value of 0.036 was calculated. Tukey's Studentized range test supported the null hypothesis of no significant differences between any treatments at an alpha value of 0.05. The data from secondary phloem did not require transformation and the two-way ANOVA had an overall p-value of 0.1172. The data from secondary xylem did not require

transformation. The two-way ANOVA had an overall p-value of 0.0425 and the Tukey's Studentized range test failed to show significant differences between treatments. The data from roots did not require transformation and the two-way ANOVA had an overall p-value of 0.0744. Although these results show marginal statistical significance, EF1α–1 performed better than any other gene tested, and thus it was decided to proceed with this gene as a reference gene for the nitrogen availability experiment.

2.3.3.3. Selection of candidate reference gene for dormancy acquisition experiments

Several sources were used to identify candidate reference genes for the dormancy acquisition experiment, beginning with reference genes tested in the tissue survey experiment and the nitrogen availability experiment and through a search of the literature. Furthermore, a list of putative reference genes was derived from *Populus* dormancy acquisition microarray data. A list of genes which showed no differential expression during dormancy acquisition in cambium tissue and during bud set was obtained from Schrader *et al.* (2004) and Ruttink *et al.* (2007), respectively. Genes on this list that were found to be not differentially expressed in only one data set were discarded. Additional genes on this list found to be differentially expressed during dormancy acquisition in the cambium by Druart *et al.* (2007) were also discarded, given that Druart *et al.* did not provide a list of genes which did not show differential expression. These analyses led to a

list of candidate reference genes for testing.

Second, microarray data of dormancy acquisition in spruce (El Kayal, in review) were filtered by Chelsea Ju for genes which were stably expressed in shoot tip, stem, roots and needles across several time points ranging from Day 0 to 10 weeks of short day treatment. For shoot tips, dormant tissue was also included. A list of 30 genes was derived, and the spruce EST sequences were used to BLAST the *Populus* genome v1.1 Jamboree Models to determine the most similar poplar gene model. This list of gene models was compared to the list derived from poplar microarray data, which yielded 7 candidate reference genes to test for the dormancy experiment.

For shoot tip, mature leaves and roots, *VHA-A* was selected as the most suitable candidate (Figure 2.29). An ANOVA of the reference gene C_t values in each of the tissues across all experimental plates was performed. In shoot tip, the data were $\log_{10}$ transformed to meet the assumption of normality. However, the assumption of homogeneity of variance was not met (Bartlett's test $p = 0.460$). The ANOVA had a p-value of 0.0351 but the Tukey's Studentized range test did not show significant differences between the different weeks of exposure to short day. The data from mature leaves were not transformed and the ANOVA had a p-value of 0.0737. In roots, an outlier in week 2 was removed in order to meet the assumption of normality. Attempts to transform the data to meet the assumption of homogeneity of variance failed ($p=0.0065$). The ANOVA had a p-value of 0.0464 and the Tukey's Studentized range test did not show significant differences

between the different weeks of exposure to short day.

After screening candidate reference genes, no single gene was identified as suitable for secondary phloem and secondary xylem across all time points. Because the expression profile of the reference genes being tested tended to show a trend of decreasing expression in phloem and xylem, a list of potential genes which showed a trend of increasing expression during dormancy acquisition (Group 6 from Park *et al.*, 2008) was acquired. Genes on this list which correspond to genes which were found to be invariantly expressed in microarrays run by Ruttink *et al.* (2007) and Schrader *et al.* (2004) were removed from the list. This yielded four genes which were tested. After testing of these additional candidates, it was found that the geometric mean of the expression of *VHA-A* and a phosphorylase was suitable for use as a reference for secondary phloem and secondary xylem during dormancy acquisition (Figure 2.30). The data from secondary phloem were log-transformed to meet the assumption of normality and the ANOVA had a p-value of 0.8132. In secondary xylem, an outlier was removed from week 4 in order to meet the assumption of normality and the ANOVA had a p-value of 0.5390.

2.4. Discussion

2.4.1. Cloned sequences of *PtdSnRK1* and *PtdAKINγ* gene family members

In general, alignment of the *P. trichocarpa* gene models from both v1.1 and v2.0 of the genome sequence and of the cloned cDNAs from *P. trichocarpa* × *deltoides* showed high similarity in sequence. In the case of *PtdAKINγ2.6,* where there is a notable difference between the gene models, the cloned sequence includes the region of 363 bases predicted by fgenesh4_pg.C_LG_XIX000546 which is not predicted by POPTR_0019s07980.1, indicating that POPTR_0019s07980.1 is a mistakenly truncated gene model. Unfortunately, in the case of *PtdSnRK1.2,* the cloned sequence was of insufficient length to determine if the 285 bases of coding sequence predicted by POPTR_0017s12380.1 is actually part of the mature transcript.

The 42 bp sequence in *PtdAKINγ2.2* corresponds to sequence predicted to be genomic between the second and third exons in the gene models, indicating that the gene models mistakingly exclude the sequence from the predicted transcript. In the case of *PtdAKINγ2.4*, the extra 6 bases were not predicted to be in the genomic sequence. As the *Populus* genome was sequenced from *P. trichocarpa* while the tissue used for cDNA cloning was from the hybrid *P. trichocarpa* × *deltoides*, it is possible that the extra 6 bases could represent an allele from *P. deltoides.*

2.4.2. Validation of qRT-PCR primers

qRT-PCR is the method of choice for accurate, sensitive and specific

analysis of transcript abundance in biological material. Because of the number of genes for which I planned to conduct gene expression profiling, SYBR Green I-based qRT-PCR was a more economical option than probe-based qRT-PCR. SYBR Green I is an intercalating dye which will bind to any double-stranded DNA molecule, fluorescing upon excitation with a light source. Because the SYBR Green I assay is not sequence specific, i.e. will bind to any double-stranded DNA, it is necessary to pay particular attention to appropriate primer design (to mitigate the formation of primer dimers) and the analysis of melting curves (to determine if non-target amplicons are being formed), in order to increase the robustness of SYBR Green I (Ginginzer, 2002; Bustin *et al.*, 2005). Thus, a major objective of this component of my study was to design and validate qRT-PCR primers for the *PtdSnRK1*, *PtdAKINβ*, and *PtdAKINγ* gene family members that did not generate primer dimers or other secondary structure that reduces amplification efficiency or amplicon detection, and also were gene specific so as not to generate amplicons representing multiple transcripts. In the case of this study, it is necessary to differentiate between closely related family members, and thus specific experiments were carried out to validate the specificity of the primers.

Gene specificity of the primers was intially tested on agarose gels under the assumption that gene specificity would be demonstrated through lack of amplification of product except when the desired gene was present (data not shown). However, due to the close sequence similarity between gene family

members, it was discovered that amplification could still occur when the template was part of the same gene family. The inability to quantify the amount of product made it difficult to determine if there was a significant difference in the amount of amplicon or the efficiency of the reaction. Since standard curves can be used to determine the efficiency of the primers, they were used to determine if the qRT-PCR primers were sufficiently gene specific, as poor amplification would lead to a standard curve plot with poor efficiency and r^2 values. As can be seen, when specific primer pairs are used in PCR reactions with the desired target template, amplification of the desired product lead to standard curve plots with relatively high efficiency and high r^2 values. On the other hand, the same is not true when the same primer pairs are used in conjunction with closely related sequences as templates. Furthermore, examination of the melting curves showed that there primer-dimers were not formed.

2.4.3. Reference genes

Results from a qRT-PCR assay can be normalized to either an internal control (reference gene), to a standard curve or both. Using a reference gene allows for the normalization of the data to reduce variability arising from technical sources such as cDNA synthesis or potential inhibitors which may be present in the sample (Bustin *et al*., 2005). However, validation of the reference gene chosen is necessary, as variability within the reference gene can cause

misleading results should the reference gene be expressed variably across all samples of interest.

As part of developing reliable, sensitive and robust qRT-PCR assays for *PtdSnRK1*, *PtdAKINβ,* and *PtdAKINγ* genes, it was necessary to identify genes that were constitutively expressed across biological samples that were to be compared to use as reference genes. Candidate reference genes were initially selected based on whether the difference in transcript abundance between treatments was less than two-fold. This criterion was chosen because a difference of a single PCR cycle will result in a two-fold difference in the number of amplicons, assuming 100% efficiency. It was judged that a two-fold difference was acceptable given the technical variability of the system. Statistical analyses were also carried out to test whether these small differences in expression were statistically significant or not.

Until recently, most published qRT-PCR studies have used a single reference gene. Ideally, this reference gene is assayed on the sample plate – preferably in the same well – as the target gene, to minimize technical variation between the target and reference genes (VanGuilder *et al.*, 2008). In most cases, I was able to identify a single reference gene for use in most of the qRT-PCR assays. While there has been an increasing shift towards the use of multiple reference genes in order to ensure stability (Vandesompele *et al.*, 2002; Gutierrez *et al.*, 2008; Bustin *et al.*, 2009), this approach must be coupled with technical and experimental considerations. For instance, space limitations associated with the 96-well plate

format makes the use of multiple reference genes problematic when many biological samples are to be assayed. One way to circumvent this issue is to assay reference genes on independent plates, but this may increase technical variation. Furthermore, the use of a single reference gene is valid as long as it serves the same purpose as the use of multiple reference genes – namely, that the expression level between treatments is relatively stable. As is shown, statistical analysis of the reference genes in this study across all experimental plates showed that the reference genes are not significantly differentially expressed across biological samples to be compared.

In the case of secondary phloem and secondary xylem in the dormancy acquisition experiment, two reference genes were chosen in order to ensure stable expression between all treatments. The geometric mean was chosen as a way of combining the quantified data, as this controls better for outlying values and abundance differences than an arithmetic mean (Vandesompele et al, 2002).

2.5. Conclusion

In order to develop a senstive, robust and reliable qRT-PCR assay for the members of the *PtdSnRK1*, *PtdAKINβ*, and *PtdAKINγ* gene families, I used rigorous methods to design and validate gene specific primers. I also employed a rigorous and thorough method to test candidate reference genes in order to ensure that I can have confidence that the results of the qRT-PCR assays will reflect differences in the genes of interest rather than in the reference genes.

2.6. Tables

Table 2.1. Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) checklist of data to report when using qRT-PCR assays. All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If primers are from RTPrimerDB, information on qPCR target, oligonucleotides, protocols, and validation is available from that source.

Reproduced with minor modifications for clarity from Bustin *et al.*, 2009.

Item to check	Importance	Item to check	Importance
Experimental Design		qPCR oligonucleotides	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or Investigator's laboratory?	D	Probe sequences	D[b]
Acknowledgement of author's contributions	D	Location and identity of any modifications	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D
Volume/mass of sample processed	D	qPCR protocol	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg^{2+}, and dNTP concentrations	E
If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
Sample storage conditions and duration (especially for formalin-fixed, paraffin-embedded samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact chemical composition of the buffer	D
Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Source of additional reagents used	D	Complete thermocycling parameters	E
Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
Nucleic acid quantification	E	qPCR validation	

Instrument and method	E	Evidence of optimization (from gradients)	D
Purity (A_{260}/A_{280})	D	Specificity (gel, sequence, melt, or digest)	E
Yield	D	For SYBR Green I, C_t of the no-template control	E
RNA integrity: method/instrument	E	Calibration curves with slope and y intercept	E
RNA integrity number/RNA quality indicator or C_t of 3' and 5' transcripts	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	Confidence intervals for PCR efficiency or standard error	D
Inhibition testing (C_t dilutions, spike, or other)	E	R^2 of calibration curve	E
Reverse transcription		Linear dynamic range	E
Complete reaction conditions	E	C_t variation at limit of detection	E
Amount of RNA and reaction volume	E	CIs throughout range	D
Priming oligonucleotide (if using gene-specific priming) and concentration	E	Evidence of limit of detection	E
Reverse transcriptase and concentration	E	If multiplex, efficiency and limit of detection of each assay	E
Temperature and time	E	Data analysis	
Manufacturer of reagents and catalogue numbers	D	qPCR analysis program (source, version)	E
C_ts with and without reverse transcription	D[a]	Method of C_t determination	E
Storage conditions of cDNA	D	Outlier identification and disposition	E
qPCR target information		Results for no-template controls	E
Gene symbol	E	Justification of number and choice of reference genes	E
Sequence accession number	E	Description of normalization method	E
Location of amplicon	D	Number and concordance of biological replicates	D
Amplicon length	E	Number and stage (reverse transcription or qPCR) of technical replicates	E
In silico specificity screen (BLAST, and so)	E	Repeatability (intraassay variation)	E
Pseudogenes, retropseudogenes, or other homologs?	D	Reproducibility (interassay variation, CV)	D
Sequence alignment	D	Power analysis	D
Secondary structure analysis of amplicon	D	Statistical methods for results significance	E
Location of each primer by	E	Software (source, version)	E

exon or intron (if applicable)			
What splice variants are targeted?	E	C_t or raw data submission with Real-Time PCR Data Markup Language	D

[a] Assessing the absence of DNA with a no-reverse transcription assay is essential when first extracting RNA. Once the sample has been validated as DNA free, inclusion of a no-reverse transcription control is desibrable but no longer essential.

[b] Disclosure of the probe sequence is highly desirable and strongly encouraged; however, because not all vendors of commercial predesigned assays provide this information, it cannot be an essential requirement. Use of such assays is discouraged.

Table 2.2. Primers designed to clone *PtdSnRK1* and *PtdAKINγ* gene family members. In cases where there are multiple potential forward or reverse primers, primers which were ultimately used for cloning are indicated with a *.

cDNA	Primer name	Sequence (5'-3')	Tm	GC content (%)
PtdSnRK1.1	PtdSnRK1.1_left_primer	GCAGATAATTTCCGGTGTGG	60.33	50
	PtdSnRK1.1_right_primer	GGTCCTCTCAAAAGCCTACCTAC	59.68	52.17
PtdSnRK1.2	PtdSnRK1.2_left_primer	TGCTCTTCTCTGCGGTACAC	60.21	50
	PtdSnRK1.4/1.2_right_primer	TCTCGAAAGGCTACCTACAGGAC	61.12	52.17
PtdSnRK1.3	PtdSnRK1.3_left_primer	GTATGCTGGGCCTGAAGTGG	62.91	60
	PtdSnRK1.3_right_primer	AAGGACTCGGAGCTGTACAAGG	62.03	54.55
PtdAKINγ1.1	cPtdAKINg1.1_L1	CTGGTTTCGGGATCTTAGGC	60.95	55
	cPtdAKINg1.1_R1	GATACATCAACGTCGGAGTGG	60.39	52.38
	cPtdAKINg1.1_R2*	ACGTCGGAGTGGAGGAGAAC	61.65	60
PtdAKINγ1.2	cPtdAKINg1.2 _L1*	GACACTAGTCGACCTTTCTATACCG	59.6	48
	cPtdAKINg1.2_L2	GTTCATGAGGTTACGCAAGAC	57.3	47.6
	cPtdAKINg1.2_R1	CAAGCCTTGATAGTATATCCCTCAG	59.6	44
	cPtdAKINg1.2_R2*	CGTCAGTTATTAGGAAATGACCAGA	60.7	40
PtdAKINγ1.3	cPtdAKINg1.3_L1	ATGTTTCTTGATCACATCCCCATT	62.8	37.5
	cPtdAKINg1.3_R1	TCAATGATCACAAATGACAGTTCC	61.1	37.5
PtdAKINγ1.4	cPtdAKINg1.4_L1*	GTCGGTTGCTGATGCTGTTAG	60.84	52.38
	cPtdAKINg1.4_L2	CTTCATCATTCCCACCAGTTCC	62.87	50
	cPtdAKINg1.4_R1	ACCTTGCCCTCACCACTTTC	61.45	55
	cPtdAKINg1.4_R2*	TCCTGTGACCTGACCTGGAAG	62.62	57.14
PtdAKINγ2.1	cPtdAKINg2.1_L1	TCTCGCACTCACCACAACAATAG	62.5	47.8
	cPtdAKINg2.1_R1*	GATCAGGATTGAGCCTAGCAGAT	61.1	47.8
	cPtdAKINg2.1_R2	TTCCCTGAAAACTTTTAGCATATCAT	60.5	30.8
PtdAKINγ2.2	cPtdAKINg2.2_L1*	CTCTCTCACTCACCACAACGATAA	60.7	45.8
	cPtdAKINg2.2_L2	GCACACAGCAAAAACTAATAAAGAC	58.6	36
	cPtdAKINg2.2_R1	CGTTAACGATAAAGCCATCAG	57.5	32.9
	cPtdAKINg2.2_R2*	CCTGAAAACTTTCAACACATCAC	58.7	39.1
PtdAKINγ2.3	cPtdAKINg2.3_L1*	GCATGGCAGCAAGTATTTTATCTC	61.27	41.67
	cPtdAKINg2.3_L2	GAACCTCACGCCAGCTTAC	58.46	57.89
	cPtdAKINg2.3_R1	GCATGATCACACCATTGACC	59.78	50
PtdAKINγ2.4	cPtdAKINg2.4_L1	GTCTGTGCATGGCAGTCAGTATC	62	52.2

	cPtdAKINg2.4_R1*	GAATGCTCACGCCATTGATT	61	45
	cPtdAKINg2.4_R2	AAATCATAGTTTTGGCTCCTCTC	58	39.1
PtdAKINγ2.5	cPtdAKINg2.5_L1	GATTCGGGCGATTGGAGAG	63	57.9
	cPtdAKINg2.5_R1	CCACAAGCTTATATCTCTAGTACAATG	57	37
	cPtdAKINg2.5_R2*	AAGAAACCTATAAATTCCCACAAGC	60.1	36
PtdAKINγ2.6	cPtdAKINg2.6_L1	GGGATAAGGCTATCAAGACTCCA	60.8	47.8
	cPtdAKINg2.6_L2*	GATTCGGGCAATAGGAGAA	57.7	47.4
	cPtdAKINg2.6_R1	GTTCAACACAAAGTTGCAAAAG	57.5	36.4

Table 2.3. *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene family members with corresponding gene models from v1.1 (provided by Fedosejevs, 2008) and v2.0 of the *Populus trichocarpa* genome. For those genes which were cloned, full cDNA sequence length and the length of any 5' and 3' UTR are included. *PtdAKINβ* genes were cloned by Fedosejevs (2008). The uncertainty in the length of *PtdAKINγ2.1* is due to the presence of a T repeat sequence in the 3' UTR which, when sequenced, has varied in length from 14 to 17 bases.

cDNA	Corresponding *P. trichocarpa* v1.1 gene model	Corresponding *P. trichocarpa* v2.0 gene model	plasmid	*P. trichocarpa* × *deltoides* cDNA length (bp)	full length CDS?	5' UTR	3'UTR
PtdSnRK1.1	estExt_fgenesh4_pg.C_LG_IV1177	POPTR_0004s11500.1 POPTR_0004s11500.2	pGEM-T	1196	no	none	19
PtdSnRK1.2	eugene3.00170430 estExt_fgenesh4_pg.C_9860004 gw1.9913.5.1	POPTR_0017s12380.1 POPTR_0017s12380.2	pGEM-T Easy	939	no	none	14
PtdSnRK1.3	gw1.XIII.3230.1	POPTR_0013s09420.1	pGEM-T	1340	no	none	none
PtdAKINγ1.1	estExt_Genewise1_v1.C_LG_XV2076 eugene3.183700001	POPTR_0015s10680.1	pGEM-T Easy	1002	no	none	169
PtdAKINγ1.2	eugene3.00120902	POPTR_0012s09900.1	pGEM-T	1384	no	none	41
PtdAKINγ1.3	fgenesh4_pm.C_LG_IX000242	POPTR_0009s12130.1					
PtdAKINγ1.4	eugene3.00170150 grail3.1838000101	POPTR_0017s08070.1	pGEM-T Easy	1004	no	none	none
PtdAKINγ2.1	fgenesh4_pm.C_scaffold_107000027 fgenesh4_pg.C_scaffold_7795000001	POPTR_0001s41330.1	pGEM-T	1244-1247	no	none	73-76
PtdAKINγ2.2	fgenesh4_pg.C_LG_XI000996	POPTR_0011s12260.1	pGEM-T	1160	no	none	none
PtdAKINγ2.3	eugene3.00040536	POPTR_0004s04120.1	pGEM-T Easy	1193	yes	2	6
PtdAKINγ2.4	eugene3.01240024	POPTR_0011s05010.1	pGEM-T	1196	yes	8	6

PtdAKINγ2.5	eugene3.00870063	POPTR_0013s08520.1	pGEM-T	1309	no	none	129
PtdAKINγ2.6	fgenesh4_pg.C_LG_XIX000546	POPTR_0019s07980.1	pGEM-T	1161	no	none	none
PtdAKINβ1.1	gw1.I.735.1	POPTR_0001s22800.1	pGEM-T	1008	yes		
PtdAKINβ1.2	gw1.IX.4510.1	POPTR_0009s02670.1	pGEM-T	923	yes		
PtdAKINβ2.1	grail3.0024005201	POPTR_0006s00760.1	pGEM-T	981	yes		
PtdAKINβ2.2	gw1.XVI.605.1	POPTR_0016s00810.1 POPTR_0016s00820.1	pGEM-T	1070	yes		
PtdAKINβ3.1	eugene3.00660287	POPTR_0004s22360.1	pGEM-T	523	yes		
PtdAKINβ3.2	grail3.0001135601	POPTR_0009s01430.1 (primary) POPTR_0009s01430.2	pGEM-T	641	yes		
PtdAKINβ4.1	gw1.XIV.3789.1	POPTR_0014s16550.1	pGEM-T	1070	yes		

Table 2.4. Gene specific qRT-PCR primers for members of the *PtdSnRK1* and *PtdAKIN*γ gene families.

qPCR primer	**Sequence (5' - 3')**
qPtdAKINg1.1_L1	CTCTTTCTGATTGTTGGATACCCA
qPtdAKINg1.1_R1	CGTCGGAGTGGAGGAGAACTA
qPtdAKINg1.2_L1	GAGAAAATCCACCGGGTATATGTC
qPtdAKINg1.2_R1	AAGTAGCCACGGGGCTCG
qPtdAKINg1.4_L1	CAGGCAGCATACCGTTATGGAT
qPtdAKINg1.4_R1	GCTTCCTGTTCCTTTGGTAGTTG
qPtdAKINg2.1_L1	GGCATTGTCAGATTTTATGATATGCTA
qPtdAKINg2.1_R1	TCCTCATCTTTTTAGGCCATATCC
qPtdAKINg2.2_L1	GTCATTCACAGTCTTCATCGTCATC
qPtdAKINg2.2_R1	GCTGTACTTTCCTGGTCTCTGCA
qPtdAKINg2.3_L1	AGTGGGGAGGAGCACCG
qPtdAKINg2.3_R1	CTCTTCAATAACCCATGTGTAGCTTAA
qPtdAKINg2.4_L1	TCAGCAGGGGTGAGGGGT
qPtdAKINg2.4_R1	TCGATAACCCATGTGCAGCTC
qPtdAKINg2.5_L1	GGGGGTTTCACAACTGAATTTC
qPtdAKINg2.5_R1	TCACACGCGCACACACG
qPtdAKINg2.6_L1	GACATCTTGGCTGCGGTAACA
qPtdAKINg2.6_R1	AGCCTCAGGTCGATTCACATG
qPtdSnRK1.1_L2	TGAAGGAATGGTTAATGATCCAGTG
qPtdSnRK1.1_R1	TGACCACATTTGGTGAATTAGTGACT
qPtdSnRK1.2_L3	AATGGTTAACCATCCAGCACACTA
qPtdSnRK1.2_R3	ACATTTGGTGAGTTAATGATTCCATTA
qPtdSnRK1.3_L2	GCTGCCCCAGAGGTCCTAG
qPtdSnRK1.3_R2	TCATCATCAAATGGTAGTGAACCA

Table 2.5. Potential reference genes derived from the literature and qRT-PCR primers. Though gene models from the v1.1 release of the poplar genome was used to design primers, gene models from the v2.0 release of the poplar genome are included for reference. References used: Langer *et al.*, 2004; Schrader *et al.*, 2004; Czechowski *et al.*, 2005; Druart *et al.*, 2007; Loivamaki *et al.*, 2007; Ruttink *et al.*, 2007; Park *et al.*, 2008; El Kayal, in review.

[1] Primers were designed by Adriana Almeida-Rodriguez.

[2] Primers were used as cited in the literature.

Candidate reference gene	Sequence used as BLAST query	Source	*P. trichocarpa* gene model (v1.1)	*P. trichocarpa* gene model (v2.0)	Primer name	Primer Sequence
Actin	-	Langer *et al.*, 2004	estExt_fgenesh4_kg.C_LG_I0082	POPTR_0001s31700.1	PtACT2fwd[2] PtdACT2rev[2]	CCCAGAAGTCCTCTT ACTGAGCACAATGTTAC
Cdc2	-	Cooke, pers comm.	grail3.0056004702	POPTR_0004s14080.1	cdc2popF[2] cdc2popR[2]	TGAAACCTCAGAATTTGCTTA TACCACAGGGTAACAACCTC
chitinase	-	Park *et al.*, 2008	estExt_Genewise1_v1.C_LG_III2334	POPTR_0003s17160.1	qPtchitinase_L1 qPtchitinase_R1	TGGGTGTTGGTTGAAACATGA GATATGAGAAAAAGGGTCCGCTG
clathrin adaptor complex medium subunit	At5g46630 (*Arabidopsis*)	Czechowski *et al.*, 2005	estExt_fgenesh4_pm.C_290079	POPTR_0001s02640.1	qPtclathrin_L1 qPtclathrin_R1	TCACTGCTTCATTAGCCTTGTCAA ATCAAGGAAATCAGCGGCCT
EF1α–1	At5g60390 (*Arabidopsis*)	Czechowski *et al.*, 2005; Almeida-Rodriguez, pers comm.	grail3.0028013201	POPTR_0006s13310.1	QPEF1_F[1] QPEF1_R[1]	TTTCTGCCTATCCTCCTCTTGGT CAACCGCCACGGTCTGA
EF1α-3	At5g60390 (*Arabidopsis*)	Czechowski *et al.*, 2005; Almeida-Rodriguez, pers comm.	eugene3.00102124	POPTR_0010s22620.1 (primary); POPTR_0010s22620.2	QPEF1-3_F[1] QPEF1-3_R[1]	TGGTCCATTTCTTGGATGTCTATC GCCTTGCAATGAAGGTGATGA
expressed protein	At4g26410 (*Arabidopsis*)	Czechowski *et al.*, 2005	grail3.0116001202	POPTR_0001s03860.1	qPtdgrail3.0116001202_L1 qPtdgrail3.0116001202_R1	AACTGCAGATTTGATGGATGGA CCTCAGCTCGAAGTTTCAAAGC
expressed	At4g33380	Czechowski *et al.*,	estExt_fgenesh4_pg.C_	POPTR_0002s12910.1	qPexpressedgene_L1	TCATTTGGTTTTTTTGGAAAGAGAG

protein	(*Arabidopsis*)	2005	LG_II1155		qPexpressedgene_R1	ATATCATTAGCGCCAGGACTTCC
gw1.29.252.1 (unknown)	GQ02012_I18 (white spruce)	El Kayal, pers comm.	gw1.29.252.1	POPTR_0001s02090.1	qPtgw1.29.252.1_L1 qPtgw1.29.252.1_R1	GGCTGCTTGGGTCACTGGT CTGCCTTTTCTTTGGCCTTCT
histone2A	GQ0194_J05 (white spruce)	El Kayal, pers comm.	estExt_fgenesh4_pg.C_LG_V1315	POPTR_0005s23810.1 (primary); other transcripts: POPTR_0005s23810.2, POPTR_0005s23810.3, POPTR_0005s23810.4	qPt_histone2A_L1 qPt_histone2A_R1	TTGGTGACTGTATCTGGGTTTAGG CACAAACGCAAGCACAACATT
latex protein	At1g14930 (*Arabidopsis*)	Almeida-Rodriguez, pers comm.	grail3.0010060001	POPTR_0008s13050.1	QPUBQ10_F[1] QPUBQ10_R[1]	CTGCCGTTGCTGCTTCCT AAGCCCGTGAATGGCACTT
latex protein	At1g14930 (*Arabidopsis*)	-	estExt_fgenesh4_pg.C_LG_X1001	POPTR_0010s12110.1	qPlatexprotein_L1 qPlatexprotein_R1	TGAGCCCTGCCAAGATACAGA CCGGCTTCCCCCATTC
latex protein	At1g14930 (*Arabidopsis*)	-	estExt_Genewise1_v1.C_LG_VIII0155	POPTR_0008s13040.1	Qplatexprotein_L2 Qplatexprotein_R2	GCGCTTTCGTGCAACGA CAAGATCTCCCCACAAATCACA
MSI1	MNC5694908 (spruce)	El Kayal, in review; Schrader *et al.*, 2004; Ruttink *et al.*, 2007; Druart *et al.*,2007	estExt_fgenesh4_pg.C_LG_XIV1179	POPTR_0014s17790.1	qPtMSI1_L1 qPtMSI1_R1	TTTTCCTCCAATAGATCCCGAAC ATCCAAACAACGCACAAGCAC
MSI1	MNC5694908 (spruce)	El Kayal, in review; Schrader *et al.*, 2004; Ruttink *et al.*, 2007; Druart *et al.*,2007	estExt_fgenesh4_pg.C_LG_XIV1179	POPTR_0014s17790.1	qPtMSI1_L2 qPtMSI1_R2	GATATACCAGCAGATGAATCAACAAAA GTTCGGGATCTATTGGAGGAAAA
nicotinate phosphoribosyltransferase family protein/ NAPRTase family protein	MNC5693726 (spruce)	El Kayal, in review; Schrader *et al.*, 2004; Ruttink *et al.*, 2007; Druart *et al.*,2007	estExt_fgenesh4_pg.C_LG_V0531	POPTR_0005s18300.1	qPtNAPRTase_L1 qPtNAPRTase_R1	AGGCGGCTGAACCCAAC GTACAAAGTTCCATGCACCAAATC

phosphorylase	-	Park *et al.*, 2008	grail3.0018037001	POPTR_0003s13440.1	qPtphosphorylase_L1 qPtphosphorylase_R1	TGTGAAGAAGAATCAGCTACTGGC CTTAGCCTTGTTCATACTCGTGACAC
PP2A	At1g13320 (*Arabidopsis*)	Czechowski *et al.*, 2005; Almeida-Rodriguez, pers comm..	estExt_fgenesh4_pm.C_LG_VIII0497	POPTR_0008s11730.1 (primary); POPTR_0008s11730.2	QPPP2A_F[1] QPPP2A_R[1]	CGAATGCGCGCTCTCAT CACGGGATACAAACAAAGCAAA
PP2A	At1g59830 (*Arabidopsis*)	Czechowski *et al.*, 2005	grail3.0009039502	POPTR_0008s19590.1	qPPP2A_L1 qPPP2A_R1	CATCATTCTTTATTTGGAATCTGCTGT GGCCATTGGTGCCTTCTGT
PP2A-2	At1g13320 (*Arabidopsis*)	Czechowski *et al.*, 2005; Almeida-Rodriguez, pers comm..	fgenesh4_pg.C_scaffold_203000013	POPTR_0012s06210.1	QPPP2A-2_F[1] QPPP2A-2_R[1]	CCCACACTATCTGTATCGGATGAC CGACCCCATTACAGGAGAGAGT
putative diacylglycerol kinase	MNC5698225 (spruce)	El Kayal, in review; Schrader *et al.*, 2004; Ruttink *et al.*, 2007; Druart *et al.*,2007	grail3.0020019502	POPTR_0018s10570.1	qPtDGK_L1 qPtDGK_R1	GCTGAAATCACTACCAAGCATTGT AGGAGCTCTCCAAAACTAAAAAGGA
Ran GTPase binding / chromatin binding / zinc ion binding	MNC5696159 (spruce)	El Kayal, in review; Schrader *et al.*, 2004; Ruttink *et al.*, 2007; Druart *et al.*,2007	gw1.IX.3062.1	POPTR_0009s07610.1	qPtGTPasebinding_L1 qPtGTPasebinding_R1	CATGCGAAGAACCAGACACG CAATGCGGTTGCTGGTGA
REF	-	Park *et al.*, 2008	estExt_Genewise1_v1.C_410611	POPTR_0013s10350.1	qPtREF_L1 qPtREF_R1	GCCGATACAGCAAGTGCTTCT GCTAACTCCTGAGTCTGAACTTGTTTT
ribosomal protein L15e	GQ0256_J04 (white spruce)	El Kayal, pers comm.	grail3.0096009401	POPTR_0003s07630.1	qPt_ribprotL15e_L1 qPt_ribprotL15e_R1	CTGAGTCCAGTGATGTGGCTGT AGAAGAACACGTTCCAAGATTTCC
RNase H domain-containing protein	MNC5692232 (spruce)	El Kayal, in review; Schrader *et al.*, 2004; Ruttink *et al.*, 2007; Druart *et al.*,2007	estExt_Genewise1_v1.C_LG_IV4343	N/A (scaffold_4:13548632..13554608)	qPtRNasedomcont_L1 qPtRNasedomcont_R1	AAACCTGGCTGCTAATCTTAGGG AGGGCCTGAATTACTTTCTGATACA
SAND	At2g28390 (*Arabidopsis*)	Czechowski *et al.*, 2005; Almeida-	eugene3.00091499	POPTR_0009s01980.1 (primary); other	QPSAND_F[1] QPSAND_R[1]	TGTCACCAGAAATTCTCAACGAA TTCCCAACTTATACCCAATTCCA

		Rodriguez, pers comm..		transcripts: POPTR_0009s01980.2		
SAND	At2g28390 (*Arabidopsis*)	Czechowski *et al.*, 2005	eugene3.00091499	POPTR_0009s01980.1 (primary); other transcripts: POPTR_0009s01980.3	qPtdSAND_L2 PtdSAND_R2	GAAGGACGACAACAAGATCAAGG GACACTCCTGACGAGGCCAA
TIF5A	-	Czechowski *et al.*, 2005; Almeida-Rodriguez, pers comm.	estExt_Genewise1_v1.C_LG_VI0968	POPTR_0006s19870.1	QPTIF5A_F[1] QPTIF5A_R[1]	AACTCGCAAGGCATGTAATGG AACTCGCAAGGCATGTAATGG
TIF5A-2	-	Czechowski *et al.*, 2005; Almeida-Rodriguez, pers comm.	estExt_fgenesh4_pm.C_LG_XVIII0351	POPTR_0018s11660.1	QPTIF5A-2_F[1] QPTIF5A-2_R[1]	CCCTGATGAAGGGAACTAGGTTT TTAAGTAGCACAGACAAATGTGAAGTAGAT
TIF5A-3	estExt_Genewise1_v1.C_LG_VI0968	-	estExt_fgenesh4_pm.C_LG_VIII0372	POPTR_0008s09150.1	qPTIF5A_L3 qPTIF5A_R3	TTTATGTGGGTTTGAGAACTGGG CCAAGAACCACAAGAATATCATTCATT
TIF5A-4	estExt_Genewise1_v1.C_LG_VI0968	-	estExt_Genewise1_v1.C_LG_X0940	POPTR_0010s17020.1	qPTIF5A_L4 qPTIF5A_R4	TTTGATTGGGAGTTTTATCCGTG GTGCCAAGAAGTGTATGCAGTCTTAC
TIF6	GQ0257_J02 (white spruce)	El Kayal, pers comm.	gw1.V.3551.1	POPTR_0005s10150.1	qPtTIF6_L1 qPtTIF6_R1	CAAGGGAACAGAGGAAATGATTG GTGTGGGGATGGACCAGG
TIP41	At4g34270 (*Arabidopsis*)	Czechowski et al., 2005	estExt_fgenesh4_pm.C_LGIX0344	POPTR_0005s10150.1	qPTIP41_L1 qPTIP41_R1	AGGCTGTAAATTATCTGTGCATGAAG CCAGTGAATATCGTTTCCTTTTTCTC
TUB	-	Loivamaki *et al.*, 2007	gw1.I.1980.1, gw1.6820.2.1, gw1.I.1974.1	POPTR_0001s27960.1	PcTUB_F[2] PcTUB_R[2]	GATTTGTCCCTCGCGCTGT TCGGTATAATGACCCTTGGCC
UBA	MNC5694437 (spruce)	El Kayal, in review; Schrader *et al.*, 2004; Ruttink *et al.*, 2007; Druart *et al.*,2007	gw1.XVI.1249.1	POPTR_0016s03500.1 (primary)	qPtUBA_L1 qPtUBA_R1	GGTTTCTATGGGCTTTGATAGGAG CCAATTCACAGCACCCATGA

UBA		El Kayal, in review; Schrader *et al.*, 2004; Ruttink *et al.*, 2007; Druart *et al.*,2007	estExt_fgenesh4_pg.C_LG_VI0320	POPTR_0006s03850.1	qPtUBA_L2 PtUBA_R2	TCTTGAAGCACAGTCCCACTGA GTTTATCTCCAAACTAACAGCCTGAA
UBC	At5g25760 (*Arabidopsis*)	Czechowski *et al.*, 2005	estExt_Genewise1_v1.C_LG_VI0122	POPTR_0006s25760.1	qPUBC_L3 qPUBC_R3	AAAGAAAGGATGAATCTGTGCAAA TCCGTGGCATTCATCAAACTT
UBC	At5g25760 (*Arabidopsis*)	Czechowski *et al.*, 2005	estExt_fgenesh4_pm.C_LG_XVIII0083	POPTR_0018s00610.1	qPUBC_L2 qPUBC_R2	CAACCATTTGCTGTGCTTTGAA TGACAAATGTCCATTTGGTCTCA
UBC 9	At4g27960 (*Arabidopsis*)	Czechowski *et al.*, 2005	estExt_Genewise1_V1.C_LG_I8663	POPTR_0001s10290.1	qPUBC_L4 qPUBC_R4	CAGAGACCACTTGGAGGATGAAC ACTTCAACATTTCCGGTAATGGA
UBC 9	At4g27960 (*Arabidopsis*)	Czechowski *et al.*, 2005	eugene3.00041353	POPTR_0004s18090.1	qPUBC_L1 qPUBC_R1	GCGTGCTGAATAAAAACAAGGA TGTCCGAGAAATGAGAGACTCAAAT
UBC5	At4g27960 (*Arabidopsis*)	-	estExt_fgenesh4_kg.C_LG_III0041	POPTR_0003s13600.1 (primary); POPTR_0003s13600.2	qPUBC_L5 qPUBC_R5	TTGATGTCCAGGAACAGGGTTA CCCATGCAAAGAATCATCCAT
UBC6	At4g27960 (*Arabidopsis*)	-	eugene3.01970019	POPTR_0015s06060.1	qPUBC_L6 qPUBC_R6	TTCCCCTATGTATCAATGCTTGC GCCAGTTTTGCCCCGTTT
UBQ10-3	At4g05320 (*Arabidopsis*)	Czechowski *et al.*, 2005	grail3.0064002701	POPTR_0017s06450.1 (primary); POPTR_0017s06450.2	UBQ10-3F[1] UBQ10-3R[1]	CCTCCGCGGTGGTTTCTAA GGGACACAAACACCTGACCAT
UBQ10-4	At4g05320 (*Arabidopsis*)	Czechowski *et al.*, 2005	estExt_fgenesh4_pg.C_LG_I1883	POPTR_0001s27020.1	qPUBQ10_L4 qPUBQ10_R4	TTGGATGGTTTCAAATAAGATTGC AGGATGTGACATAAAATGACCAAAAG
UBQ10-5	At4g05320 (*Arabidopsis*)	-	estExt_fgenesh4_pg.C_188960001	POPTR_0001s44440.1 (primary); POPTR_0001s44440.2-5	qPUBQ10_L5 qPUBQ10_R5	GATGTGCTGTTCATGTTGTCCAA AAGACTGCTACTGAACACACACAAGAA
UBQ10-6	At4g05320 (*Arabidopsis*)	-	estExt_fgenesh4_pm.C_LG_XI0348	POPTR_0011s13770.1 (primary); POPTR_0011s13770.2-8	qPUBQ10_L6 qPUBQ10_R6	CTCCGTGGTGGTTTTTAAGCTTC CGAAAATGGCTACTGAGCACAC

UBQ10-7	At4g05320 (*Arabidopsis*)	-	eugene3.00002208	POPTR_0017s02410.1	qPUBQ10_L7 PUBQ10_R7	TGGTTTCTGAGTCGTTATTCTGTGA CTCGTTCCGGCCAATTCA
UBQ10-8	At4g05320 (*Arabidopsis*)	-	eugene3.00280152	POPTR_0006s13160.1	qPUBQ10_L8 qPUBQ10_R8	CGTGCTCCGTCTTCGAGG AAAAAAGAAAAGAAGAGACATAACCA CC
UBQ10-9	At4g05320 (*Arabidopsis*)	-	estExt_Genewise1_V1. C_LG_XI1809		qPtUBQ10_L9 qPtUBQ10_R9	CTAAGGGTCTCTGGTTCTGCTCAA GCTGAGACTTTTATTCAATCATTAGGAA
UBQ11	At4g05050 (*Arabidopsis*)	Czechowski *et al.*, 2005	estExt_fgenesh4_pg.C_ 188960001	POPTR_0001s44440.1 (primary); POPTR_0001s44440.2-5	QPUBQ1_F[1] QPUBQ1_R[1]	GCTGTTCATGTTGTCCAAGATAATG AGACTGCTACTGAACACACACAAGAA
VEP1	-	Park *et al.*, 2008	estExt_Genewise1_v1. C_LG_IX0905	POPTR_0009s14420.1	qPt_VEP1_L1 qPt_VEP1_R1	CCTTCAAGTTTTACGCCCACTG GTTCCCGACTCTGTTATTCTCCA
VHA-A	MNC5697026 (spruce)	El Kayal, in review; Schrader *et al.*, 2004; Ruttink *et al.*, 2007; Druart *et al.*,2007	estExt_Genewise1_v1. C_LG_X3071	POPTR_0010s26000.1	qPtVHA-A_L1 qPtVHA-A_R1	AGTTGCATGAGGATTTGACTGCT TGCTGTTGGTCTCATGCTGC

Table 2.6. Reference genes tested under conditions of differential nitrogen availability and dormancy acquisition. For the nitrogen availability experiment, the C_ts represented are for the mean C_ts of three biological replicates grown in low and high nitrogen availability for the tissue indicated. For dormancy acquisition experiment, the C_ts represented are for the mean C_ts of three biological replicates grown in short day conditions during week 0 and week 8 for the tissue indicated. Standard deviations are indicated in brackets. ST = shoot tips,YF = young leaves, MF = mature leaves, 2P = secondary phloem, 2X = secondary xylem, R = roots.

Putative reference gene	Name of Primer	Nitrogen Availability					Dormancy Acquisition					
		YF	MF	2P	2X	R	ST	YF	MF	2P	2X	R
Actin	PtACT2fwd		19.86 (0.27)	19.92 (0.36)	21.06 (0.98)	19.81 (0.41)			18.48 (0.23)	16.73 (0.16)		16.68 (0.19)
	PtdACT2rev		23.9 (0.48)	21.62 (0.58)	21.15 (0.14)	19.78 (0.46)			18.15 (0.17)	18.2 (0.33)		17.05 (0.93)
Cdc2	cdc2popF/R	19.56 (0.16)	22.71 (0.25)	22.67 (0.27)	24.31 (0.74)	23 (0.32)		26.79 (0.68)	21.75 (0.23)	20.75 (0.14)		21.36 (0.22)
		20.67 (0.43)	26.04 (0.57)	24.1 (0.5)	24.24 (0.73)	23.09 (0.29)		29.23 (0.11)	22.37 (0.11)	22.5 (0.25)		21.86 (0.56)
chitinase	qPtchitinase_L1/R1										17.86 (0.3)	
											15.66 (0.51)	
clathrin adaptor complex medium subunit	qPtclathrin_L1/R1								23.04 (0.44)			
									24.79 (0.1)			
EF1α-1	QPEF1_F/R	16.84 (0.25)	18.47 (0.39)	17.34 (0.17)	18.5 (0.48)	17.8 (0.26)		25.75 (0.1)	18.25 (0.49)			26.41 (1.06)
		17.08 (0.39)	22.07 (0.24)	17.99 (0.43)	18.8 (0.66)	17.05 (0.17)		27.75 (0.56)	19.96 (0.8)			25.7 (0.32)
EF1α-3	QPEF1-3_F/R		19.63 (0.25)	19.57(0.28)	21.3 (0.88)	19.94 (0.25)		24.46 (0.97)	18.07 (0.23)			26.22 (0.92)
			23.12 (0.13)	20.18 (0.29)	21.92 (2.12)	19.28 (0.42)		26.98 (0.39)	20.68 (0.59)			26.47 (0.14)
expressed protein	qPtdgrail3.011600120 2_L1/R1	21.02 (0.4)	23.82 (0.33)	24.34 (0.36)	25.96 (0.69)	24.91 (0.3)	21.21 (0.18)	28.31 (0.81)	22.25 (0.27)	21.81 (0.07)	22.51 (0.84)	22.38 (0.25)
		20.94 (0.51)	27.09 (0.27)	25.05 (0.4)	27.14 (2.05)	24.2 (0.25)	21.76 (0.06)	29.66 (0.24)	21.55 (0.08)	22 (0.29)	23.62 (0.4)	22.25 (0.34)
expressed protein	qPexpressedgene_L1/ R1		24.76 (0.26)	25.54 (0.24)	26.53 (0.49)	25.82 (0.28)	22.68 (0.2)		22.59 (0.2)	22.62 (0.33)		
			28.28 (0.27)	27.26 (0.51)	27.61 (0.26)	25.95 (0.28)	23.56 (0.16)		22.26 (0.28)	23.65 (0.27)		
gw1.29.252 .1	qPtgw1.29.252.1_L1/ R1		21.68 (0.51)	22.71 (0.34)	22.06 (0.62)	22.57 (0.3)	20.94 (0.32)		20.63 (0.22)	20.27 (0.22)		
			25.81 (0.42)	24.74 (0.52)	23.49 (0.35)	23.34 (0.53)	22.21 (0.39)		20.89 (0.05)	22.23 (0.48)		

(unknown)													
histone2A	qPt_histone2A_L1/R1		21.1 (0.22) 24.94 (0.31)	22.09 (0.15) 23.69 (0.27)	22.44 (0.65) 23.52 (0.45)	22.65 (0.26) 22.57 (0.47)	18.74 (0.74) 21.24 (0.31)		20.22 (0.22) 20.32 (0.13)	20.05 (0.14) 21.65 (0.26)		
latex protein	QPUBQ10_F/R	19 (0.39) 18.13 (0.3)	21.56 (0.46) 21.62 (0.32)	21.17 (0.19) 21.93 (0.27)	22.91 (0.37) 22.33 (1.58)	19.53 (0.26) 17.79 (0.34)		26.59 (1.1) 28.57 (0.39)	19.14 (0.54) 24.36 (0.2)			26.07 (1.06) 26.16 (0.19)
latex protein	qPlatexprotein_L1/R1		29.88 (0.52) 29.18 (0.89)	25.18 (0.29) 24.31 (0.58)	32.6 (0.68) 30.92 (0.95)	20.56 (0.42) 19.05 (0.31)			28.25 (0.64) 32.73 (2.08)			
MSI1	qPtMSI1_L1/R1									36.46 (1.06) 35.77 (0.51)		36.05 (0.44) 35.38 (0.13)
MSI1	qPtMSI1_L2/R2										poor amplification	
nicotinate phosphoribosyltransferase family protein / NAPRTase family protein	qPtNAPRTase_L1/R1									20.53 (0.23) 21.52 (0.21)	20.97 (0.55) 22.52 (0.49)	20.82 (0.3) 20.87 (0.37)
phosphorylase	qPtphosphorylase_L1/R1						22.66 (0.16) 18.41 (0.57)		24.26 (0.52) 24.48 (0.35)	18.61 (0.62) 16.58 (0.18)	21.17 (0.9) 19.19 (0.5)	19.22 (0.53) 16.72 (0.25)
PP2A	QPPP2A_F/R		24.41 (0.19) 27.44 (0.25)	24.02 (0.2) 24.9 (0.35)	24.71 (0.38) 25.63 (0.79)	23.78 (0.27) 23.89 (0.46)	21.39 (0.18) 22.02 (0.18)		22.69 (0.25) 22.37 (0.22)	21.49 (0.1) 22.7 (0.14)		
PP2A	qPPP2A_L1/R1		23.49 (0.23) 26.5 (0.22)	24.34 (0.29) 26.19 (0.26)	25.4 (0.64) 27.22 (1.02)	24.45 (0.34) 24.92 (0.37)			22.38 (0.18) 24.36 (0.49)			
PP2A-2	QPPP2A-2_F/R		23.07 (0.29) 27.27 (0.44)	23.85 (0.16) 25.32 (0.42)	24.82 (0.43) 27.28 (2.28)	24.18 (0.32) 24.17 (0.25)	21.12 (0.27) 21.94 (0.24)	26.51 (0.67) 27.25 (0.96)	20.93 (0.2) 21.11 (0.08)	20.82 (0.12) 22.17 (0.19)		27.99 (1.13) 27.79 (0.02)
putative diacylglycerol kinase	qPtDGK_L1/R1									20.22 (0.2) 22.68 (0.32)		19.87 (0.22) 21.1 (0.41)
Ran GTPase binding / chromatin binding /	qPtGTPasebinding_L1/R1									22.24 (0.36) 23.74 (0.45)		21.75 (0.27) 22.66 (0.25)

zinc ion binding												
REF	qPtREF_L1/R1									poor amplification		
ribosomal protein L15e	qPt_ribprotL15e_L1/R1		23.54 (0.33) 26.73 (0.45)	23.92 (0.31) 25.16 (0.12)	24.48 (0.64) 24.91 (0.57)	24.29 (0.37) 24.15 (0.46)	20.87 (0.07) 21.93 (0.36)		22.74 (0.24) 21.78 (0.13)	21.86 (0.15) 22.34 (0.27)	23.08 (1.02) 24.25 (0.56)	22/35 (0.22) 22.6 (0.25)
RNase H domain-containing protein	qPtRNasedomcont_L1/R1									23.38 (0.15) 24.18 (0.35)		24.1 (0.34) 24.18 (0.29)
SAND	QPSAND_F/R		poor amplification									
SAND	qPtdSAND_L2/R2		poor amplification									
TIF5A	QPTIF5A_F/R		19.11 (0.15) 21.22 (0.19)	20 (0.22) 20.2 (0.38)	21.02 (0.63) 21.24 (0.88)	20.21 (0.2) 19.65 (0.36)	17.36 (0.15) 18.05 (0.29)		17.57 (0.22) 17.45 (0.01)	17.58 (0.14) 18.62 (0.18)	17.32 (0.84) 20.04 (0.45)	17.92 (0.42) 18.14 (0.29)
TIF5A-2	QPTIF5A-2_F/R		19.03 (0.07) 20.2 (0.01)	18.96 (0.07) 19.24 (0.44)	21.39 (0.81) 22.08 (1.13)	20.33 (0.3) 20.3 (0.35)	18.17 (0.18) 18.57 (0.24)			17.55 (0.15) 18.71 (0.29)		
TIF5A-3	qPTIF5A_L3/R3	16.64 (0.52) 17.07 (0.4)	18.24 (0.27) 20.48 (0.22)	18.07 (0.3) 18.38 (0.12)	19.8 (0.6) 19.55 (0.4)	18.38 (0.46) 17.72 (0.28)		27.01 (0.18) 28.76 (0.59)	18.04 (0.16) 17.84 (0.07)			28.03 (1.26) 28.04 (0.35)
TIF5A-4	qPTIF5A_L4/R4		22.35 (0.41) 24.51 (0.44)	20.95 (0.22) 22.15 (0.17)	22.59 (0.71) 22.62 (0.58)	20.94 (0.63) 20.93 (0.47)		27.65 (0.93) 32.05 (1.5)	21.29 (0.32) 24.26 (0.55)			29.35 (0.9) 29.65 (0.1)
TIF6	qPtTIF6_L1/R1								28.36 (0.45) 30.1 (1.32)			
TIP41	qPTIP41_L1/R1		23.75 (0.25) 26.69 (0.25)	23.89 (0.24) 25.07 (0.22)	25.58 (0.56) 27 (0.94)	24.42 (0.39) 24.5 (0.48)	21.36 (0.24) 21.85 (0.25)		21.8 (0.2) 21.55 (0.04)	21.2 (0.21) 22.12 (0.29)	21.9 (0.75) 23.94 (0.46)	21.09 (0.36) 21.4 (0.33)
TUB	PcTUB_F/R		22.48 (0.68) 27.02 (0.99)	21.72 (0.47) 23.83 (0.55)	21.57 (0.75) 21.84 (0.19)	21.21 (0.68) 21.47 (0.55)			20.64 (0.08) 23.19 (0.36)			
UBA	qPtUBA_L1/R1									23.16 (0.57) 23.93 (0.79)	23.79 (0.6) 25.71 (0.49)	23.42 (0.19) 23.74 (0.32)
UBA	qPtUBA_L2/R2										22.85 (0.77) 24.8 (0.4)	

UBC	qPUBC_L3/R3	20.11 (0.23) 21.18 (0.32)	21.33 (0.12) 23.36 (0.18)	21.58 (0.29) 22.67 (0.24)	22.4 (0.46) 23.4 (0.57)	21.61 (0.43) 21.94 (0.29)		29.25 (0.27) 29.74 (0.69)	21.86 (0.3) 23.3 (0.43)			30.16 (1.23) 29.32 (0.71)
UBC	qPUBC_L2/R2		23.47 (0.31) 25.93 (0.19)	23.79 (0.29) 24.74 (0.17)	24.92 (0.58) 26.08 (0.78)	23.88 (0.28) 24 (0.47)			22.69 (0.24) 24.52 (0.6)			
UBC 9	qPUBC_L4/R4	22.84 (0.06) 23.2 (0.29)	24.62 (0.25) 26.06 (0.21)	24.4 (0.33) 24.62 (0.17)	25.76 (0.52) 26.31 (0.46)	24.26 (0.48) 24.36 (0.46)	23.87 (0.11) 24.06 (0.06)	32.16 (0.92) 32.41 (0.77)	24.63 (0.7) 26.22 (0.42)	23.3 (0.17) 24.36 (0.17)	24.2 (0.69) 25.93 (0.59)	32.51 (1.99) 32.32 (0.29)
UBC 9	qPUBC_L1/R1		21.8 (0.26) 24.01 (0.26)	22.8 (0.18) 23.23 (0.25)	32.35 (0.94) 33.84 (0.76)	22.83 (0.11) 22.6 (0.28)			20.68 (0.52) 23.15 (0.69)		30.25 (0.96) 26.32 (0.17)	
UBC5	qPUBC_L5/R5	20.6 (0.24) 21.58 (0.25)	23.14 (0.18) 24.51 (0.24)	22.75 (0.23) 23.63 (0.19)	23.74 (0.3) 24.48 (0.34)	22.62 (0.3) 22.74 (0.42)	22.19 (0.26) 22.19 (0.17)	28.62 (0.42) 29.85 (0.48)	22.85 (0.43) 23.9 (0.68)	21.6 (0.18) 22.79 (0.14)	21.54 (0.7) 23.53 (0.32)	29.43 (1.51) 28.89 (0.3)
UBC6	qPUBC_L6/R6		19.3 (0.24) 21.21 (0.25)	19.03 (0.3) 20.29 (0.06)	19.59 (0.42) 20.04 (0.37)	18.89 (0.38) 19.11 (0.43)		24.92 (0.97) 26.39 (0.48)	19.71 (0.15) 21.29 (0.51)			24.71 (1.22) 24.63 (0.51)
UBQ10-3	UBQ10-3	15.49 (0.61) 17.38 (0.22)	21.37 (0.31) 18.75 (0.13)	21.16 (0.34) 20.21 (0.2)	22.31 (1.46) 21.5 (0.53)	20.01 (0.12) 20.63 (0.33)		27.49 (0.9) 28.44 (0.46)	17.71 (0.22) 19.64 (0.27)			28.46 (0.48) 28.22 (0.13)
UBQ10-4	qPUBQ10_L4/R4		18.78 (0.27) 21.99 (0.15)	19.36 (0.29) 20.41 (0.24)	19.82 (0.58) 21.09 (0.96)	19.38 (0.3) 19.74 (0.36)			18.03 (0.15) 20.65 (0.37)			
UBQ10-5	qPUBQ10_L5/R5		17.11 (0.2) 20.16 (0.16)	17.14 (0.22) 18.61 (0.13)	17.16 (0.4) 18.42 (0.48)	16.64 (0.27) 17.17 (0.33)	16.62 (0.15) 17.35 (0.46)	25.94 (1.17) 26.7 (0.34)	17.04 (0.17) 18.41 (0.33)	15.68 (0.37) 17.31 (0.88)	14.91 (0.5) 18.54 (0.51)	26.19 (1.1) 26.31 (0.24)
UBQ10-6	qPUBQ10_L6/R6		17.8 (0.25) 22.03 (0.34)	18.43 (0.39) 19.94 (0.28)	19.15 (0.58) 20.04 (0.46)	18 (0.52) 18.66 (0.47)		26.4 (0.47) 27.63 (0.58)	17.46 (0.7) 18.62 (0.4)			26.99 (0.77) 27.06 (0.12)
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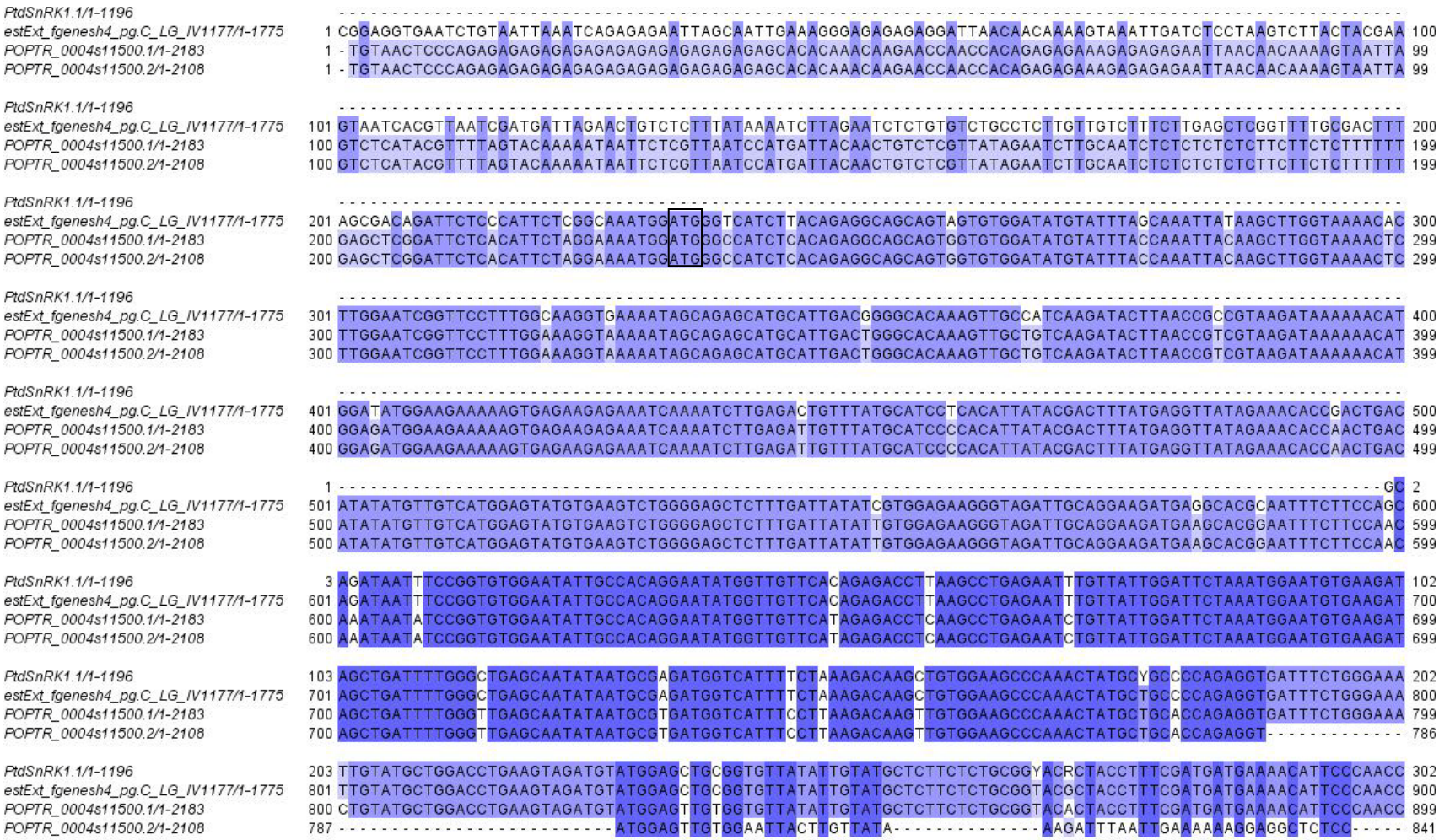
UBQ11	QPUBQ11_F/R	14.76 (0.35) 16.92 (0.31)	18.1 (0.26) 22.31 (0.24)	18.36 (0.06) 20.44 (0.34)	18.39 (0.49) 20.76 (1.33)	18.17 (0.24) 18.64 (0.28)		25.37 (0.65) 26.85 (0.99)	17.33 (0.2) 18.68 (0.41)			25.8 (0.96) 25.78 (0.39)
VEP1	qPt_VEP1_L1 /R1										20.52 (0.69) 15.22 (0.52)	
VHA-A;	qPtVHA-A_L1/R1						19.26 (0.21) 19.72 (0.19)		17.58 (0.44) 18.37 (0.24)	18.75 (0.2) 19.4 (0.33)	18.94 (0.51) 20.22 (0.41)	18.85 (0.19) 19.07 (0.34)

2.7 Figures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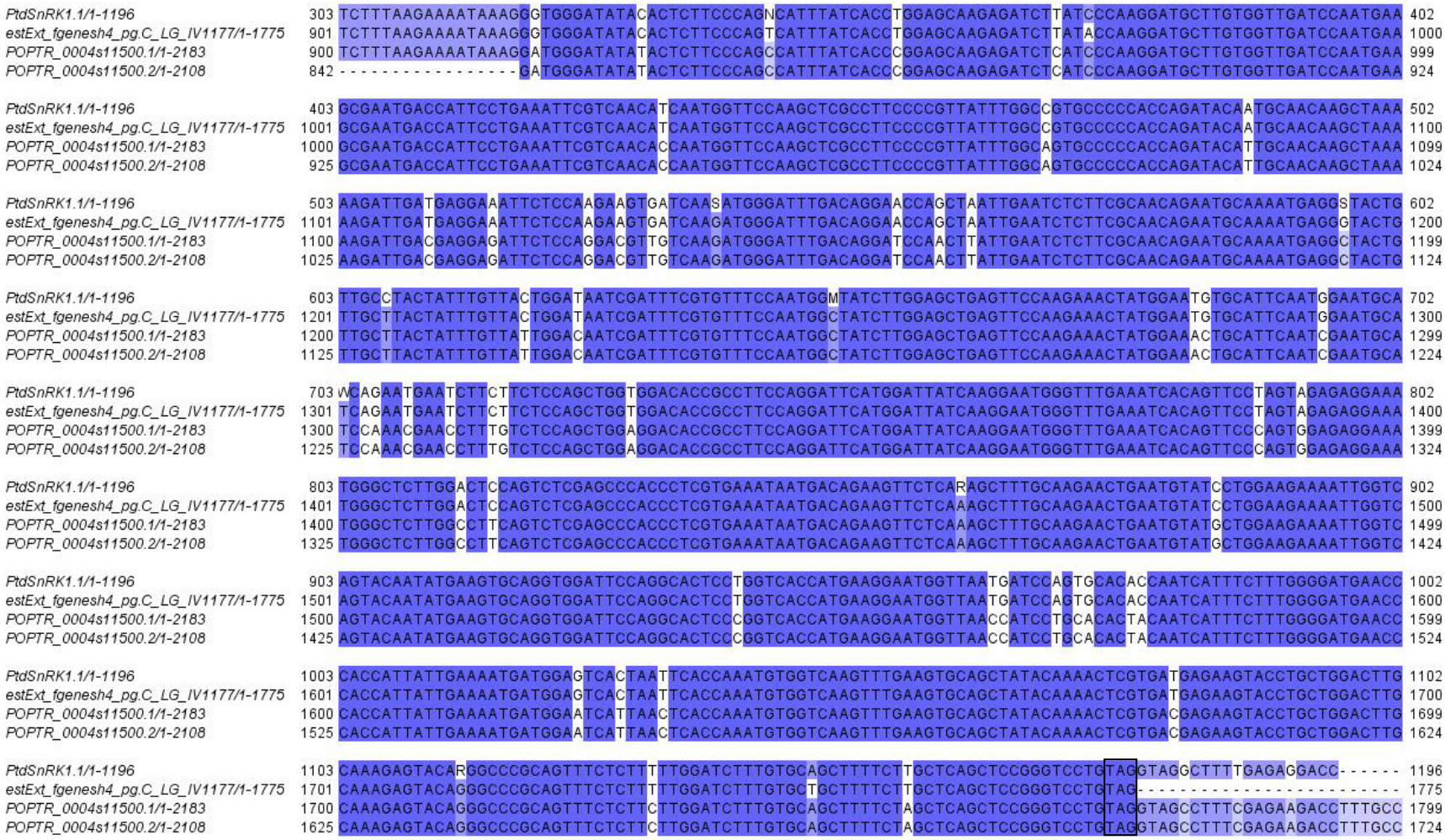
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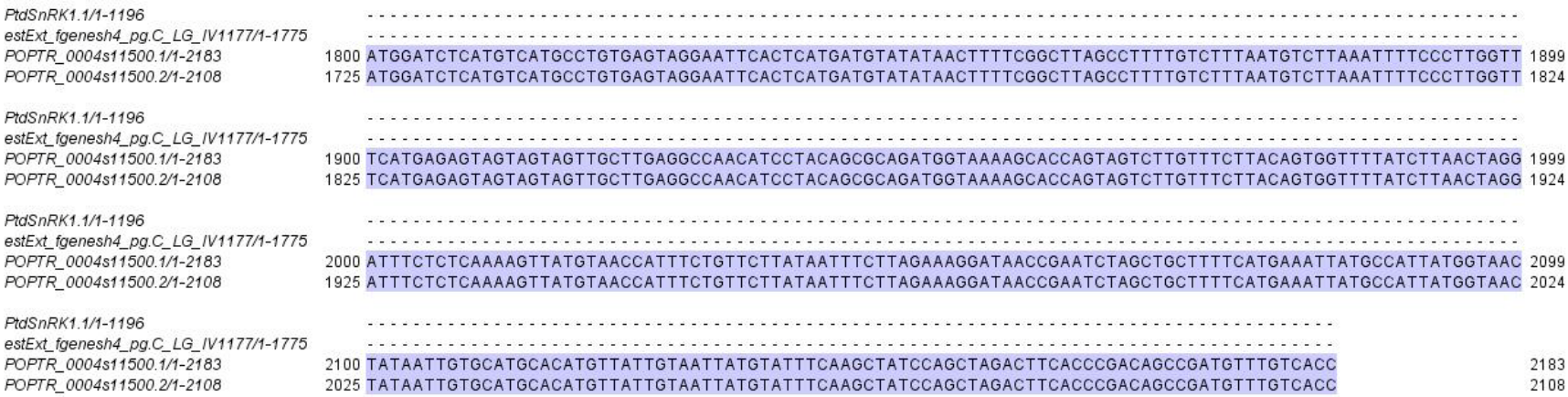
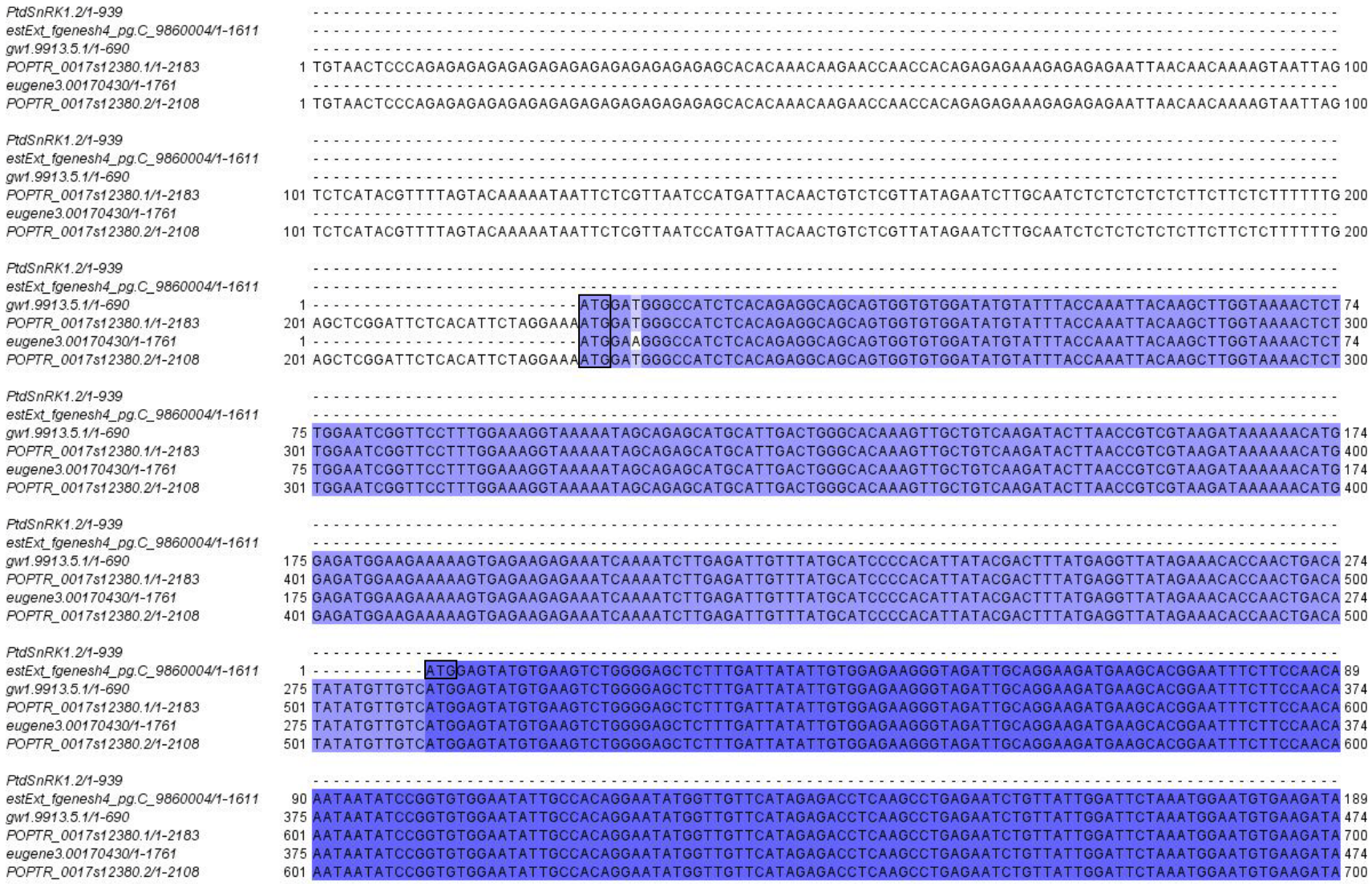


Figure 2.1. Alignment of the cloned sequence of *PtdSnRK1.1* with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al.*, 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop codons are boxed in black.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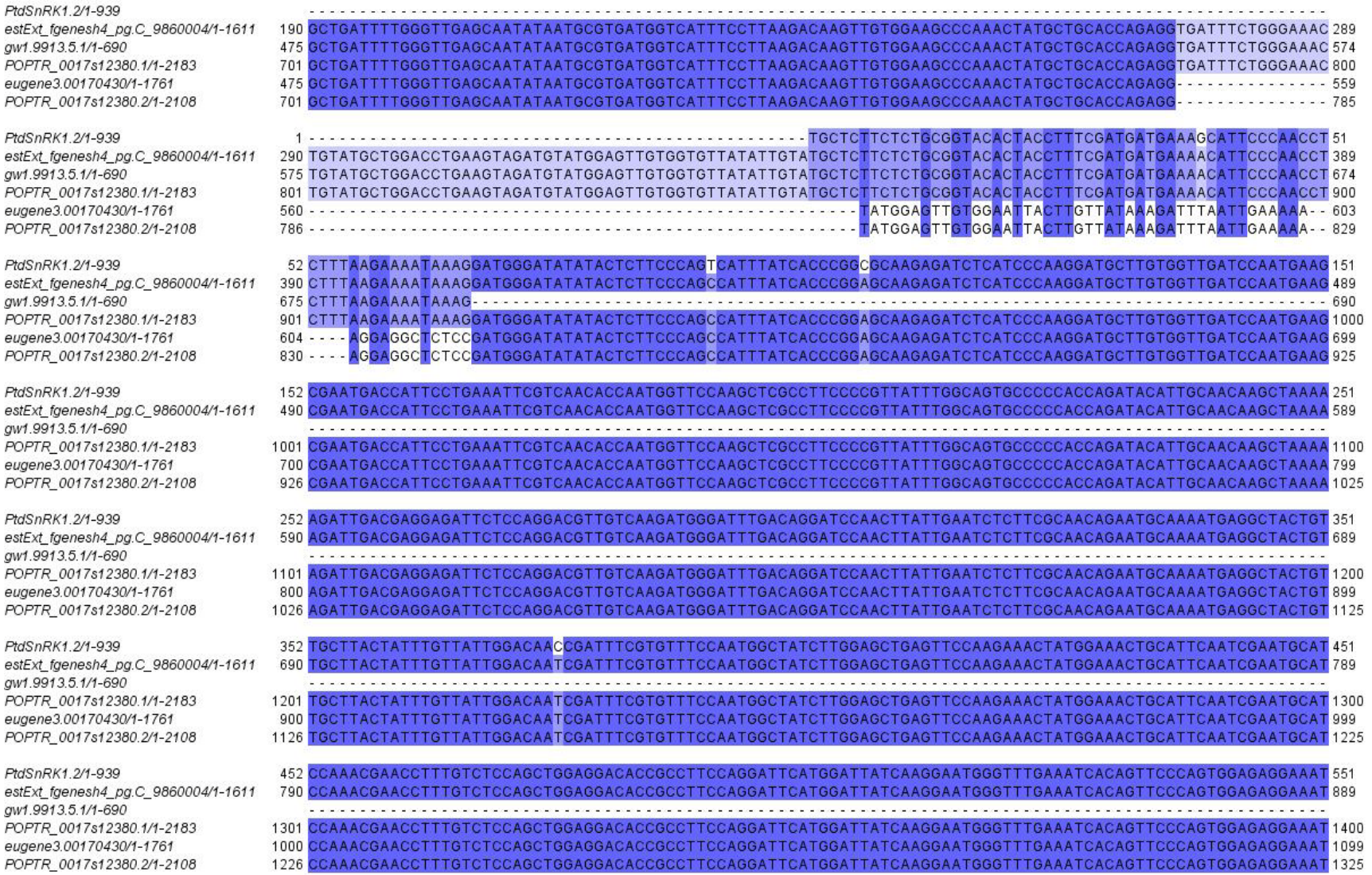
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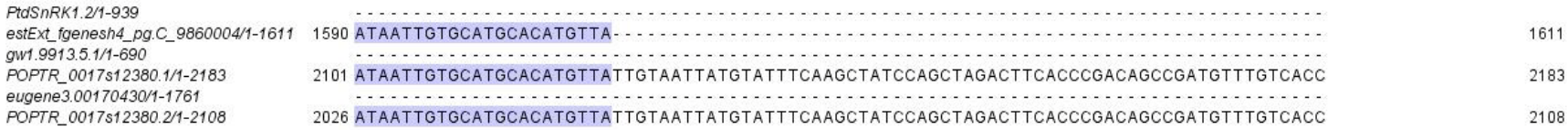

Figure 2.2. Alignment of the cloned sequence of *PtdSnRK1.2* with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al*., 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop codons are boxed in black.

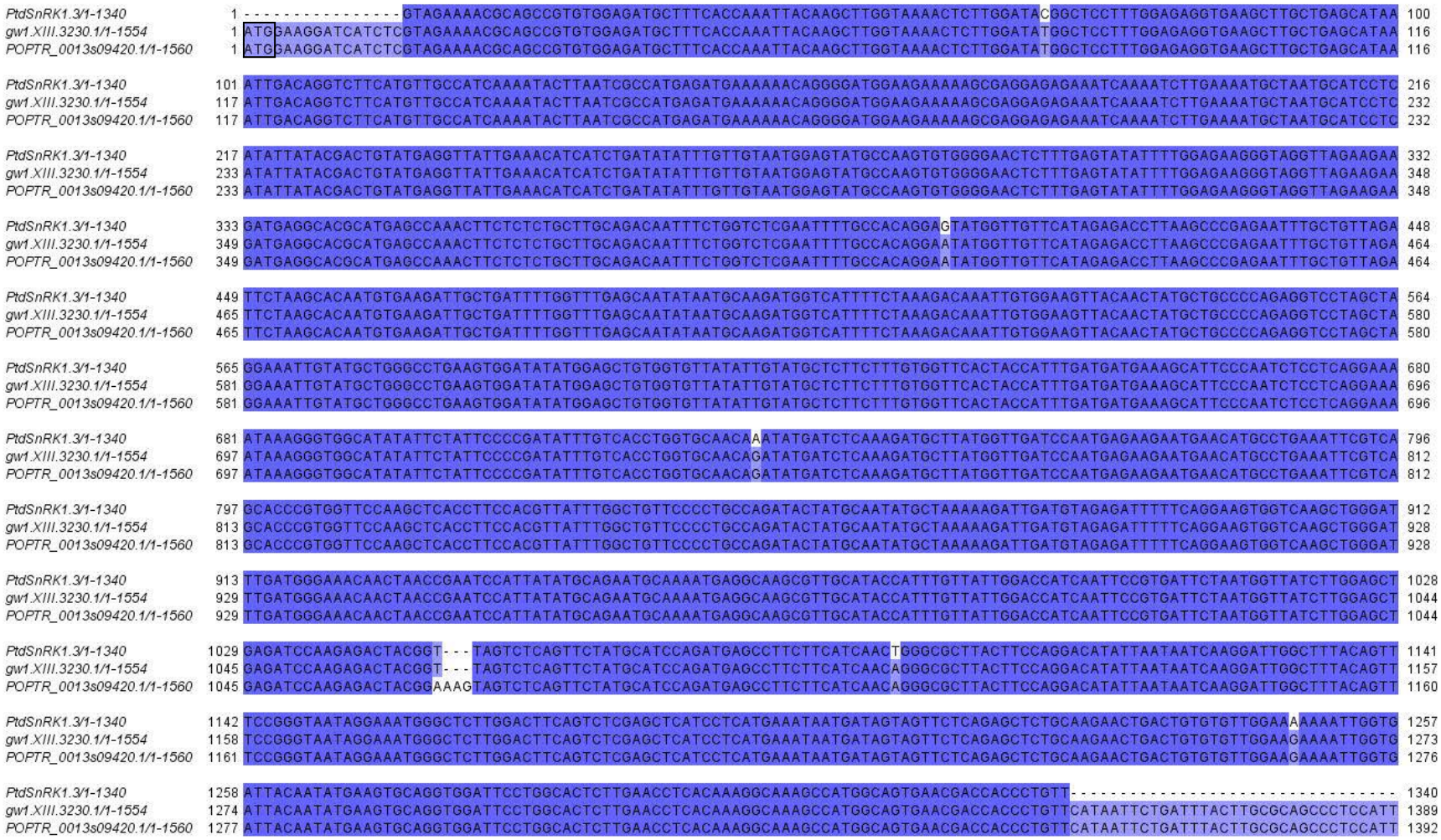
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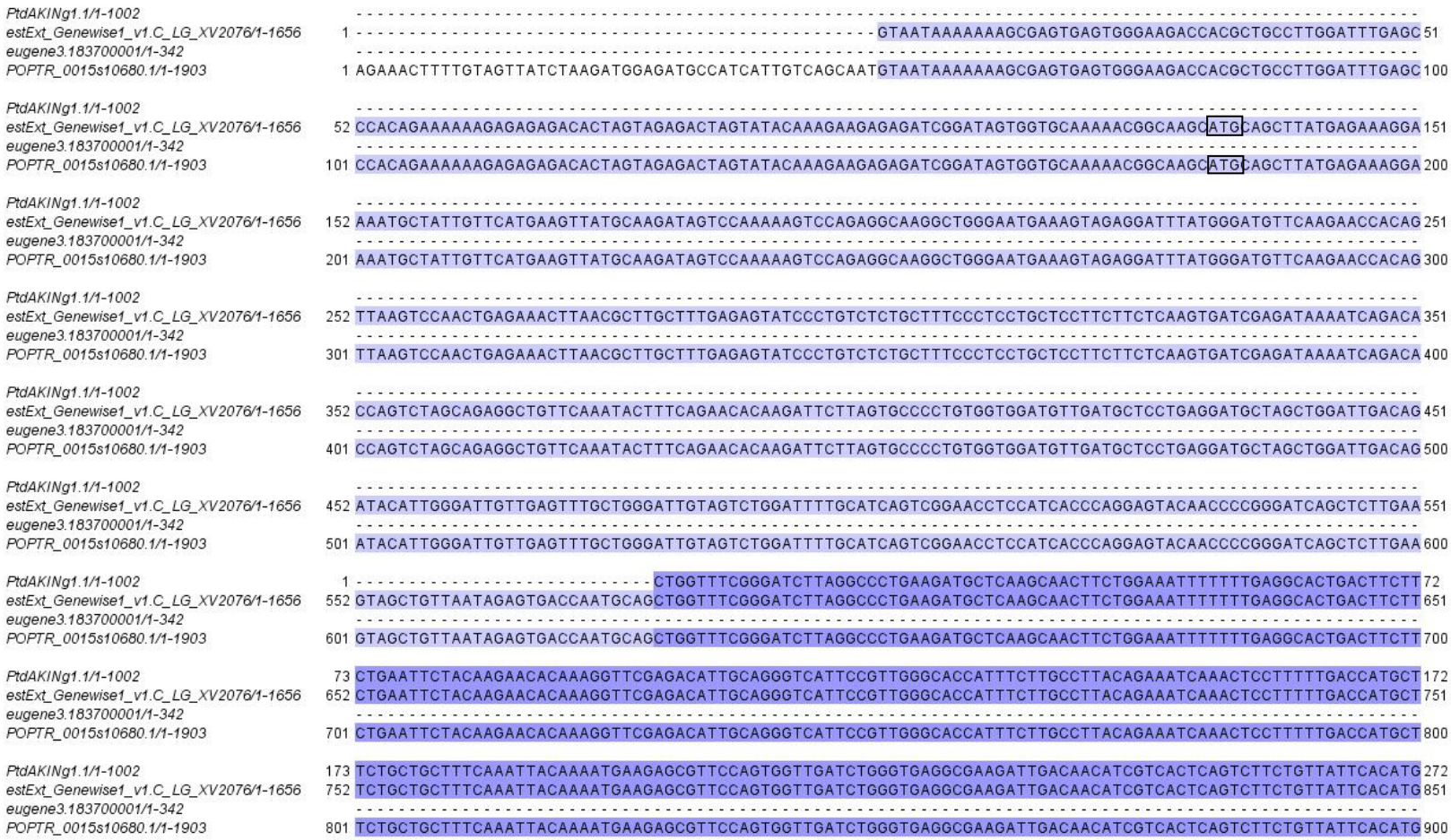
Figure 2.3. Alignment of the cloned sequence of *PtdSnRK1.3* with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al.*, 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop codons are boxed in black.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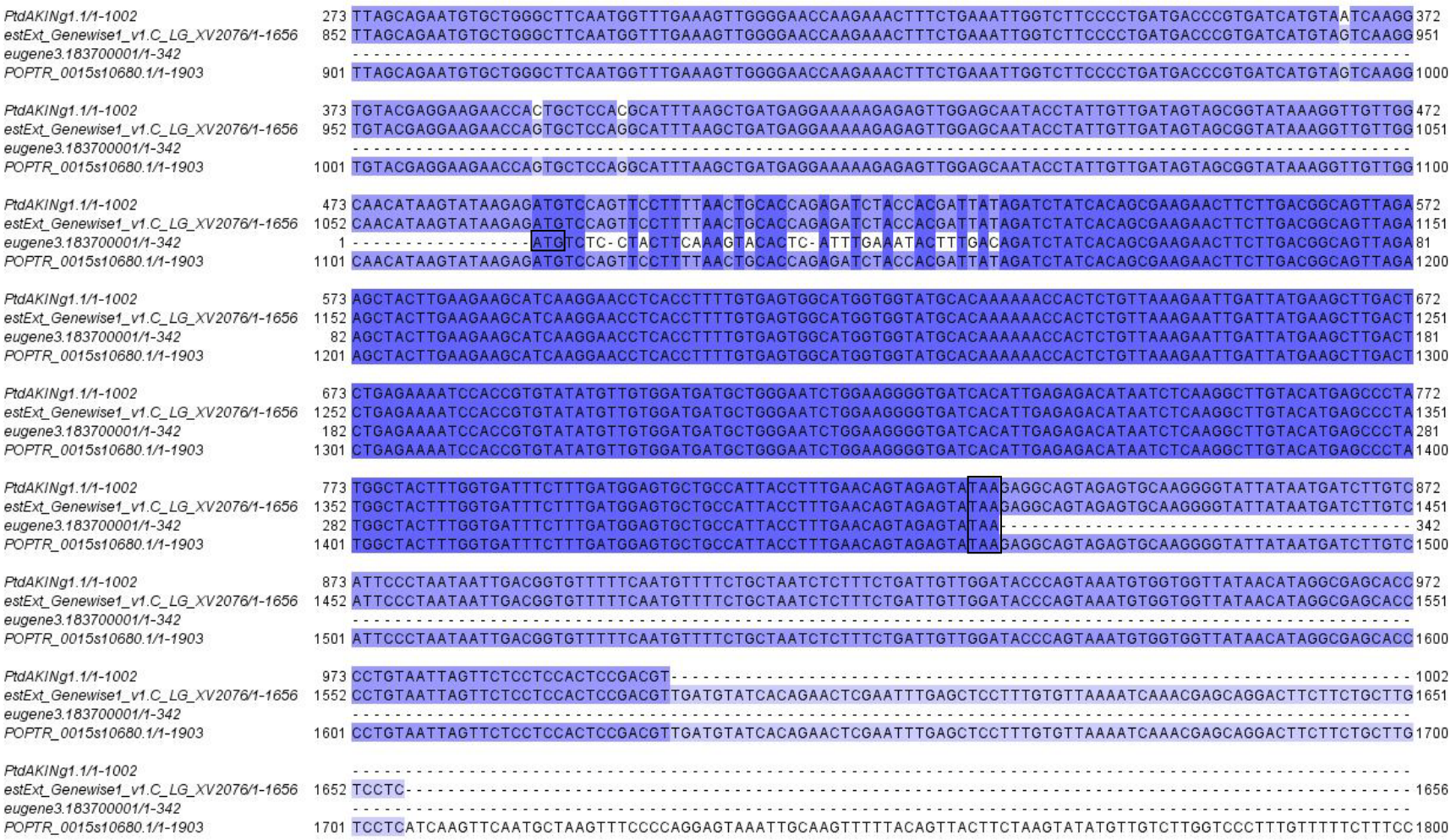
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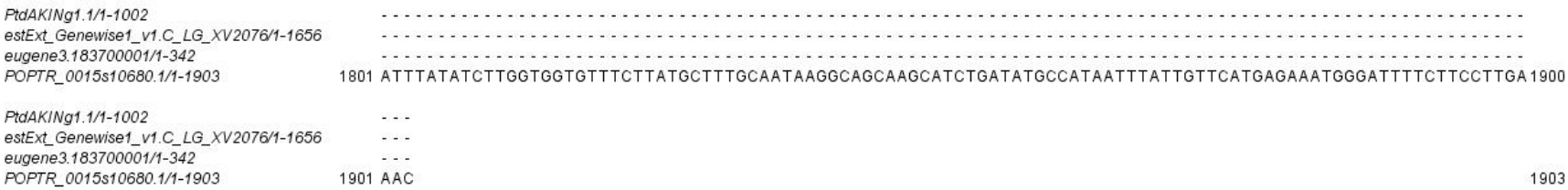

Figure 2.4. Alignment of the cloned sequence of *PtdAKINγ1.1* with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al.*, 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop codons are boxed in black.

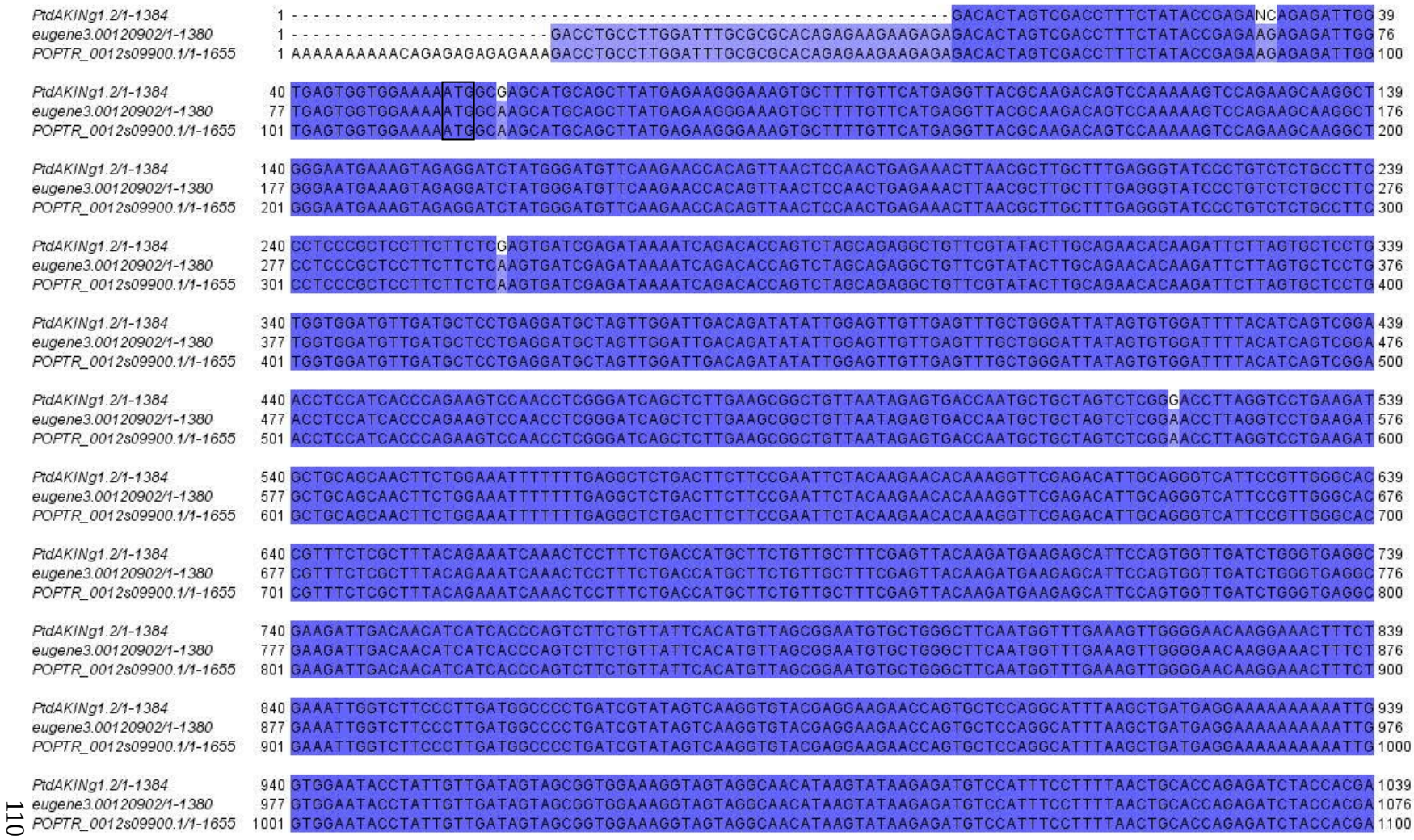
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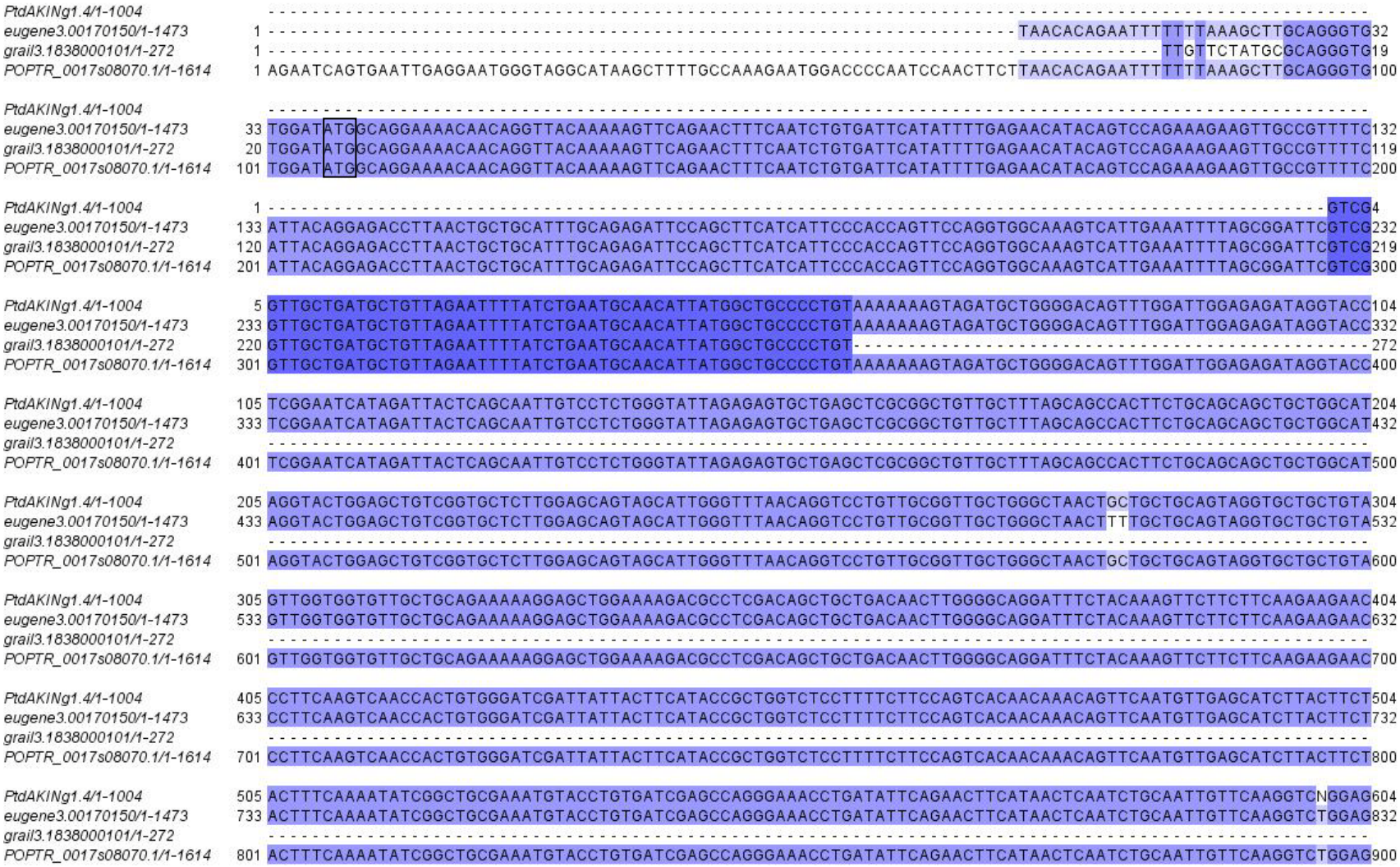
Figure 2.5. Alignment of the cloned sequence of *PtdAKINγ1.2* with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al.*, 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop codons are boxed in black.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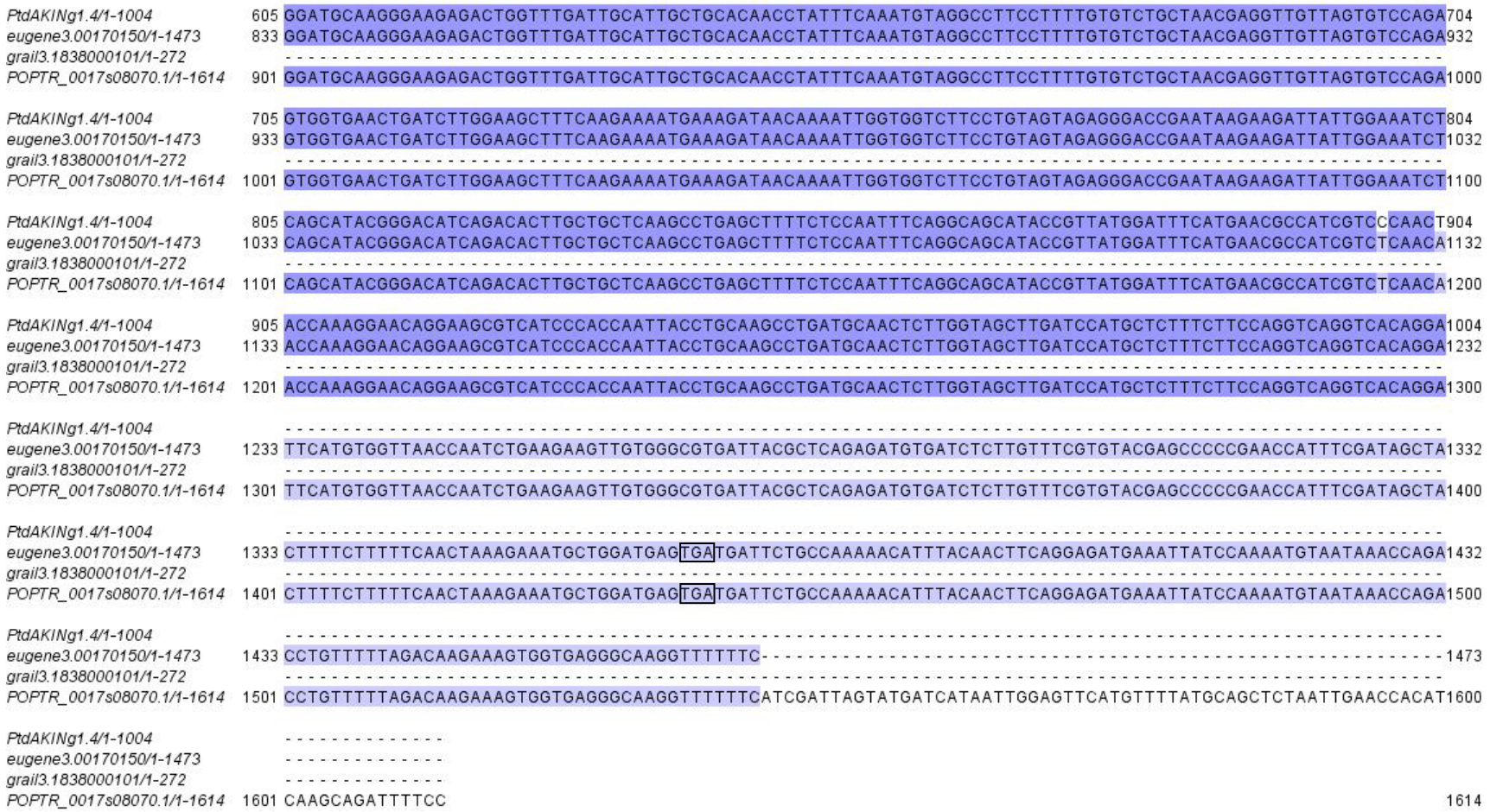
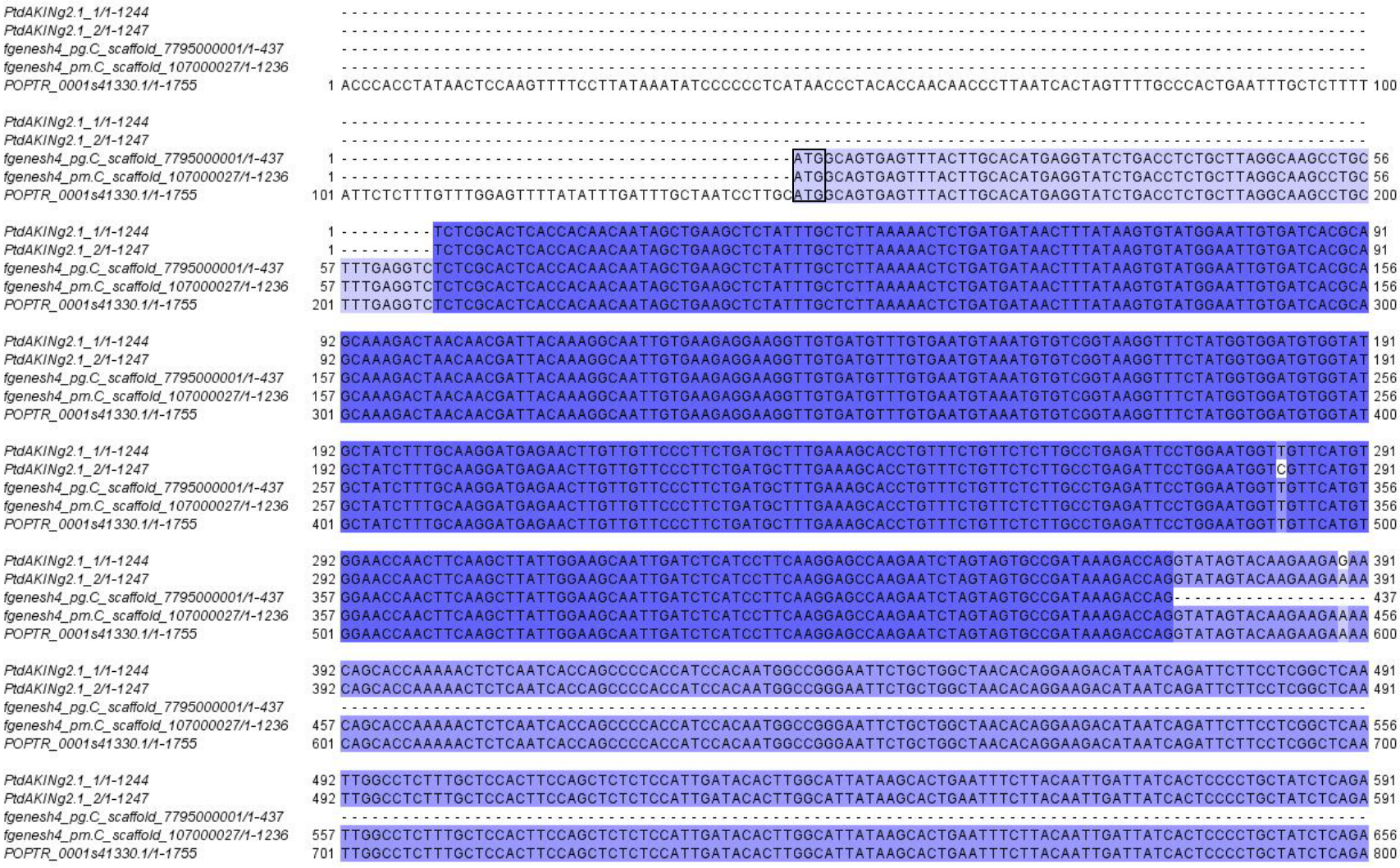


Figure 2.6. Alignment of the cloned sequence of *PtdAKINγ1.4* with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al.*, 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop codons are boxed in black.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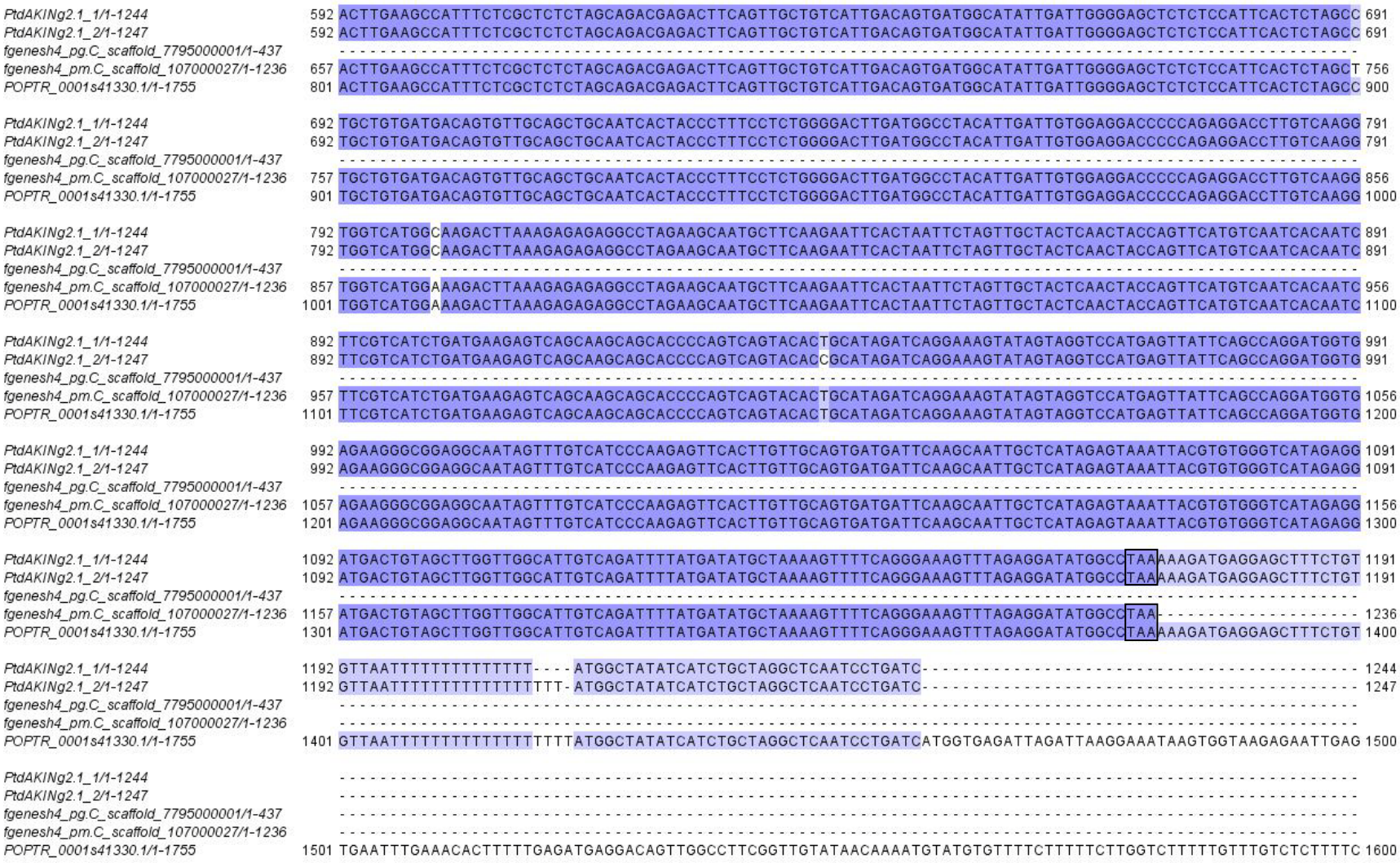
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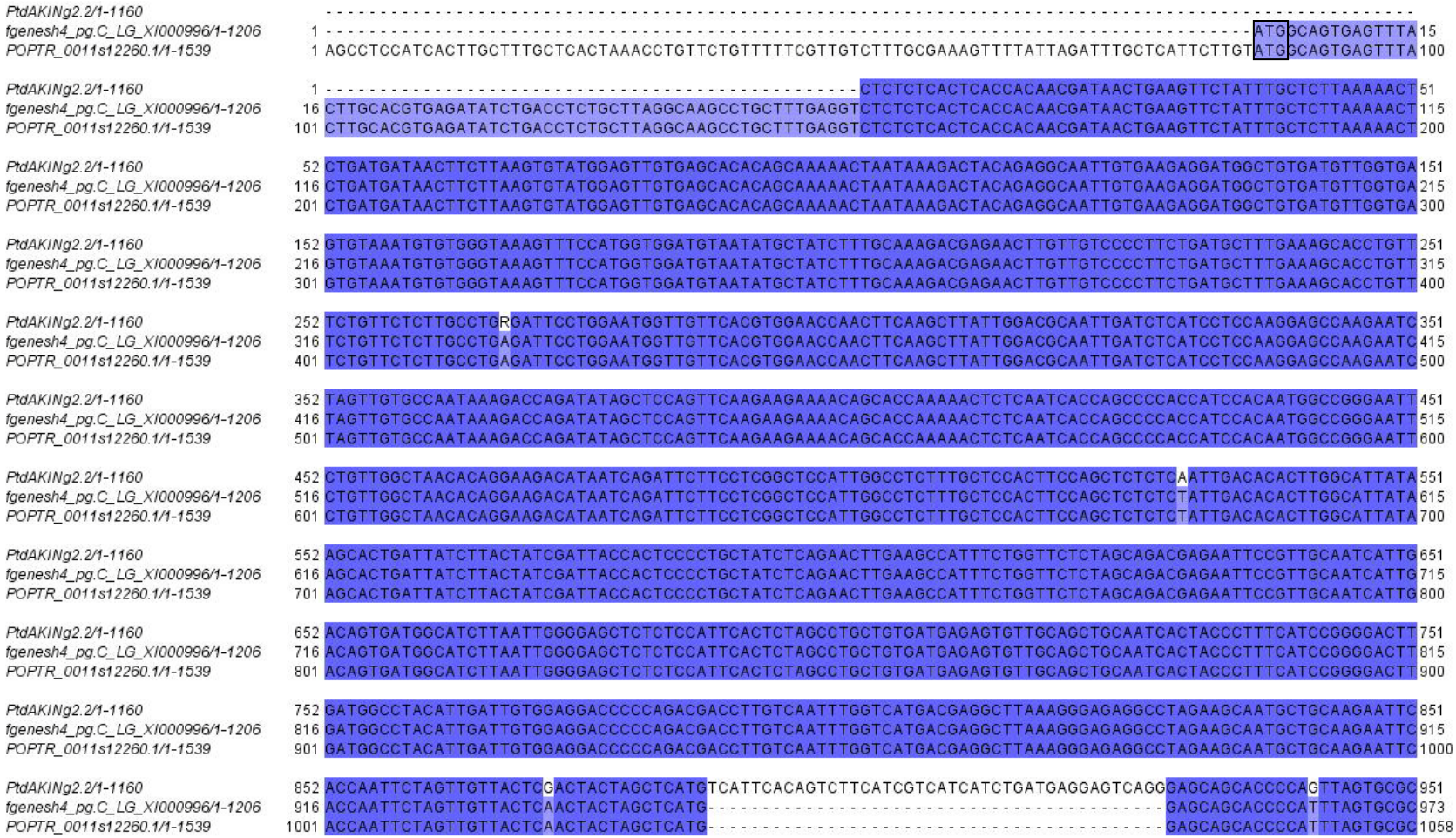
Figure 2.7. Alignment of the cloned sequence of *PtdAKINγ2.1* with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al*., 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop codons are boxed in black.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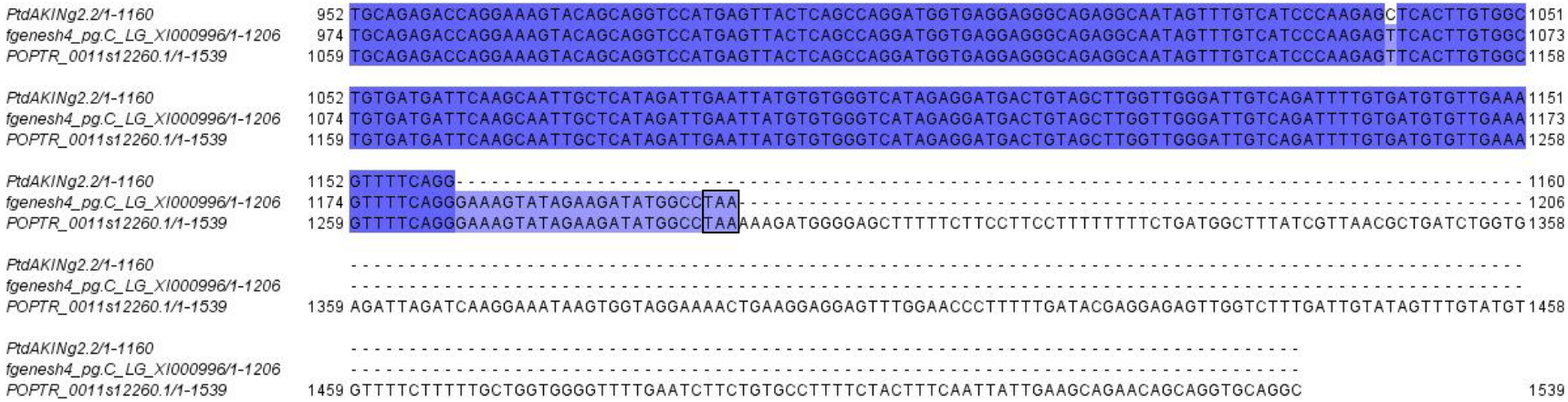
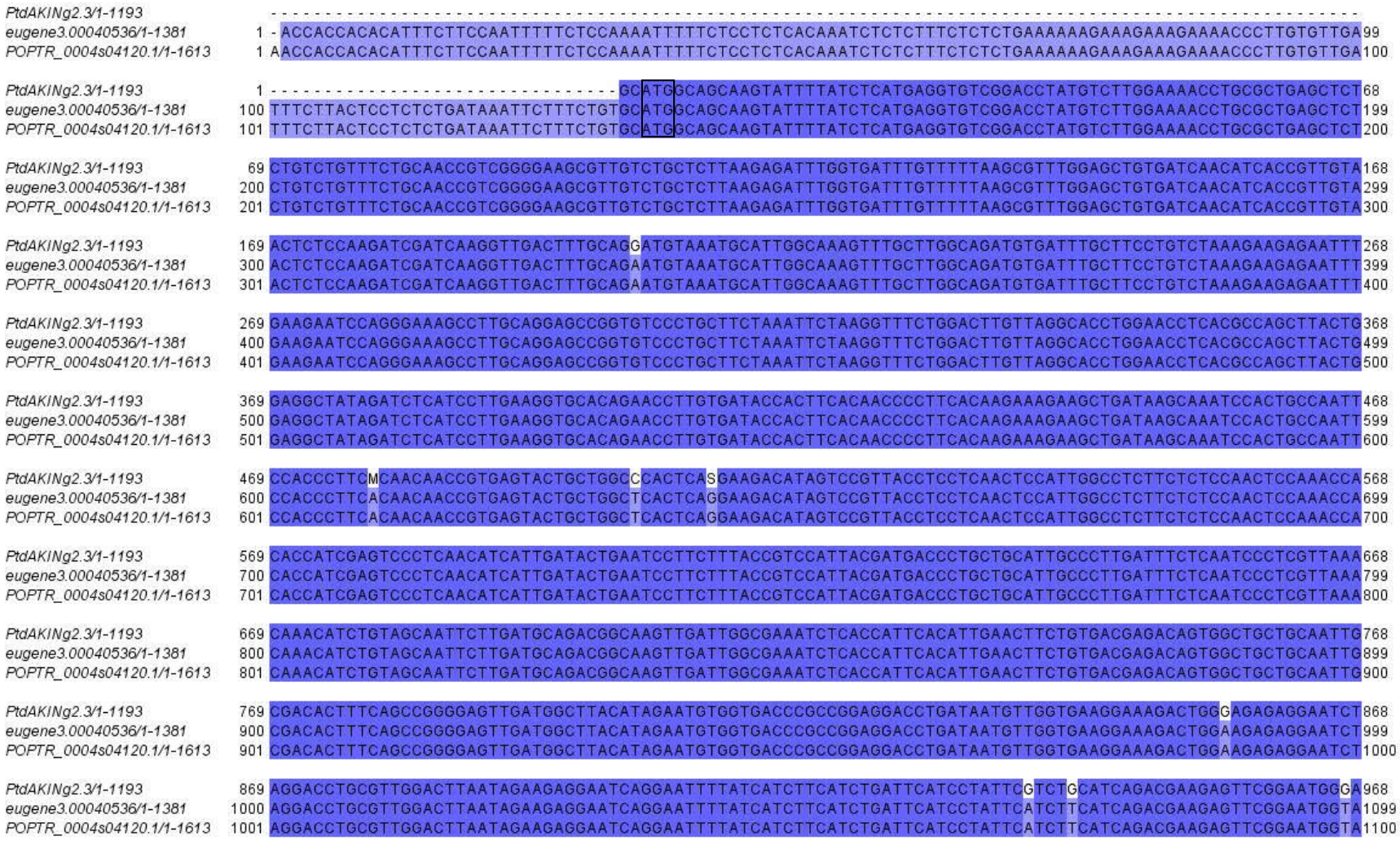


Figure 2.8. Alignment of the cloned sequence of PtdAKINγ2.2 with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al.*, 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop codons are boxed in black.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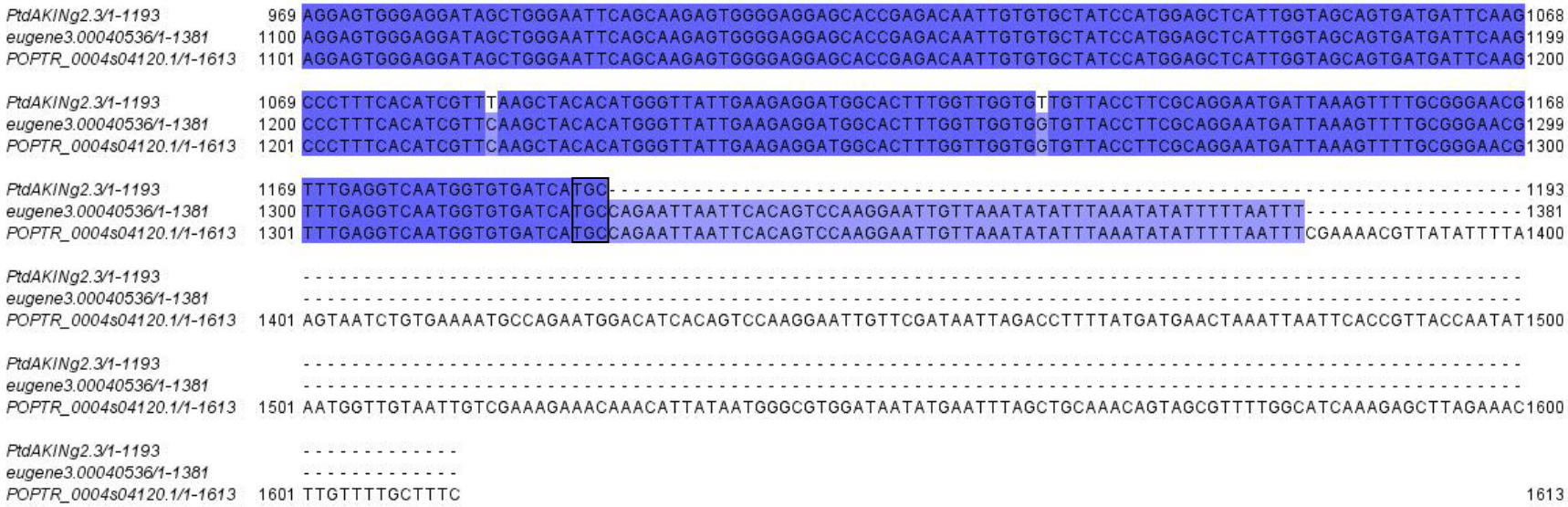

Figure 2.9. Alignment of the cloned sequence of *PtdAKINγ2.3* with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al.*, 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop codons are boxed in black.

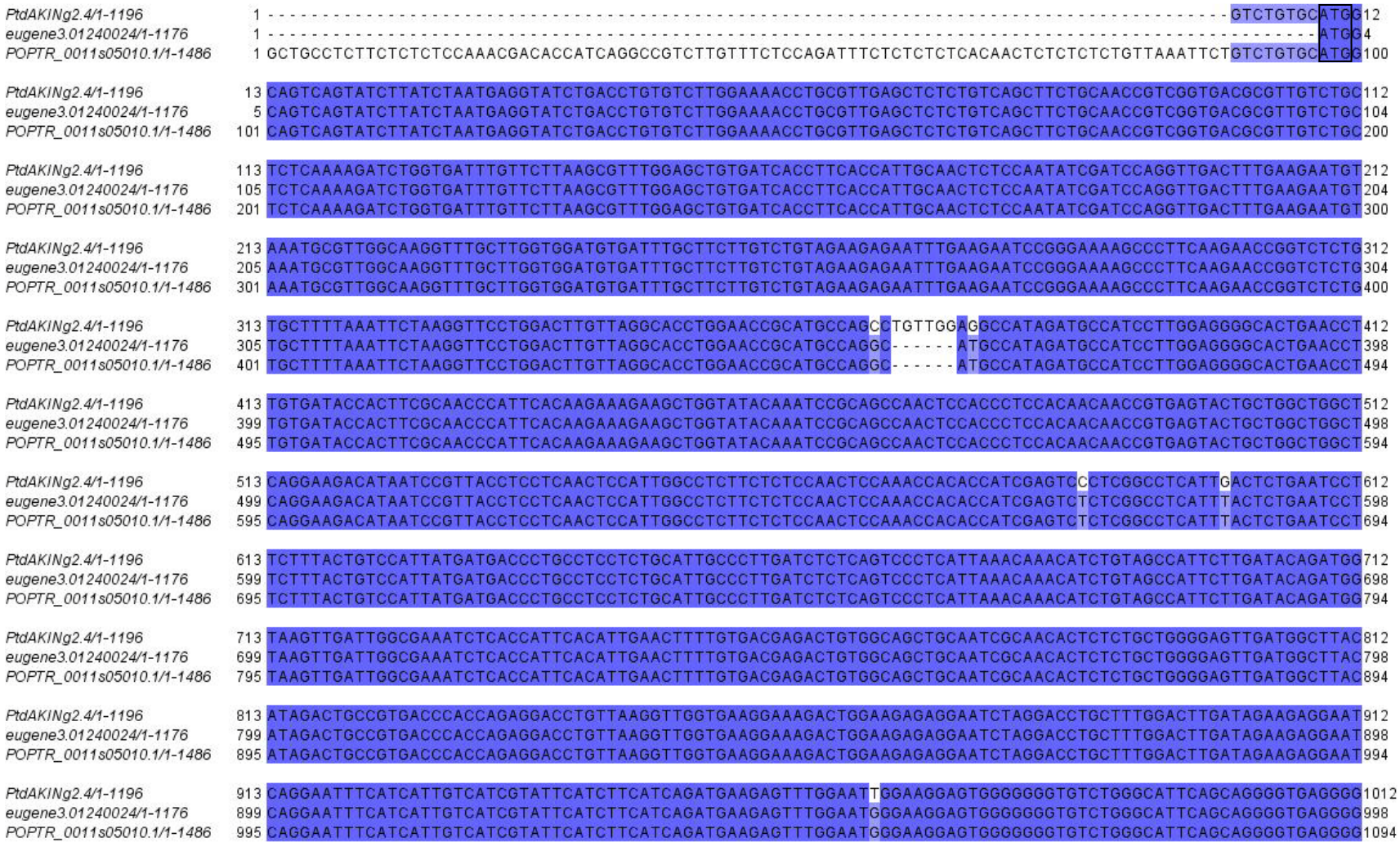
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Figure 2.10. Alignment of the cloned sequence of *PtdAKINγ2.4* with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al*., 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop codons are boxed in black.

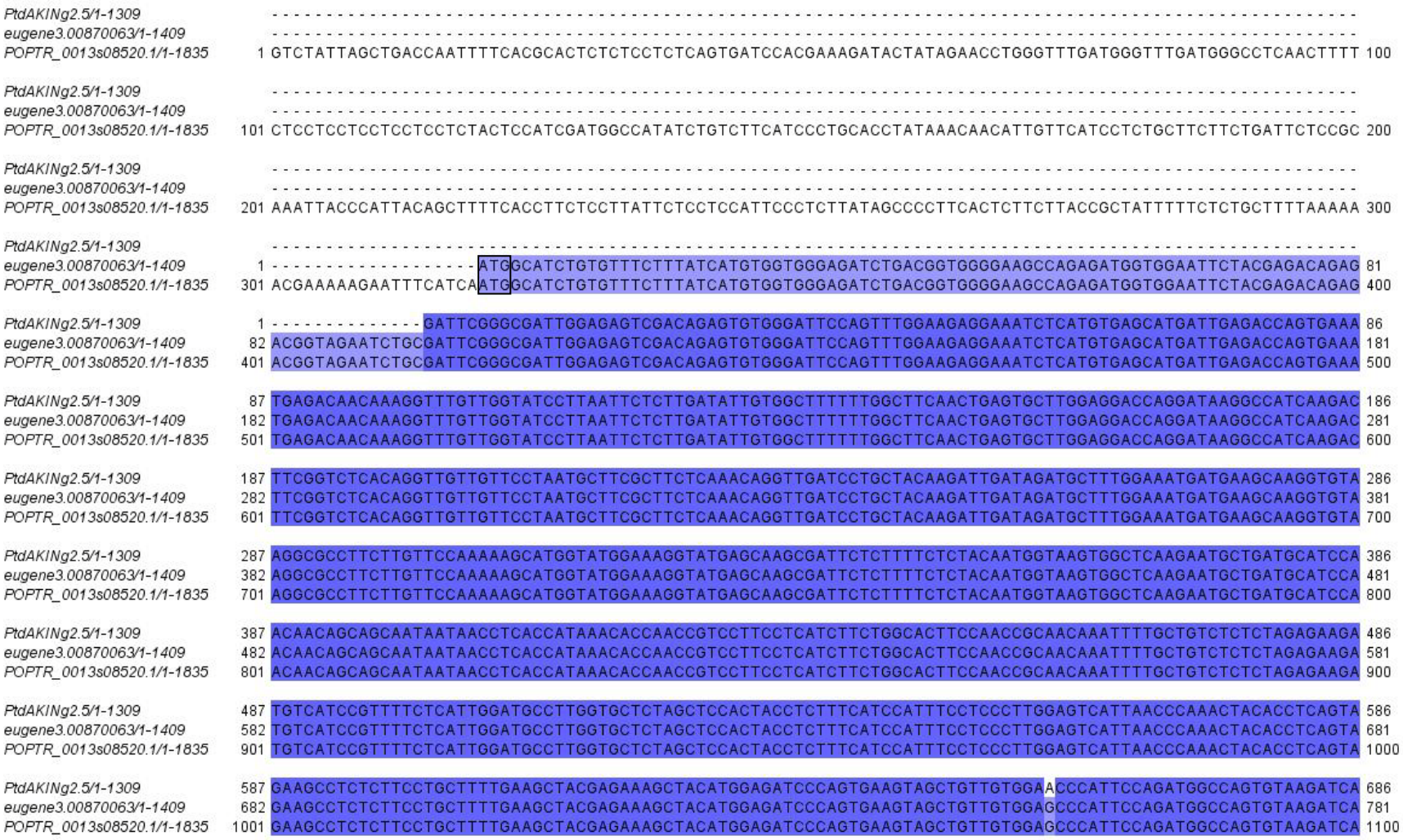
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Figure 2.11. Alignment of the cloned sequence of *PtdAKINγ2.5* with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al.*, 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop codons are boxed in black.

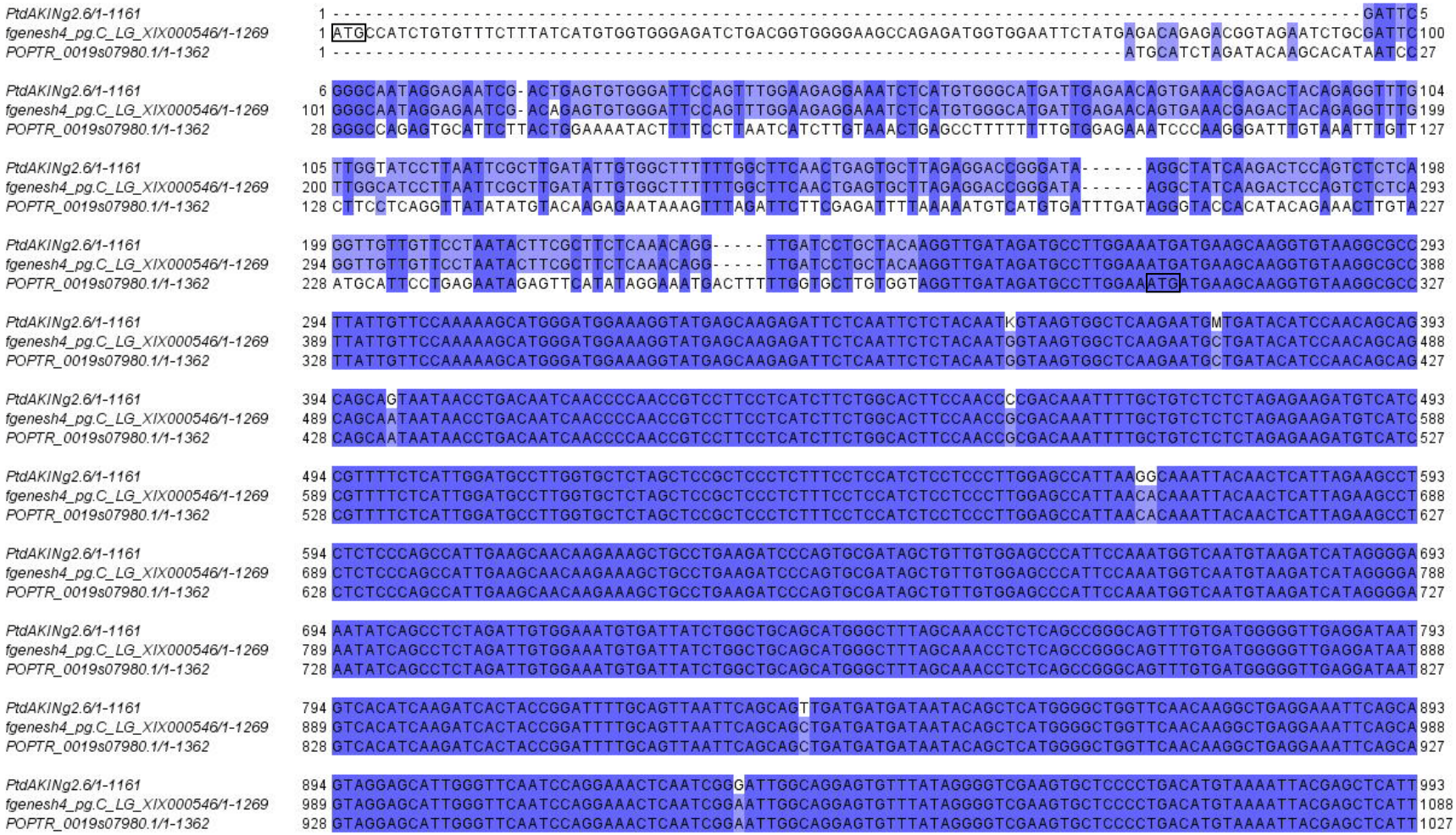
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Figure 2.12. Alignment of the cloned sequence of *PtdAKINγ2.6* with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al.*, 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop condons are boxed in black.

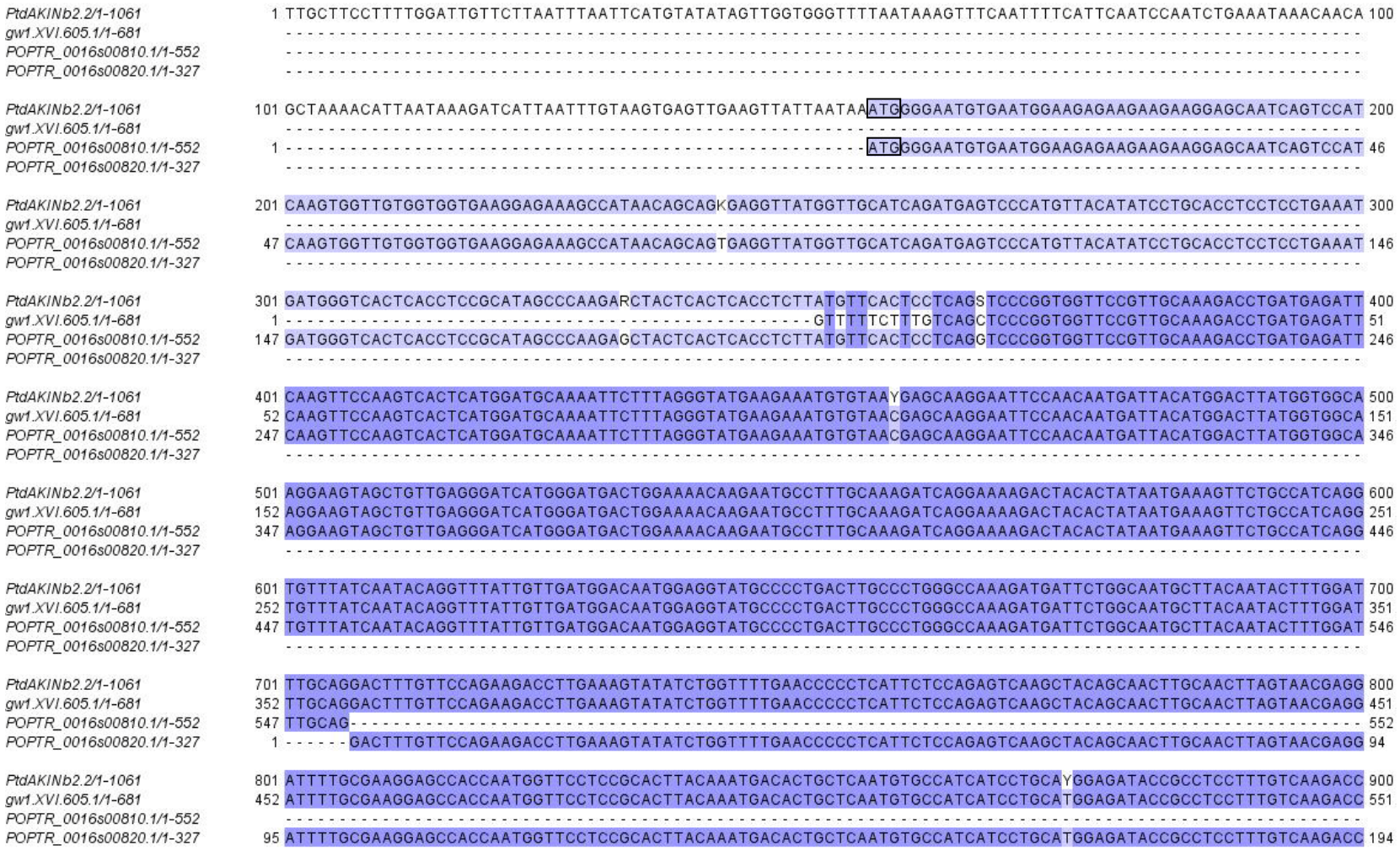
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Figure 2.13. Alignment of the cloned sequence of *PtdAKINβ2.2* with transcripts deduced from gene models from v1.1 and v2.0 of the *Populus* genome. The alignment was conducted in MEGA4 (Tamura *et al.*, 2007) and displayed using Jalview (Waterhouse *et al.*, 2009). Blue highlighting denotes consensus sequence, with lighter shades of blue indicating fewer sequences sharing consensus. Predicted start and stop codons are boxed in black.

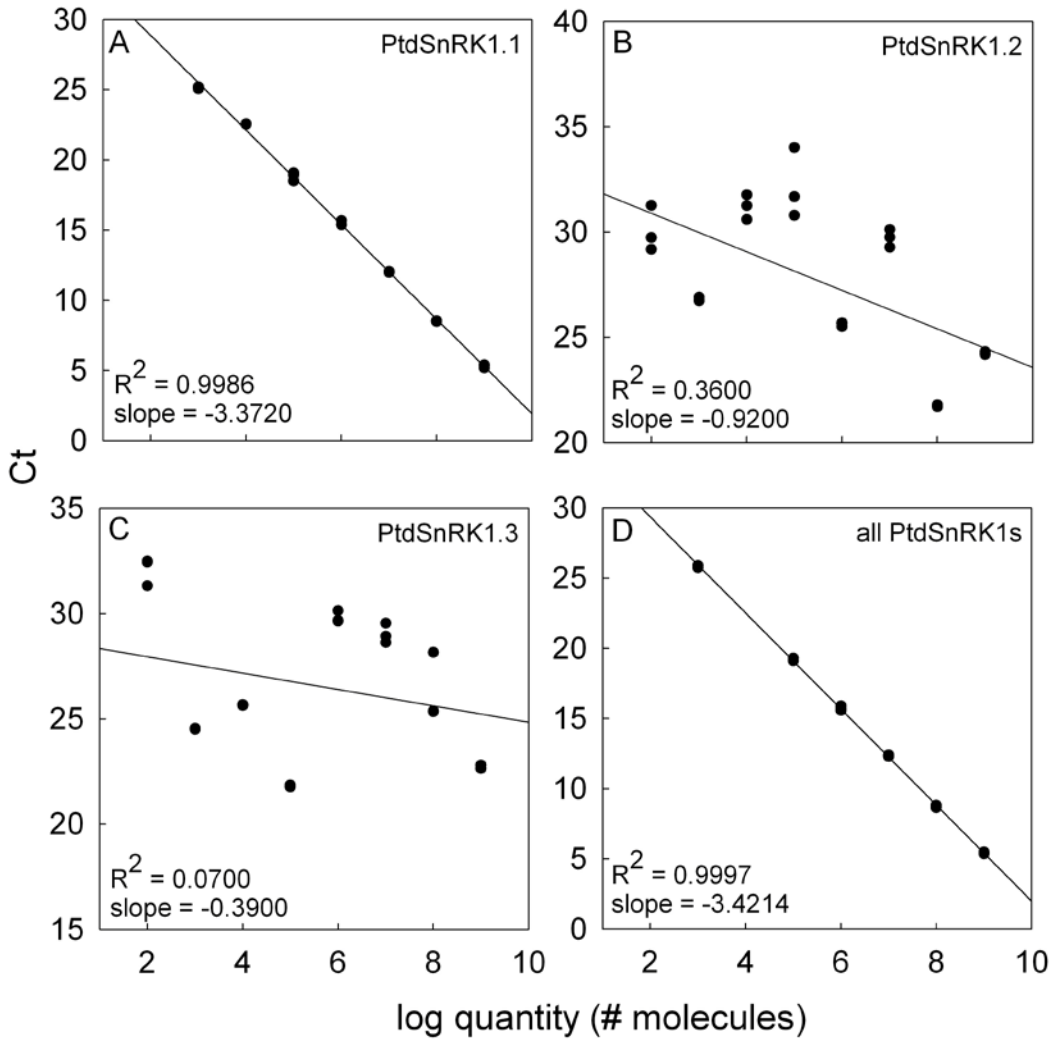

Figure 2.14. Validation of the specificity of *PtdSnRK1.1* qRT-PCR primers using dilutions series of members of the *PtdSnRK1* gene family to generate standard curves. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation. The template(s) in each standard curve are: (A) *PtdSnRK1.1* (B) *PtdSnRK1.2* (C) *PtdSnRK1.3* (D) a mix of all members of the *PtdSnRK1* gene family.

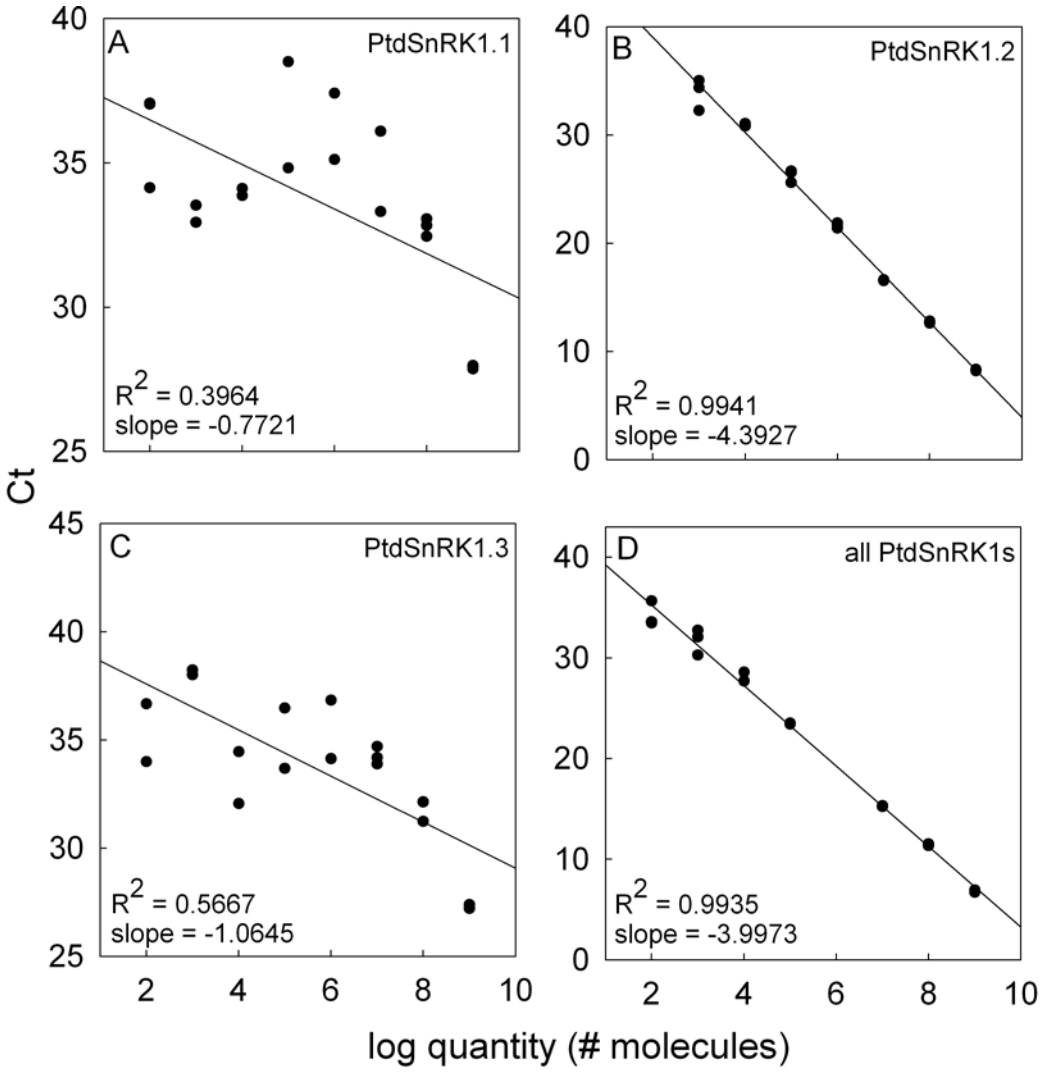

Figure 2.15. Validation of the specificity of *PtdSnRK1.2* qRT-PCR primers using dilutions series of members of the *PtdSnRK1* gene family to generate standard curves. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation. The template(s) in each standard curve are: (A) *PtdSnRK1.1* (B) *PtdSnRK1.2* (C) *PtdSnRK1.3* (D) a mix of all members of the *PtdSnRK1* gene family.

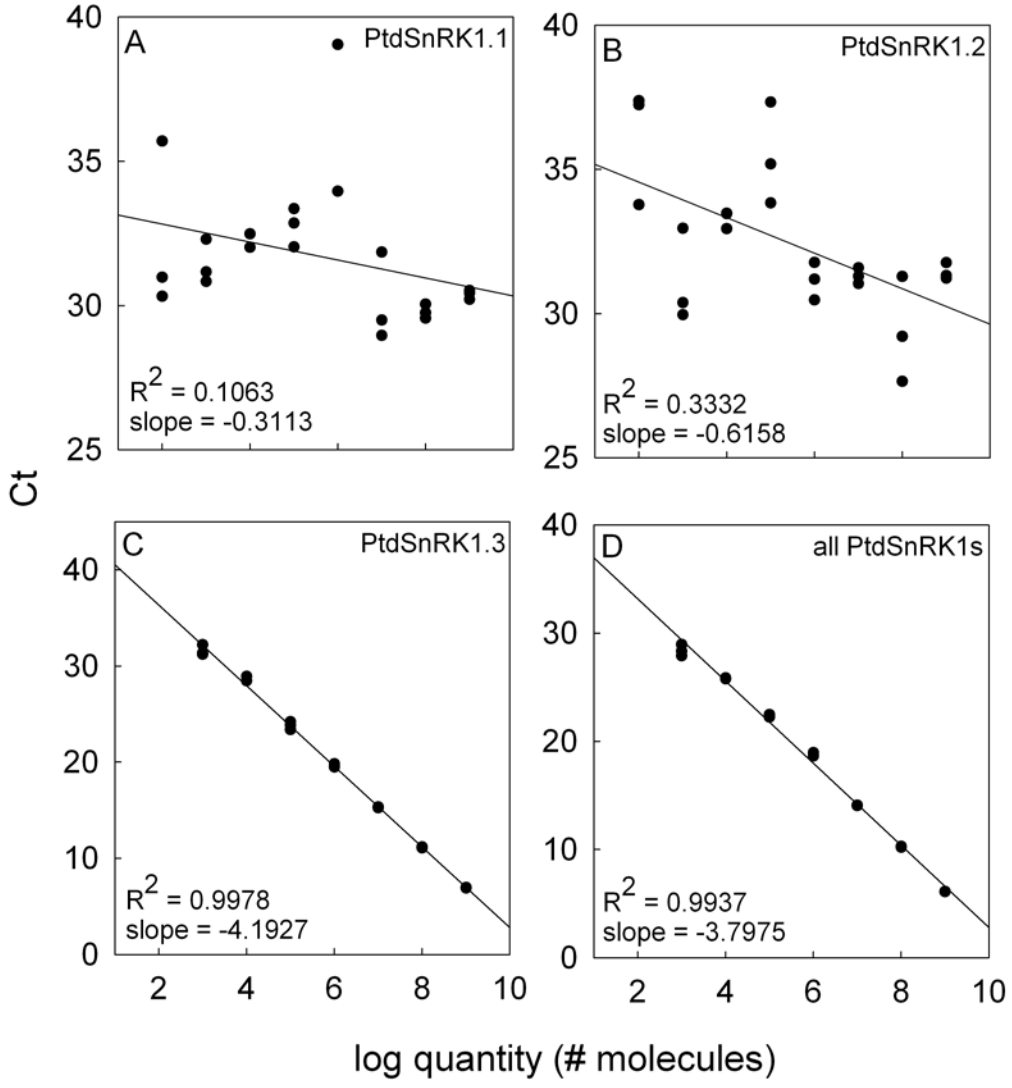

Figure 2.16. Validation of the specificity of *PtdSnRK1.3* qRT-PCR primers using dilutions series of members of the *PtdSnRK1* gene family to generate standard curves. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation. The template(s) in each standard curve are: (A) *PtdSnRK1.1* (B) *PtdSnRK1.2* (C) *PtdSnRK1.3* (D) a mix of all members of the *PtdSnRK1* gene family.

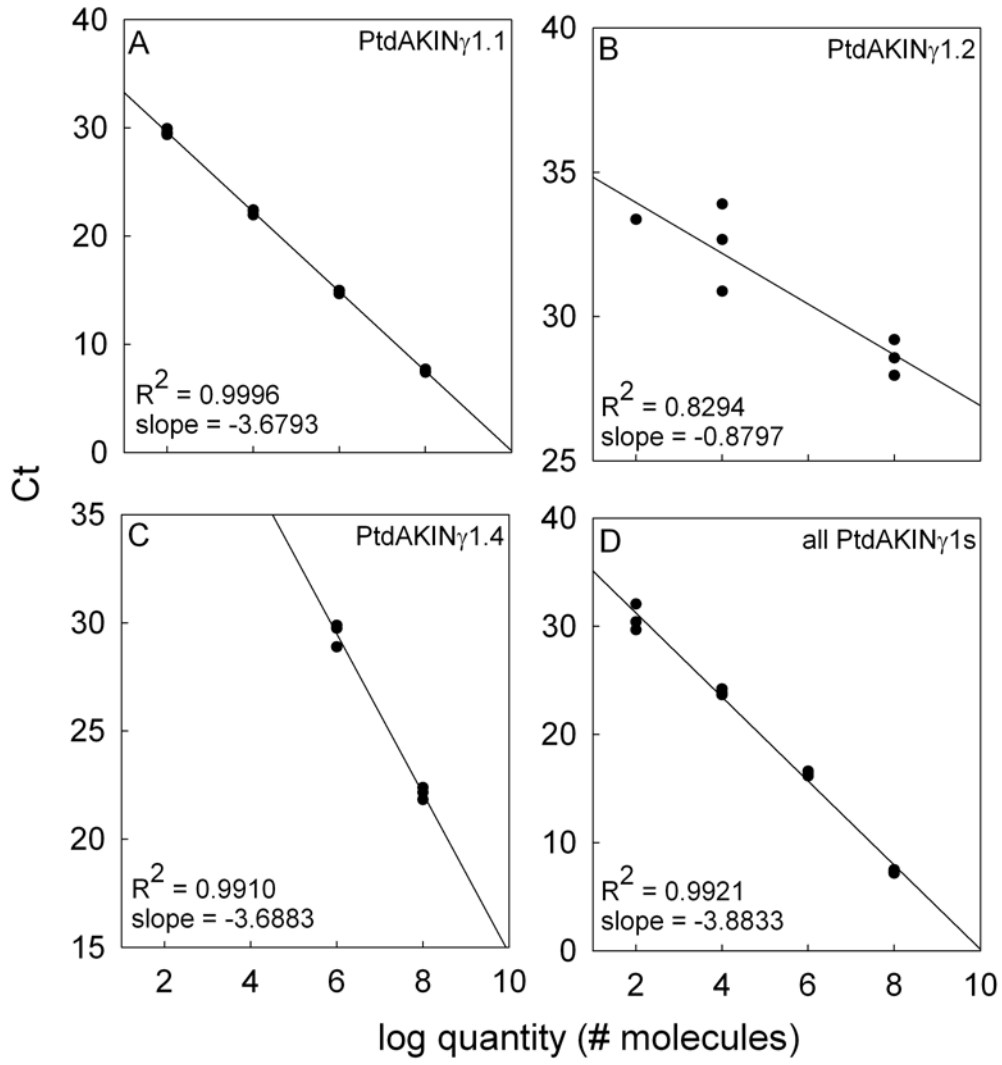

Figure 2.17. Validation of the specificity of *PtdAKINγ1.1* qRT-PCR primers using dilutions series of members of the *PtdAKINγ1* gene family to generate standard curves. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation. The template(s) in each standard curve are: (A) *PtdAKINγ1.1* (B) *PtdAKINγ1.2* (C) *PtdAKINγ1.4* (D) a mix of all members of the *PtdAKINγ1* gene family.

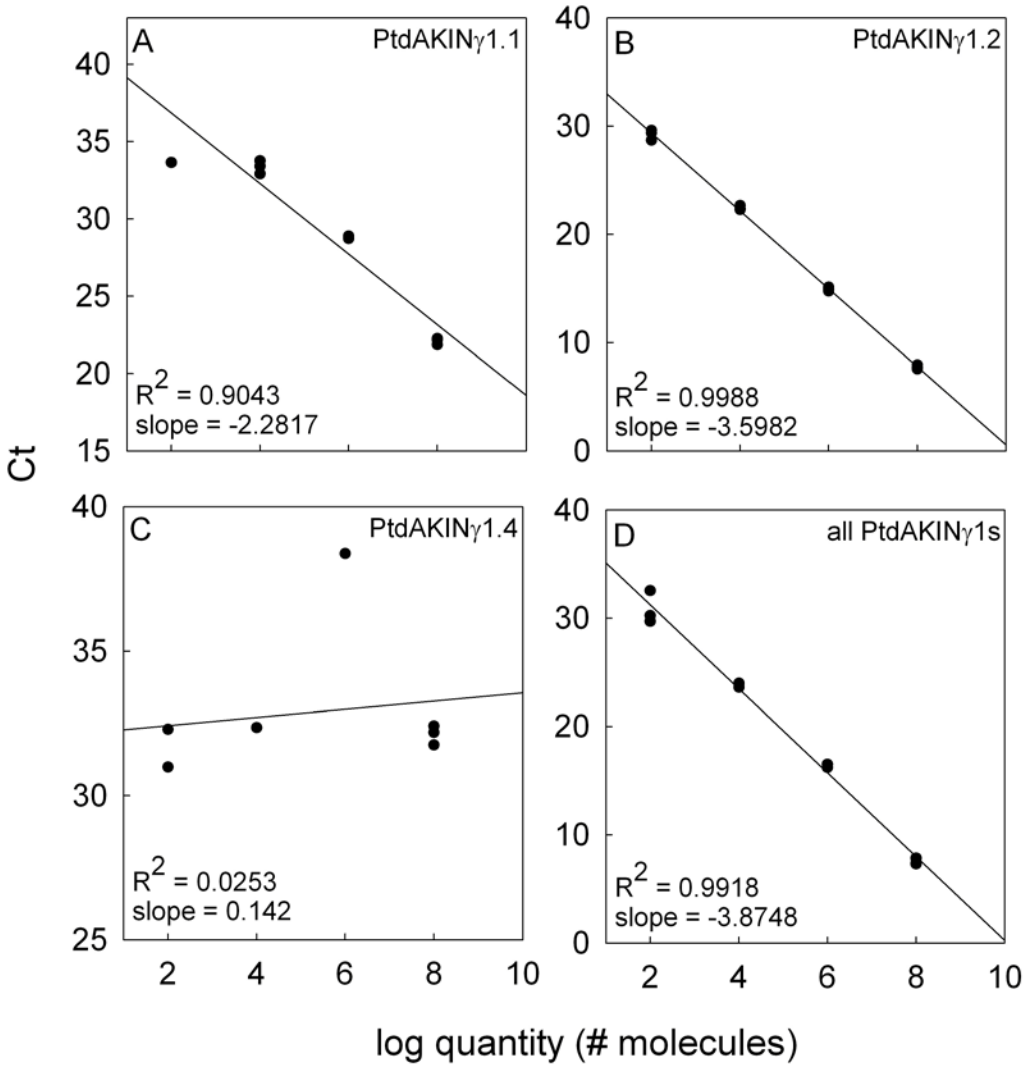

Figure 2.18. Validation of the specificity of *PtdAKINγ1.2* qRT-PCR primers using dilutions series of members of the *PtdAKINγ1* gene family to generate standard curves. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation. The template(s) in each standard curve are: (A) *PtdAKINγ1.1* (B) *PtdAKINγ1.2* (C) *PtdAKINγ1.4* (D) a mix of all members of the *PtdAKINγ1* gene family.

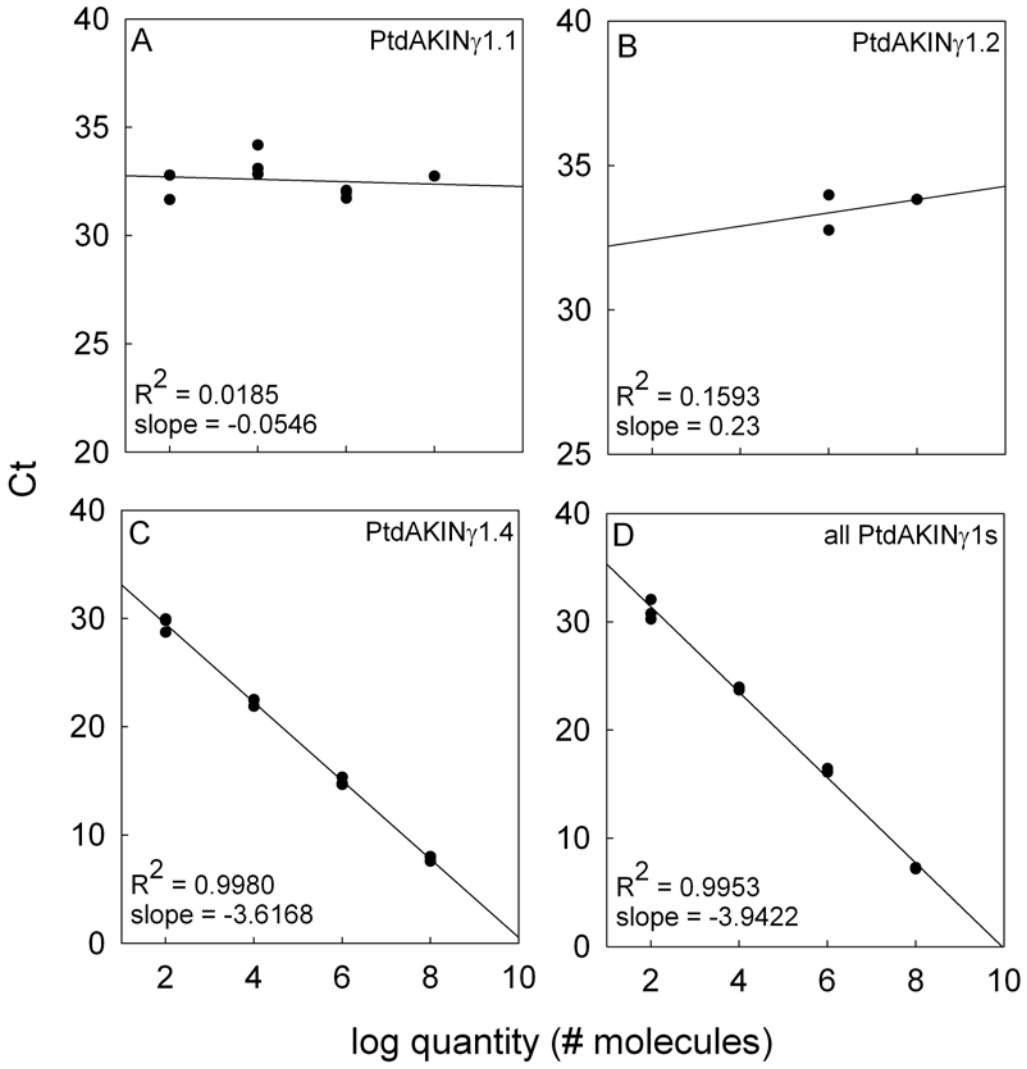

Figure 2.19. Validation of the specificity of *PtdAKINγ1.4* qRT-PCR primers using dilutions series of members of the *PtdAKINγ1* gene family to generate standard curves. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation. The template(s) in each standard curve are: (A) *PtdAKINγ1.1* (B) *PtdAKINγ1.2* (C) *PtdAKINγ1.4* (D) a mix of all members of the *PtdAKINγ1* gene family.

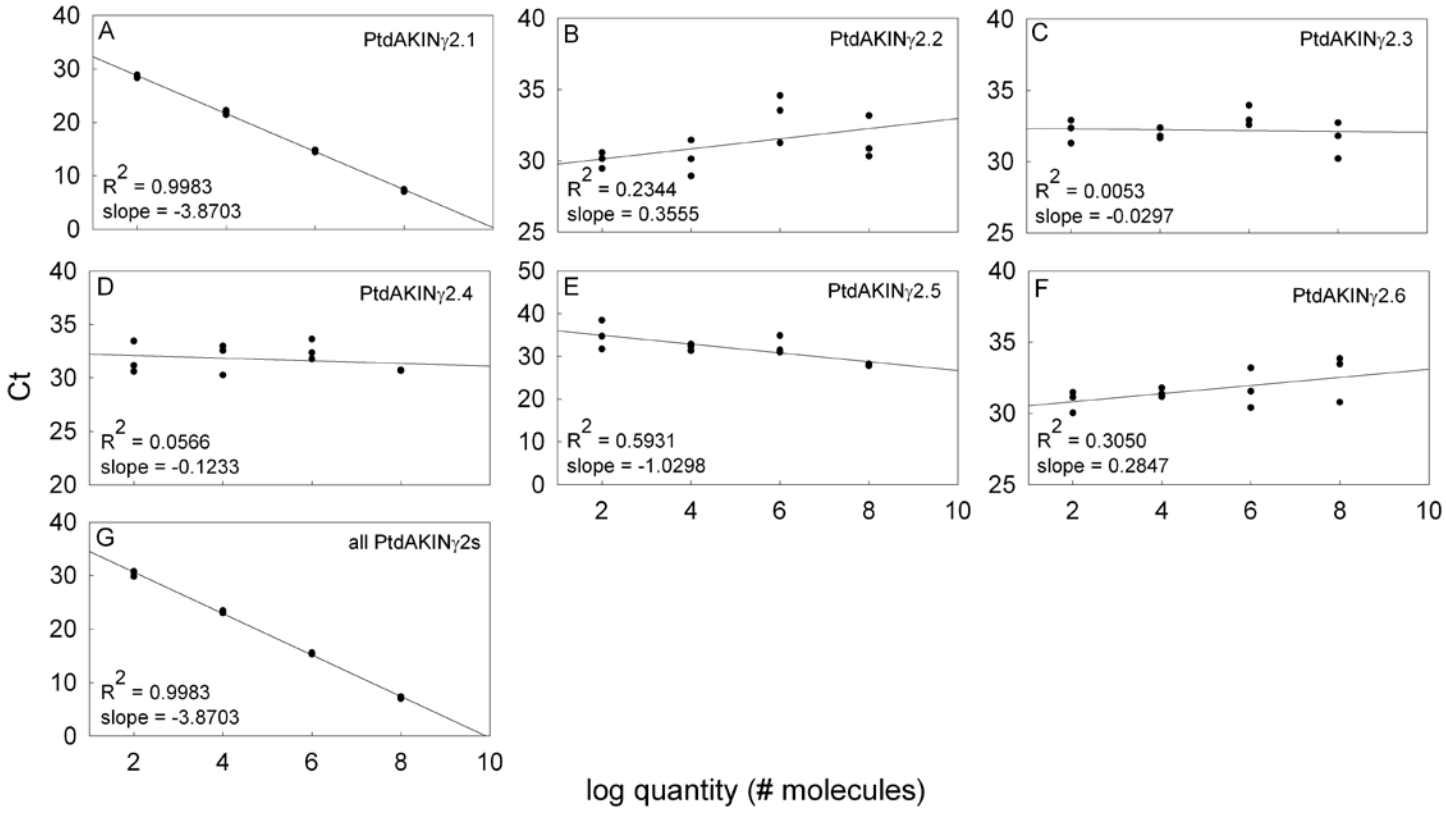

Figure 2.20. Validation of the specificity of *PtdAKINγ2.1* qRT-PCR primers using dilutions series of members of the *PtdAKINγ2* gene family to generate standard curves. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation. The template(s) in each standard curve are: (A) *PtdAKINγ2.1* (B) *PtdAKINγ2.2* (C) *PtdAKINγ2.3* (D) *PtdAKINγ2.4* (E) *PtdAKINγ2.5* (F) *PtdAKINγ2.6* (G) a mix of all members of the *PtdAKINγ2* gene family.

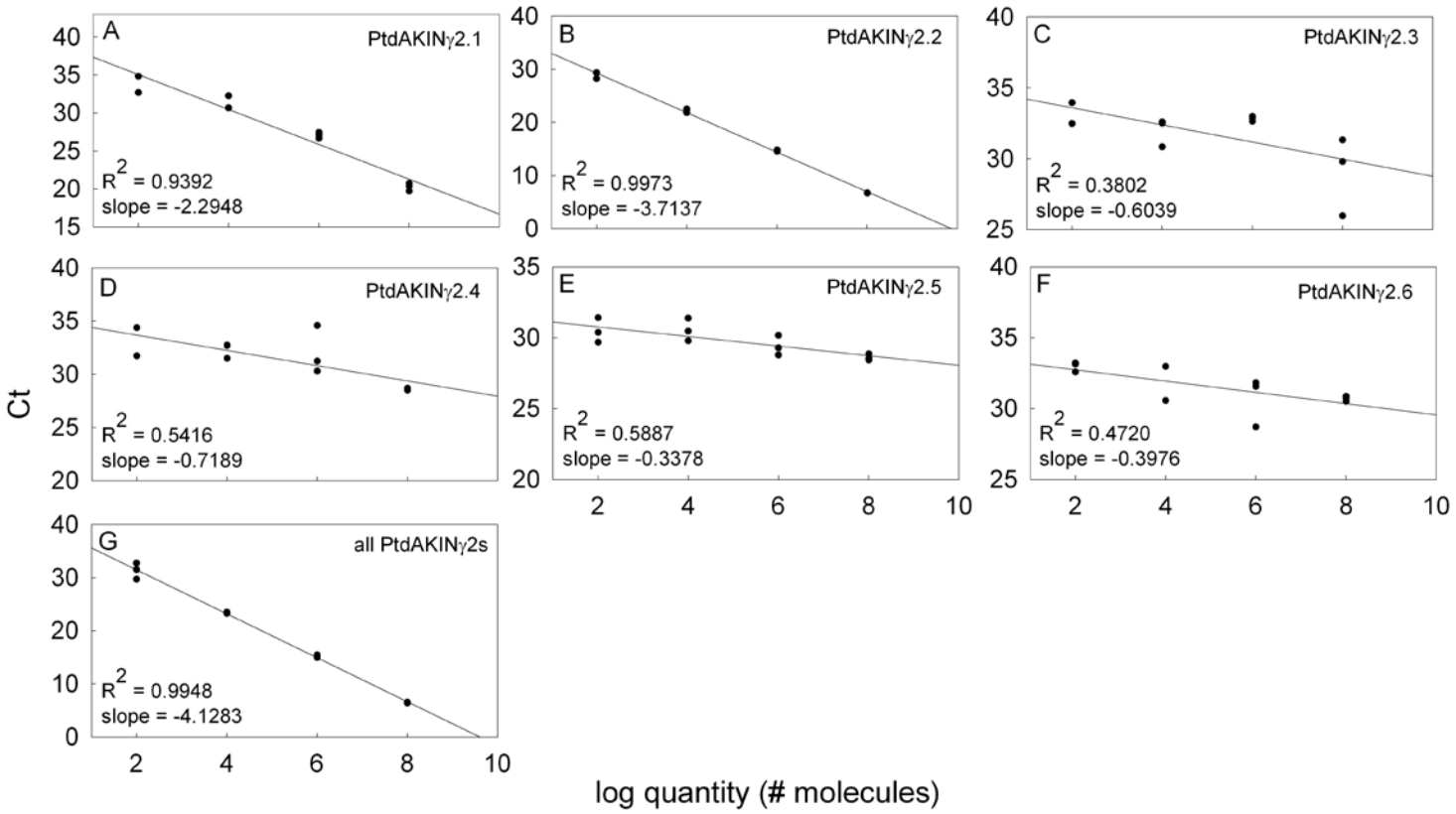

Figure 2.21. Validation of the specificity of *PtdAKINγ2.2* qRT-PCR primers using dilutions series of members of the *PtdAKINγ2* gene family to generate standard curves. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation. The template(s) in each standard curve are: (A) *PtdAKINγ2.1* (B) *PtdAKINγ2.2* (C) *PtdAKINγ2.3* (D) *PtdAKINγ2.4* (E) *PtdAKINγ2.5* (F) *PtdAKINγ2.6* (G) a mix of all members of the *PtdAKINγ2* gene family.

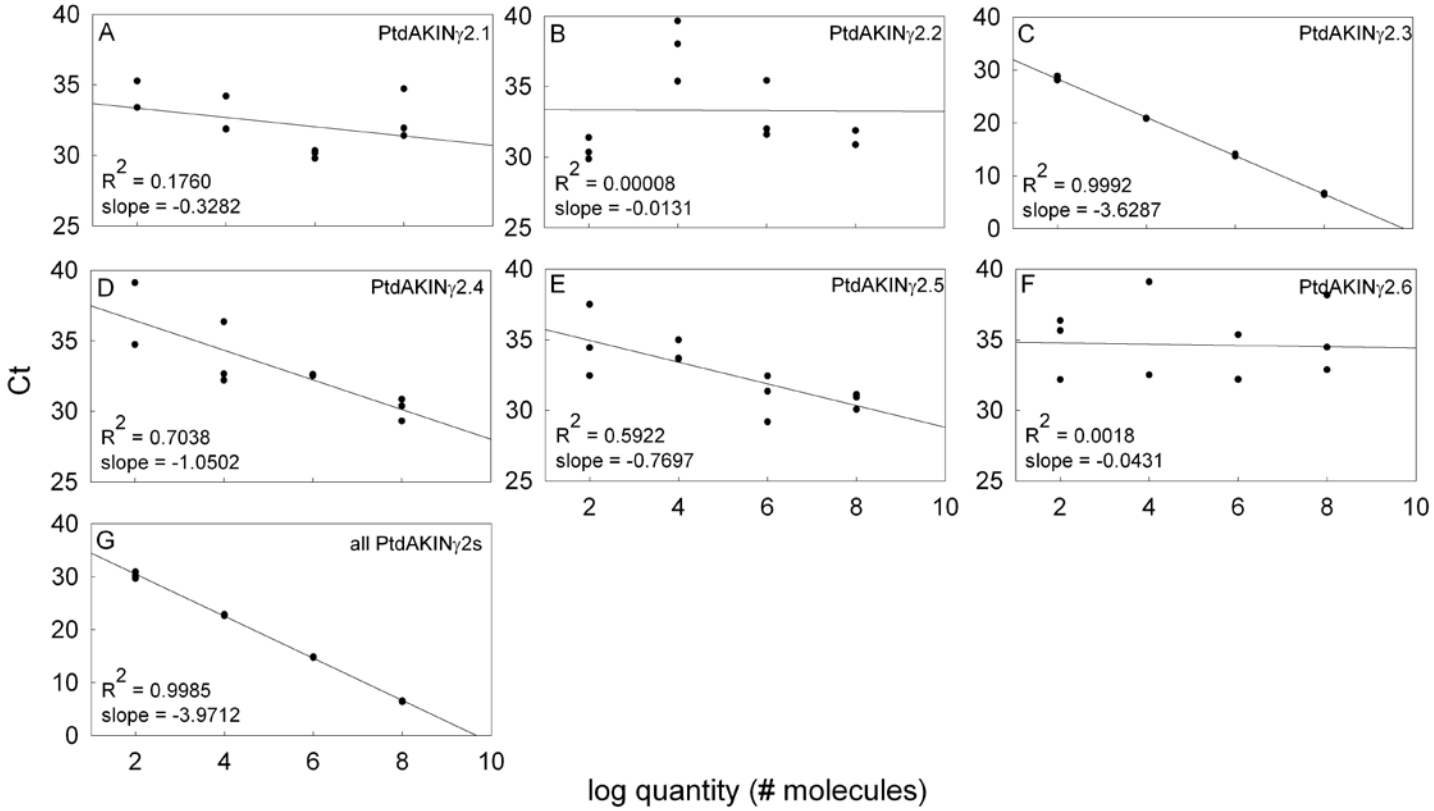

Figure 2.22. Validation of the specificity of *PtdAKINγ2.3* qRT-PCR primers using dilutions series of members of the *PtdAKINγ2* gene family to generate standard curves. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation. The template(s) in each standard curve are: (A) *PtdAKINγ2.1* (B) *PtdAKINγ2.2* (C) *PtdAKINγ2.3* (D) *PtdAKINγ2.4* (E) *PtdAKINγ2.5* (F) *PtdAKINγ2.6* (G) a mix of all members of the *PtdAKINγ2* gene family.

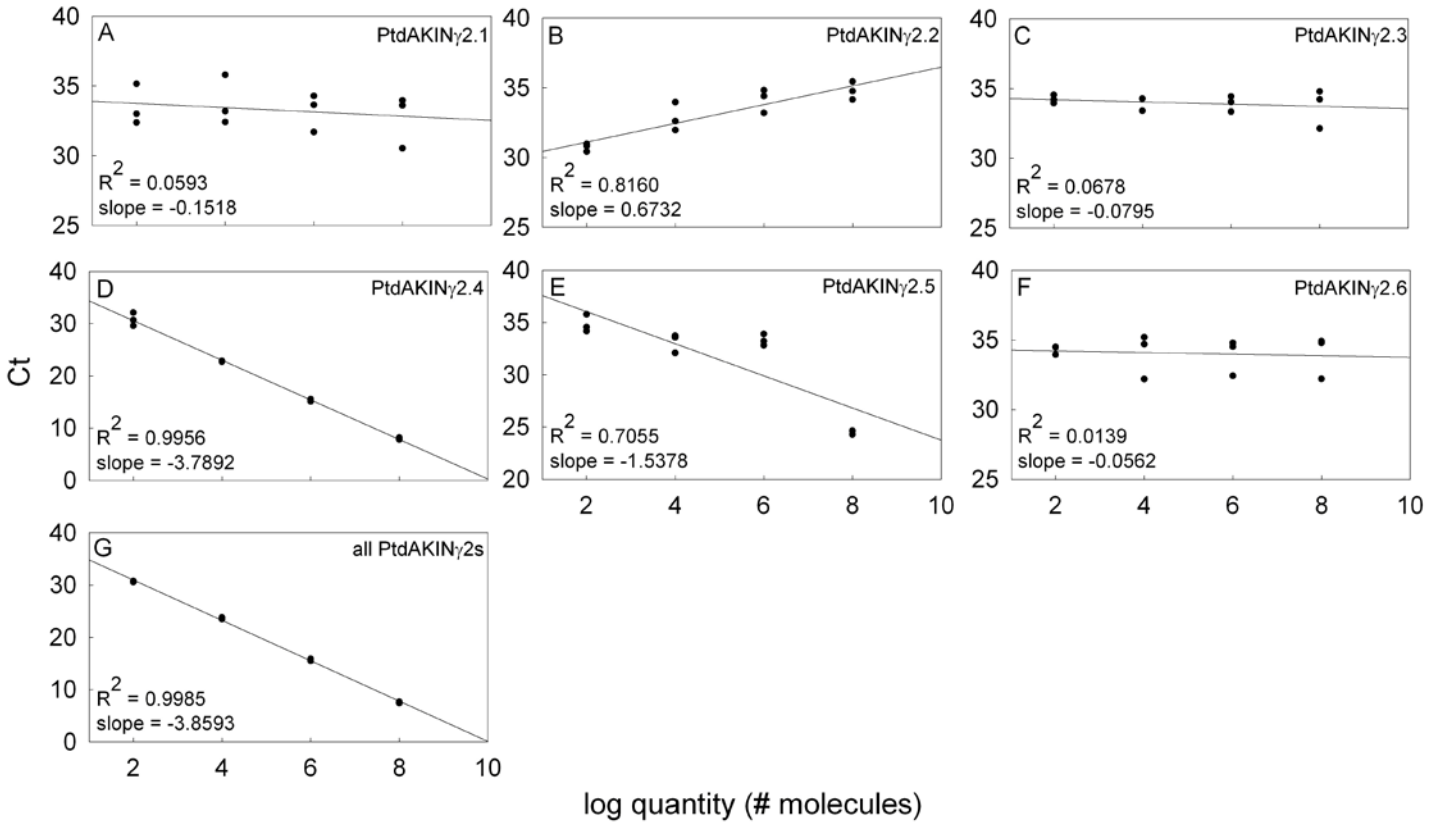

Figure 2.23. Validation of the specificity of *PtdAKINγ2.4* qRT-PCR primers using dilutions series of members of the *PtdAKINγ2* gene family to generate standard curves. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation. The template(s) in each standard curve are: (A) *PtdAKINγ2.1* (B) *PtdAKINγ2.2* (C) *PtdAKINγ2.3* (D) *PtdAKINγ2.4* (E) *PtdAKINγ2.5* (F) *PtdAKINγ2.6* (G) a mix of all members of the *PtdAKINγ2* gene family.

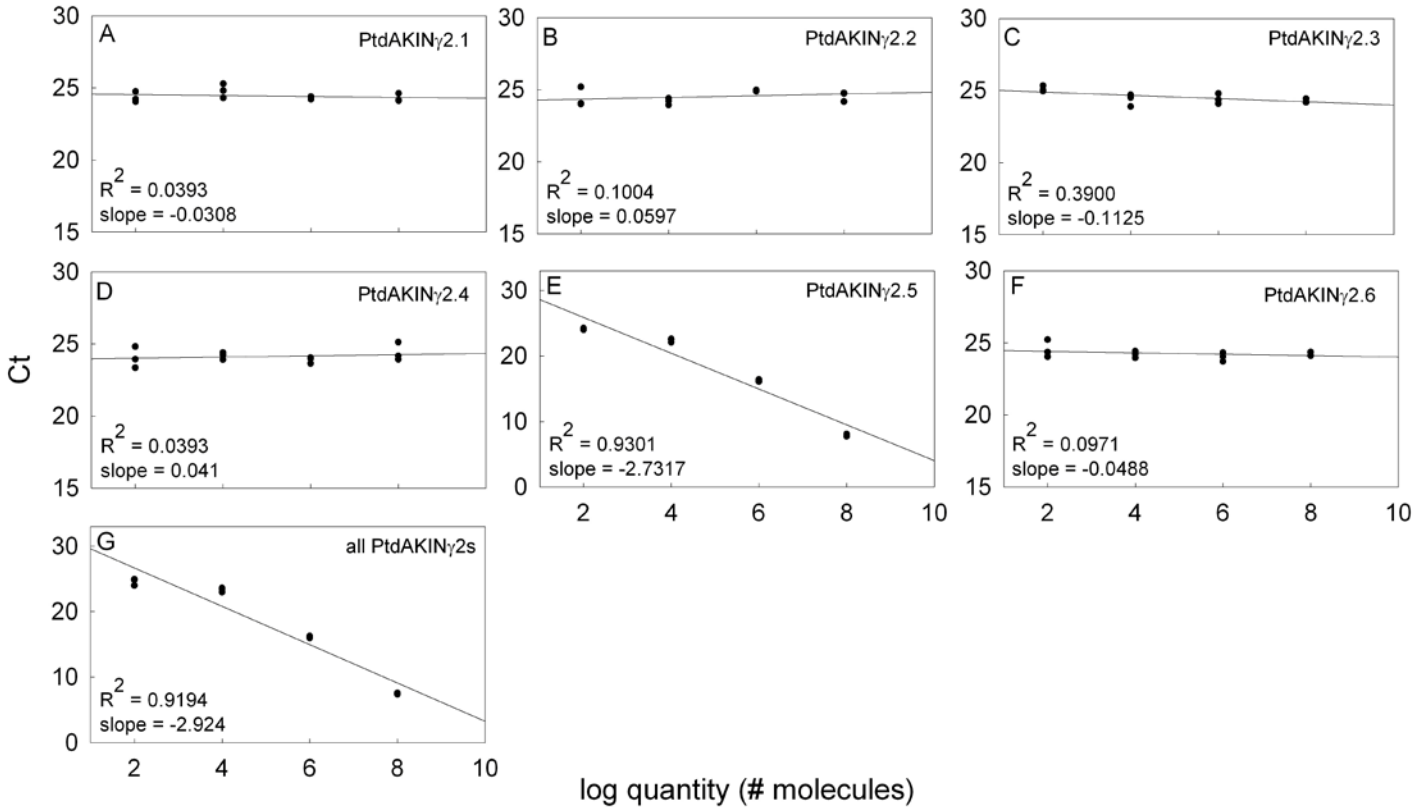

Figure 2.24. Validation of the specificity of *PtdAKINγ2.5* qRT-PCR primers using dilutions series of members of the *PtdAKINγ2* gene family to generate standard curves. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation. The template(s) in each standard curve are: (A) *PtdAKINγ2.1* (B) *PtdAKINγ2.2* (C) *PtdAKINγ2.3* (D) *PtdAKINγ2.4* (E) *PtdAKINγ2.5* (F) *PtdAKINγ2.6* (G) a mix of all members of the *PtdAKINγ2* gene family.

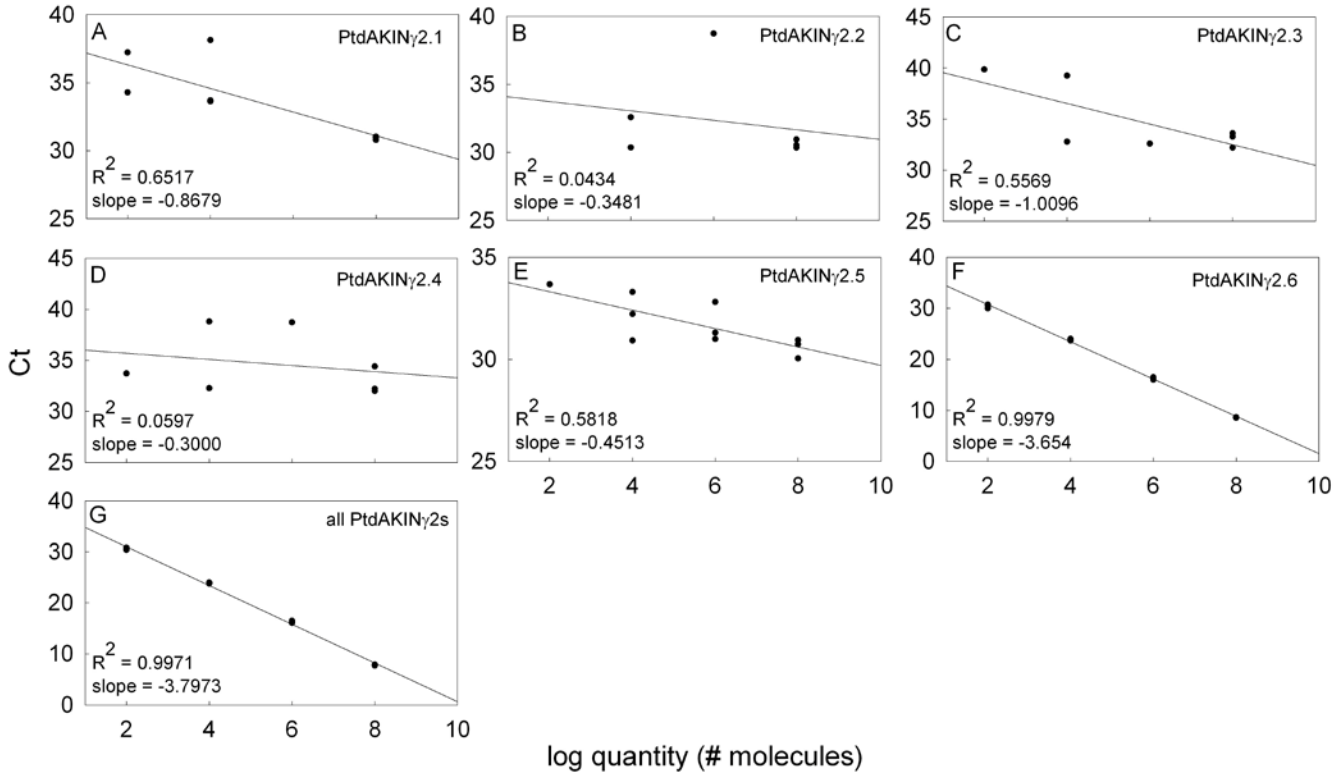

Figure 2.25. Validation of the specificity of *PtdAKINγ2.6* qRT-PCR primers using dilutions series of members of the *PtdAKINγ2* gene family to generate standard curves. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation. The template(s) in each standard curve are: (A) *PtdAKINγ2.1* (B) *PtdAKINγ2.2* (C) *PtdAKINγ2.3* (D) *PtdAKINγ2.4* (E) *PtdAKINγ2.5* (F) *PtdAKINγ2.6* (G) a mix of all members of the *PtdAKINγ2* gene family.

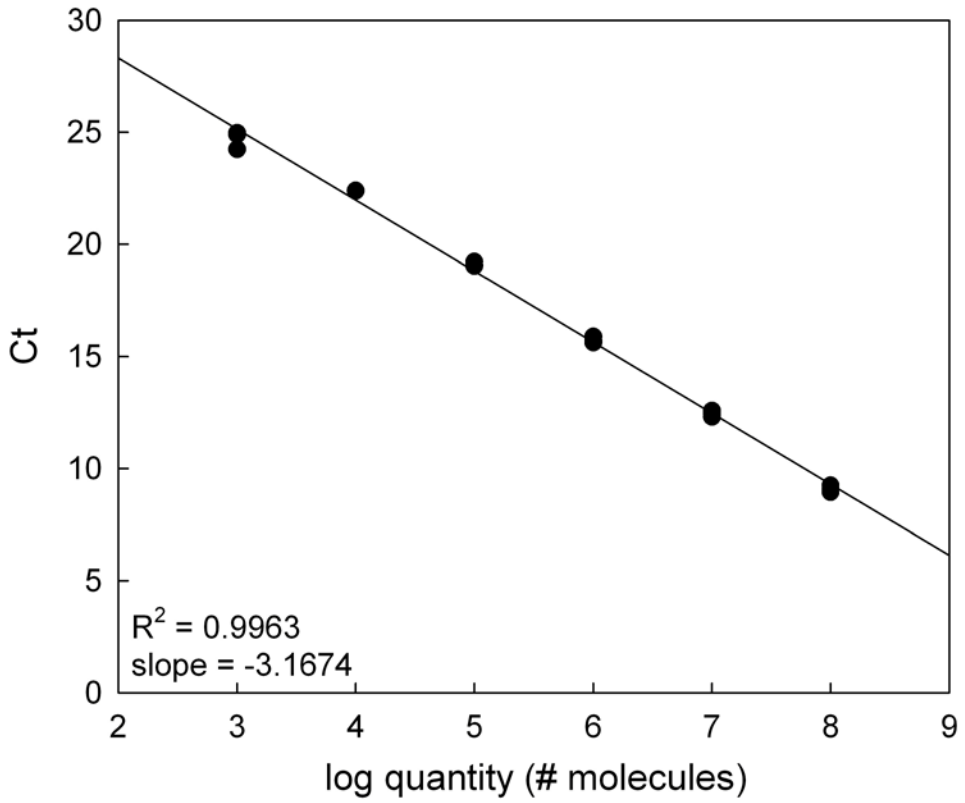

Figure 2.26. Validation of the specificity of *PtdAKINγ2.5* qRT-PCR primers using a dilution series of *PtdAKINγ2.5*. A qRT-PCR assay was used to generate standard curves in order to calculate the R^2 value and the slope of the curve. An R^2 value of 0.99 is considered strong correlation.

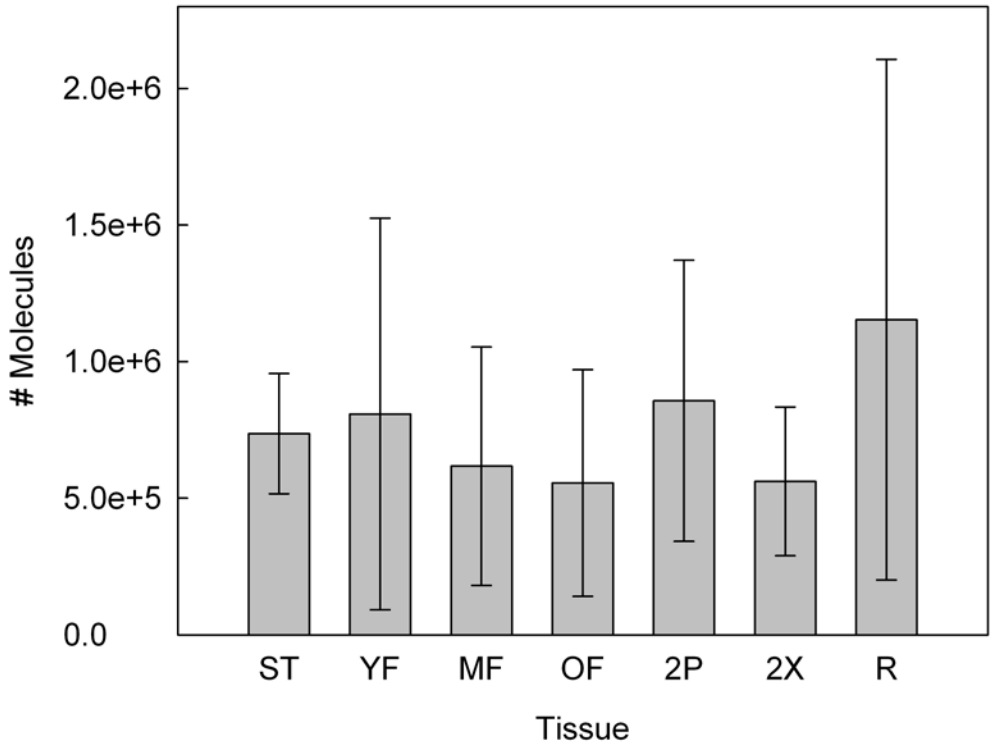

Figure 2.27. Expression profile of *EF1α1-3* in different tissues of poplar. The expression level of *EF1α1-3* in number of molecules was averaged across all experimental plates. ANOVA p = 0.8406. ST = shoot tip; YF = young foliage; MF = mature foliage; OF = old foliage; 2P = secondary phloem; 2X = secondary xylem; R = roots. Error bars indicate standard deviation.

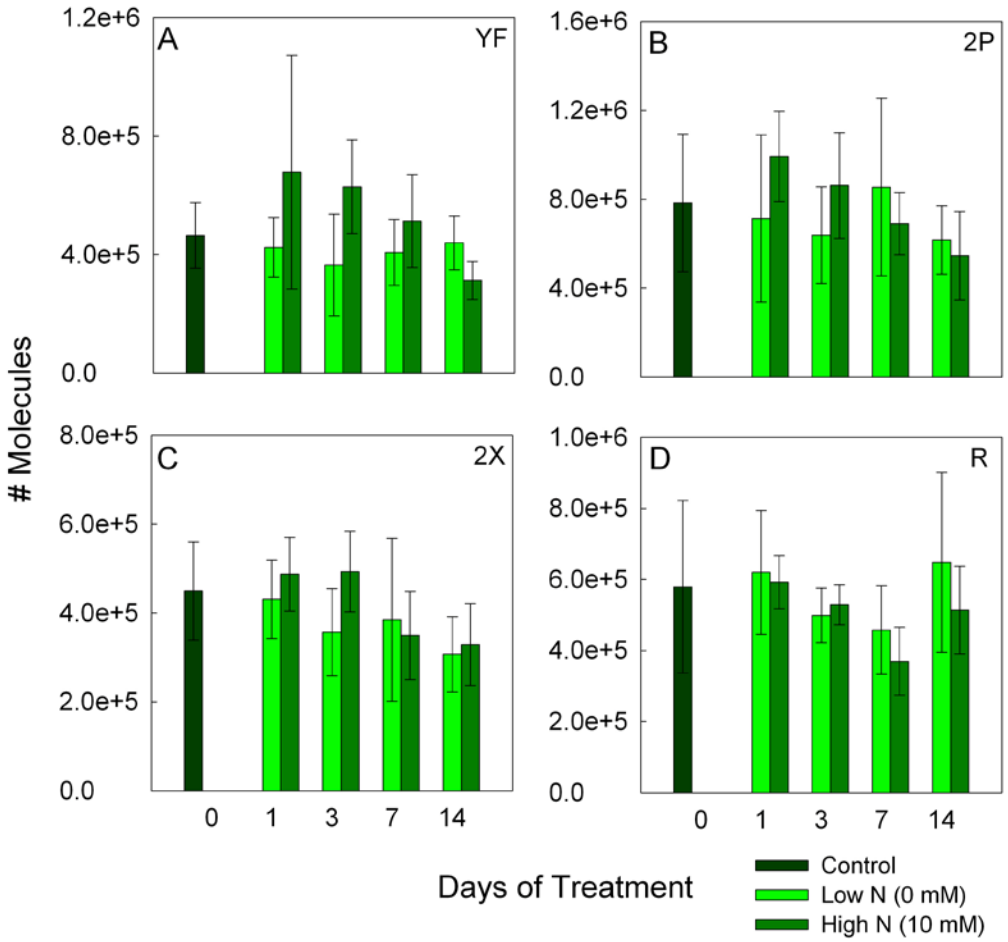

Figure 2.28. Expression profile of *EF1α–1* in several tissues of poplars treated with low versus high nitrogen for up to 14 days. The expression level of *EF1α–1* was averaged across all experimental plates. (A) Young leaves (YF) ANOVA p = 0.036. (B) Secondary phloem (2P) ANOVA p = 0.1172. (C) Secondary xylem (2X) ANOVA p = 0.0425. (D) Roots (R) ANOVA p = 0.0744. Error bars indicate standard deviation.

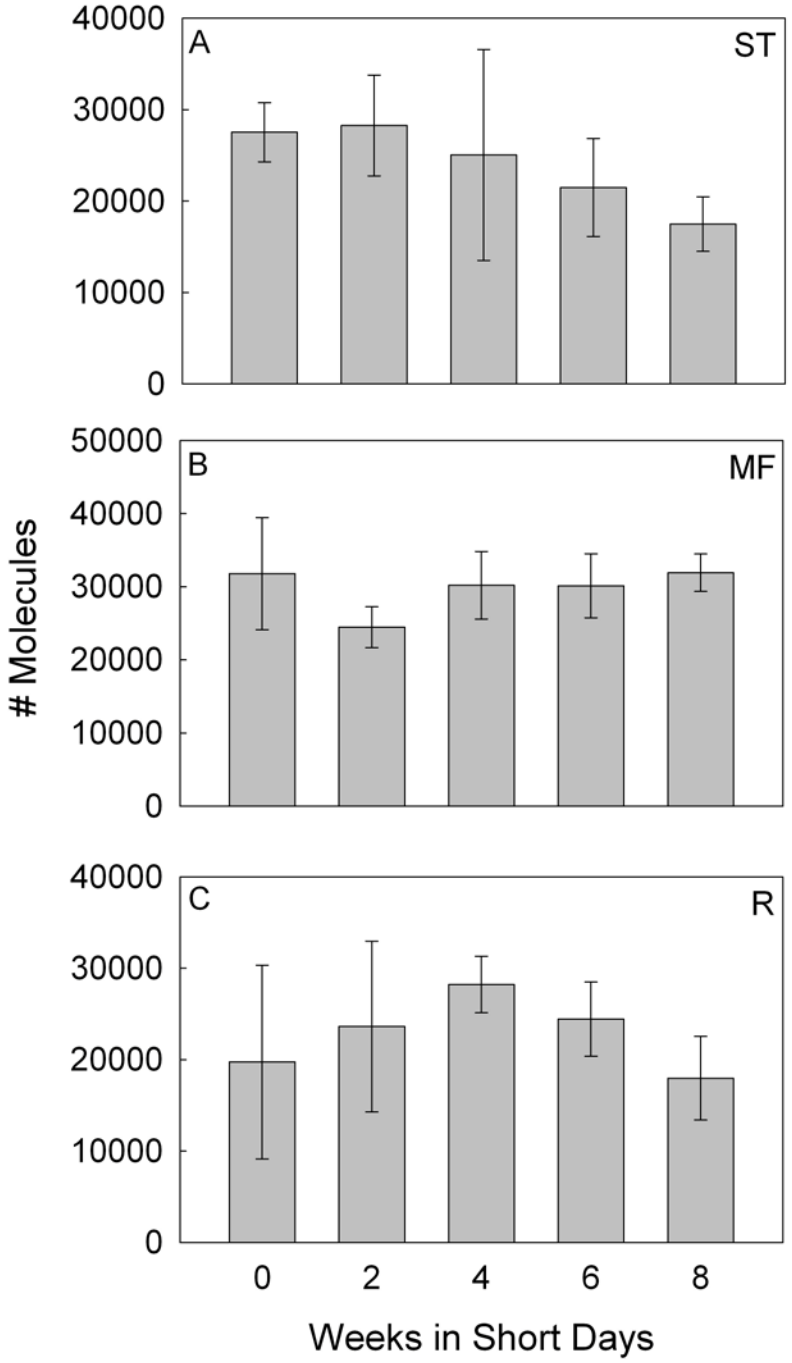

Figure 2.29. Expression profile of *VHA-A* in several tissues of poplars undergoing short day-induced dormancy. The expression level of *VHA-A* was averaged across all experimental plates. (A) Shoot tips (ST) ANOVA p = 0.0351. (B) Mature leaves (MF) ANOVA p = 0.0737. (C) Roots (R) ANOVA p = 0.0464. Error bars indicate standard deviation.

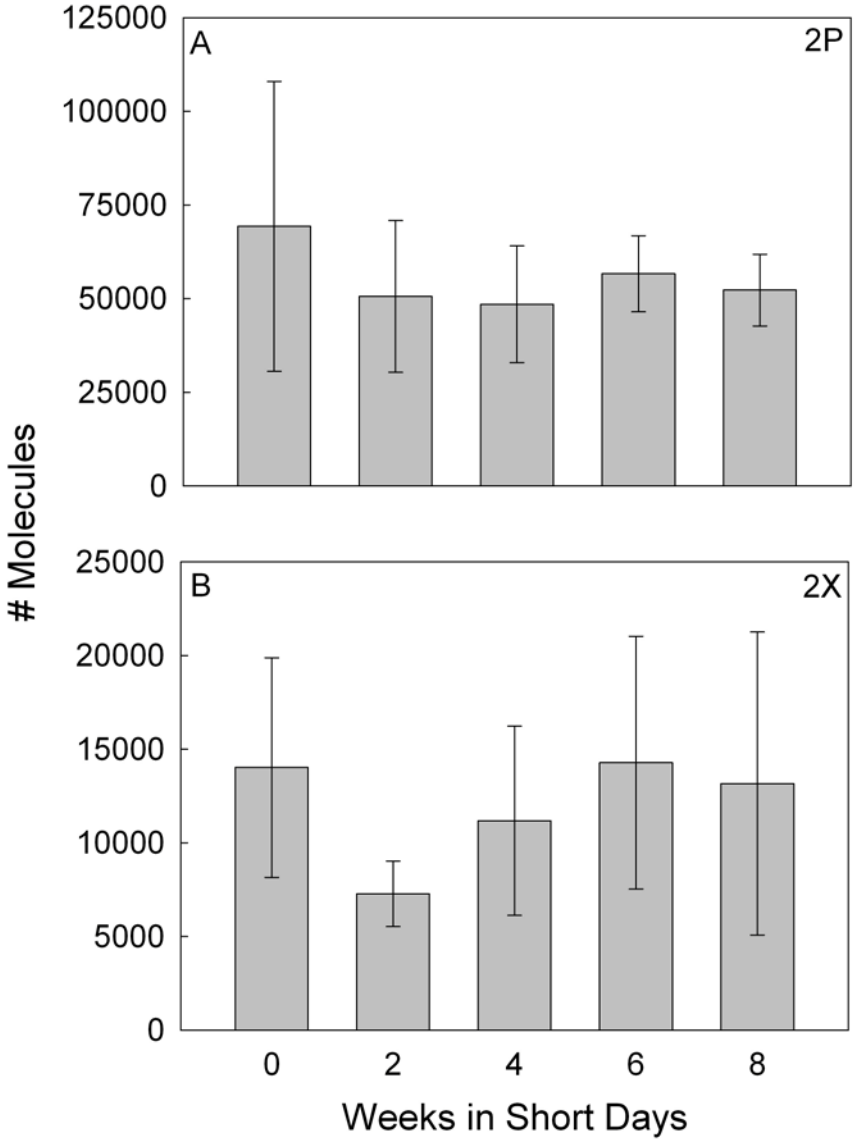

Figure 2.30. Expression profile of the genometric mean of *VHA-A* and *phosphorylase* in secondary phloem (2P) and secondary xylem (2X) of poplars undergoing short day-induced dormancy. The geometric mean of the expression of *VHA-A* and *phosphorylase* were averaged across all experimental plantes. (A) Secondary phloem ANOVA p = 0.8132. (B) Secondary xylem ANOVA p = 0.5390. Error bars indicate standard deviation.

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3.0. Expression profiling of *PtdSnRK1*, *PtdAKINβ*, and *PtdAKINγ* gene family members in poplars under different environmental conditions

3.1. Introduction

Plant growth can be viewed under the lens of biomass accumulation and resource allocation. Many factors can affect growth, ranging from the specific responses of different tissues to nutrient availability to environmental conditions such as drought, cold or disease. We are interested in understanding regulatory factors that mediate biological processes associated with biomass accumulation and carbon and nitrogen resource allocation in poplar (*Populus* spp.). Since the SNF1-related kinase (SnRK1) complex has been implicated in carbon metabolism and energy modulation (reviewed in Halford *et al.*, 2003; Halford, 2006; Polge and Thomas, 2007; Baena-González and Sheen, 2008), this protein kinase could potentially play a role in processes that affect poplar growth.

The poplar genome has undergone multiple duplications (Tuskan *et al.*, 2006) and while the conventional wisdom is that duplicated genes can often lose their function to become pseudogenes (Force *et al.*, 1999), it is also possible that paralogues will acquire a new function (neofunctionalization) or undergo a change in regulation so that the ancestral role of the gene becomes split between both paralogues (subfunctionalization; Force *et al.*, 1999; Lynch and Force, 2000; Prince and Pickett, 2002). Since the SnRK1 protein kinase complex is a

heterotrimer made up of three subunits which come from multi-member families, it is likely that different combinations of subunit members form complexes that play specific roles in the plant. In poplar, Segerman *et al.* (2007) analyzed over 90, 000 ESTs from 18 different non-normalized cDNA libraries (including dormant and stressed tissue) in order to determine if there was a connection between genes with tissue-specific expression and gene duplication. The data suggest that selective pressure for tissue-specific differential expression among duplicated genes is fairly strong, affecting genes which code for proteins involved in tissue-specific processes as well as tissue-specific responsive proteins. Given that members of the three gene families which comprise the SnRK1 protein complex appear to have members that have arisen through genome duplication (Fedosejevs, 2008), I hypothesize that while some of these genes may have functional redundancy, others may have assumed distinct functions in the plant.

Nitrogen availability and dormancy acquisition both affect poplar growth through modulating biomass accumulation and resource allocation (Cooke and Weih, 2005). The SnRK1 protein complex appears to respond to nitrogen availability. For instance, in poplar, microarray analysis indicates that an *AKINβ*-like gene is significantly upregulated ($p=0.01$) in secondary xylem under high nitrogen conditions (Cooke *et al.*, in preparation). The response of the SnRK1 protein complex may indicate that downstream targets of the SnRK1 protein complex are being regulated differently depending on nitrogen availability. We are interested in determining if the SnRK1 protein complex plays such a role during

the nitrogen response of poplar.

Microarray data imply that members of the SnRK1 complex gene families are also differentially expressed during dormancy acquisition in trees, and thus SnRK1 could also play specific roles in regulating dormancy-associated processes. In order to identify the molecular regulation of bud development and dormancy, Ruttink *et al.* (2007) used transcript and metabolite profiling of apical buds of poplar (*Populus tremula* x *alba*) during short day induction of dormancy to dissect the temporal sequence of bud formation, acclimation to dehydration and cold, and dormancy. Of particular interest was the finding that some *AKINβ* and *AKINγ* genes were shown to be significantly differentially expressed during apical bud formation, although not all SnRK1 complex gene family members were represented. Microarray data from conifers indicates that an *AKINγ*-like gene is differentially regulated when spruce enters dormancy (El Kayal *et al.*, submitted), Together, these data suggest that SnRK1 complexes involving certain subunits may be involved in regulating processes during dormancy acquisition.

The overall objective of my study is to determine if specific genes encoding subunits of the PtdSnRK1 protein complex show differential expression in poplar in response to nitrogen availability or short day-induced dormancy acquisition, as a first step in elucidating whether SnRK1 plays a role mediating aspects of these biological processes. A second objective is to use the gene expression profiles to infer which genes show co-expression, and thus potentially assemble to form functional SnRK1 complexes, or alternatively, which gene members were unlikely

to form complexes. In order to complete this objective, three different experiments were designed: (1) a tissue survey experiment, (2) a nitrogen availability experiment, and (3) a dormancy acquisition experiment. The expression patterns of the members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families were assayed using qRT-PCR and compared. Principal component analysis was used to determine which genes had particularly interesting expression profiles in the different experiments.

3.2. Materials and methods

3.2.1. Plant experiments

Plant experiments were designed and executed as described in Chapter 2.0.

3.2.2. qRT-PCR assay

cDNA synthesis and the qRT-PCR assay was done as described in Chapter 2.0. qRT-PCR for the tissue survey experiment was conducted on 96-well plates while the nitrogen availability experiments and the dormancy experiments were conducted on 384-well plates. For reference genes and standard curve production, see Chapter 2. Expression levels of SnRK1 complex subunit family members for

the tissue survey experiment were compared between all seven tissues harvested. Fedosejevs (2008) examined the expression level of the *PtdAKINβ* gene members for the tissue survey experiment. The annealing temperature in the thermal profile used for the *PtdAKINβ* gene members was 63^{o}C rather than 60^{o}C for all experiments, as per Fedosejevs' design. Expression levels of target genes for the nitrogen experiment were compared within a single tissue (young leaves, secondary phloem, secondary xylem or roots) between tissue treated with high nitrogen and tissue treated with low nitrogen and across the five time points. Expression levels of target genes for the dormancy acquisition experiment were compared within a single tissue (shoot tip, mature leaves, secondary phloem, secondary xylem or roots) across the five time points. A minimum of three and a maximum of six biological replicates were used and there were three technical replicates for each sample.

3.2.3. Data Analysis

3.2.3.1. qRT-PCR data

Technical replicates were averaged for each biological replicate. Absolute number of molecules of a particular gene of interest (as determined by standard curve) was normalized using the absolute number of molecules of the reference gene(s) selected for the experiment (see Chapter 2 for details). The expression

level for a gene of interest for a biological replicate was discarded if the expression level did not fall within the linear portion of the standard curve, i.e. could not be reliably quantified. If a minimum of three biological replicates did not have expression levels within the linear portion of the standard curve, then the expression level for the particular tissue or treatment was deemed to be below the detectable limit. Expression levels of biological replicates were averaged and standard deviation was calculated.

3.2.3.2. Statistical analysis

Expression levels of the 19 members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene familes were analyzed using SAS/STAT. Normality was tested using the Shapiro-Wilk test, and homogeneity of variance was tested using Bartlett's test. When necessary to meet the assumptions of normality and homogeneity of variance, the data was transformed, either by log transformation or square root transformation (Table 3.1-3.4). A one-way ANOVA test was performed for the tissue survey experiment and the dormancy acquisition experiment. A two-way ANOVA was used in the nitrogen availability experiment. If the p-value derived from the ANOVA was less than 0.05, Tukey's Studentized range test was used to determine if there were any significant differences at a p-value of 0.05.

3.2.3.3. Principal component analysis

Principal component analysis (PCA) was done using all members of the SnRK1 protein complex that were assayed in order to determine if the expression profiles of certain subunits was responsible for driving differences between tissues and treatments. The PCA was done in R version 2.11.0 (R Development Core Team, 2010; http://www.R-project.org) using the packages vegan (Oksanen *et al.*, 2010) and BiodiversityR (Kindt and Coe, 2005). The script used was provided by Patrick James (Appendix 5.1). Principal components were determined to be significant using the broken-stick distribution, a model of expected relative species abundance, and an equilibrium circle was used to determine if a particular gene significantly contributed to the variability of the principal components (Legendre and Legendre, 1998).

3.3 Results.

Data transformation, normality, homogeneity of variance and ANOVA results are summarized in Tables 3.1 – 3.4.

3.3.1. *PtdSnRK1*, *PtdAKINβ*, and *PtdAKINγ* expression profiles across tissues

3.3.1.1. Expression profiles of members of the *PtdSnRK1*, *PtdAKINβ* and

PtdAKINγ gene families across different tissues

Transcript abundance profiles corresponding to the *PtdSnRK1*, *PtdAKINβ*, and *PtdAKINγ* gene family members in various tissues are shown in Figure 3.1, Figure 3.2 and Figure 3.3, respectively. Data for *PtdAKINβ1.1*, *PtdAKINβ1.2*, *PtdAKINβ2.2*, *PtdAKINβ3.1* and *PtdAKINβ4.1* were obtained from E. Fedosejevs, and are used with permission to facilitate the PCA analyses and comparison with other experiments.

The members of the *PtdSnRK1* and *PtdAKINβ* gene family showed similar expression patterns. Expression levels were relatively low in shoot tips, young leaves and roots. There was a trend of increasing expression as the leaves mature, and the expression in secondary phloem and secondary xylem were similar. Statistically significant differences were not found for *PtdSnRK1.1*, *PtdAKINβ1.1* or *PtdAKINβ3.2* relative transcript abundance among different tissues within a gene using ANOVA analysis while Tukey's Studentized range test was unable to distinguish between expression levels at a p-value of 0.05 despite an ANOVA p-value of 0.0172. For *PtdSnRK1.2*, the expression level in secondary xylem and shoot tips were significantly different from each other but not from other tissues ($p=0.0188$). For *PtdSnRK1.3*, the expression level of old leaves and shoot tip were significantly different from each other but not from other tissues ($p=0.0343$). Of the other *PtdAKINβ* gene family members, shoot tips, young leaves and roots tended to be grouped in the same Tukey grouping ($p<0.0004$) although they were

not always significantly different from other tissues except those with the highest expression levels. For instance, *PtdAKINβ2.1* expression was not significantly different between shoot tips, young leaves and roots. Young leaves and roots, however, were not significantly different from secondary phloem, and secondary phloem, in turn, was only significantly different from shoot tips and old leaves.

The *PtdAKINγ* gene family members showed more variability in their expression patterns. *PtdAKINγ1.1* had significantly higher expression in secondary phloem, secondary xylem and roots in comparison to shoot tips, young leaves and mature leaves (p=0.0001). There was also a trend of increasing expression as the leaves matured. *PtdAKINγ1.2* had a similar expression pattern across tissues (p=0.0001). The expression of *PtdAKINγ1.4* tended to be below the detectable limit. *PtdAKINγ2.2* showed significantly higher expression in shoot tips in comparison to all other tissues (p=0.0001). Both *PtdAKINγ2.3* and *PtdAKINγ2.5* were more highly expressed in mature leaves and old leaves in comparison to other tissues (p=0.0001). *PtdAKINγ2.1*, *PtdAKINγ2.4* and *PtdAKINγ2.6* showed similar trends to those seen in the *PtdSnRK1* and *PtdAKINβ* gene families.

3.3.1.2. Comparison of expression profiles within a gene family

Figures 3.4 – 3.6 show the relative level of expression of each gene member in relation to other gene members within the same family in different tissues. In

the *PtdSnRK1* family (Figure 3.4), *PtdSnRK1.1* and *PtdSnRK1.2* showed similarity both in magnitude of expression and profiles across tissues, while *PtdSnRK1.3* showed a much lower level of expression as well as a different profile.

In the *PtdAKINβ* family (Figure 3.5), closely related gene members tended to show similar expression patterns. Most differences within the *PtdAKINβ* gene family lie in magnitude. *PtdAKINβ1.1* tended to be expressed more than *PtdAKINβ1.2* except in mature leaves and secondary xylem. *PtdAKINβ2.2* was expressed more than *PtdAKINβ2.1* in all tissues. *PtdAKINβ3.2* tended to be expressed at similar levels to or slightly more than *PtdAKINβ3.1*. *PtdAKINβ4.1* showed the greatest level of expression in mature leaves, old leaves, secondary phloem and secondary xylem.

In the *PtdAKINγ* family, closely related gene members showed similar expression patterns, though not always the same magnitude. *PtdAKINγ1.1*, *PtdAKINγ2.3* and *PtdAKINγ2.6* were expressed at a greater magnitude in all tissues in comparison to their most closely related genes *PtdAKINγ1.2*, *PtdAKINγ2.4*, and *PtdAKINγ2.5*, respectively. *PtdAKINγ2.3* illustrated this most notably in mature leaves and old leaves. *PtdAKINγ2.1* also shows more expression than *PtdAKINγ2.2* in nearly all tissues except for in shoot tip.

3.3.1.3. Principal component analysis

A PCA was conducted on the gene expression profiles for all of the SnRK1 complex members for each in the different tissues in order to determine if expression profiles of any *SnRK* complex genes can differentiate tissues from one another. The first principal component accounts for 89.32% of the variability in the data and is driven significantly by the expression of *PtdAKINγ2.3*, which serves to significantly separate mature and old leaves from the other tissues (Figure 3.7). Other tissues tend to cluster along the first principal component, indicating that they share similar expression patterns.

The second principal component accounts for 8.46% of the variability. Most tissues tend to separate from each other along this axis, except for shoot tip and young foliage. The separation along the second component appears to be driven primarily by *PtdAKINγ1.1*and *PtdAKINβ4.1*, although not significantly. All other genes show little effect on the variance.

3.3.2. *PtdSnRK1*, *PtdAKINβ*, and *PtdAKINγ* expression profiles under differential nitrogen availability

3.3.2.1. Expression profiles of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families under a time course of high versus low nitrogen

In all tissues assayed (Figures 3.8 – 3.19), the general trend of expression for gene members of the PtdSnRK1 protein complex is a higher level of

expression under low nitrogen availability compared to high nitrogen availability, when there appears to be any difference at all.

There are a few notable exceptions to this trend. In young leaves, the expression of *PtdAKINβ1.1* (Figure 3.9A), *PtdAKINγ1.1* and *PtdAKINγ1.2* (Figures 3.10A and B) was found to be significantly higher on day 14 when nitrogen availability was high ($p<0.0001$), although the data did not appear very normal for *PtdAKINγ1.1* and *PtdAKINγ1.2* (Shapiro-Wilk test $p=0.0007$ and $p=0029$, respectively). In secondary xylem and roots, the expression of *PtdAKINβ1.1* (Figures 3.15A and 3.18A), *PtdAKINγ1.1* (Figures 3.16A and 3.19A) and *PtdAKINγ1.2* (Figures 3.16B and 3.19B) were significantly higher in tissue from plants grown in conditions of high nitrogen availability ($p\leq0.0008$) although again the data were not normally distributed (Shapiro-Wilk test $p\leq0.0346$). In secondary phloem, the expression of *PtdAKINβ1.1* (Figure 3.12A) and *PtdAKINγ1.2* (Figure 3.13B) were significantly higher in tissue from plants grown in conditions of high nitrogen availability ($p<0.0008$).

3.3.2.2. Comparision of expression levels within a gene family

The expression levels of *PtdSnRK1.1* and *PtdSnRK1.2* in young leaves, secondary phloem, secondary xylem and roots were generally very similar in both high and low nitrogen conditions (Figures 3.20 – 3.23). The exception occurred in secondary xylem at day 14 in high nitrogen conditions, where *PtdSnRK1.1* fell

below detectable limits. The expression level of *PtdSnRK1.3* fell below the detectable limit at all time points and was therefore not included in further analyses. Note that falling below the detectable limit does not mean that the transcript is absence, only that it is not possible to accurately quantify the transcript abundance.

In all tissues, *PtdAKINβ1.1* shows the highest level of expression in comparison to other members of the *PtdAKINβ* gene family (Figures 3.24 – 3.27) Furthermore, expression levels of putative paralogues tend to be similar. The exceptions include the expression of *PtdAKINβ1.1* in all tissues.

In secondary phloem, secondary xylem and roots, the highest level of expression was found in *PtdAKINγ1.1* (Figures 3.29 – 3.31), while in young leaves the highest level of expression was found in *PtdAKINγ2.3* (Figure 3.28). The expression of *PtdAKINγ1.4* tended to be very low or below the detectable limit in all tissues at both levels of nitrogen availability.

3.3.2.3. Principal component analysis

A PCA was conducted on the gene expression in different tissues during treatment with high and low nitrogen in order to determine what gene expression, if any, drives the variation during differential nitrogen availability. In young leaves (Figure 3.32), the first principal component accounted for 76.32% of the variation and was driven significantly by the expression of *PtdAKINβ1.1*,

PtdAKINγ1.1, and *PtdAKINγ2.3*. The increased expression of these genes served to differentiate the expression pattern of young leaves under high nitrogen conditions at 14 days of treatment. The second principal component accounted for 17.59% of the variation. The increased expression of most genes served to separate the expression pattern of young leaves in low nitrogen conditions from those in high nitrogen conditions.

In secondary phloem (Figure 3.33), the first principal component accounted for 66.01% of the variation. The increased expression of *PtdAKINγ1.1* and *PtdAKINβ1.1* along with the decreased expression of the other genes contributes to the first principal component, although only the expression profile of *PtdAKINγ1.1* was considered significant. The first principal component separated the expression of secondary phloem in high nitrogen conditions compared to low nitrogen conditions. The second principal component accounted for 31% of the variation in the data and served to further separate the expression patterns in secondary phloem under low and high nitrogen conditions. The PCA of secondary xylem (Figure 3.34) showed a very strong similarity to the PCA of secondary phloem, though only the first principal component was significant.

In roots (Figure 3.35), the first principal component accounted for 95.92% of the variation in the data and separated the expression pattern in roots in low and high nitrogen conditions. It was driven significantly by increased expression in *PtdAKINγ1.1*, *PtdAKINβ1.1* and non-significantly by *PtdAKINγ1.2*. The second principal component accounted for 2.49% of the variation in the data and served

to separate the time points further.

3.3.3. *PtdSnRK1, PtdAKINβ,* and *PtdAKINγ* expression profiles during dormancy acquisition

3.3.3.1. Expression profiles of members of the *PtdSnRK1, PtdAKINβ* and *PtdAKINγ* gene families across different tissues during dormancy acquisition

In shoot tips (Figure 3.36), the expression of *PtdSnRK1.1* remained unchanged (p=0.9293) but *PtdSnRK1.2* showed a trend of increased expression after the second week (p<0.0001). In mature leaves (Figure 3.37), *PtdSnRK1.1* and *PtdSnRK1.2* were largely unchanged (p>0.2876). In secondary phloem (Figure 3.38) and secondary xylem (Figure 3.39), *PtdSnRK1.1*and *PtdSnRK1.2* expression decreased after the second week of short day exposure (p<0.0001). Secondary xylem also showed a peak of *PtdSnRK1.1* and *PtdSnRK1.2* expression in the second week. In roots (Figure 3.40), the expression levels of *PtdSnRK1.2* was unchanged while the expression of *PtdSnRK1.1* decreased at week 8 of short day conditions (p=0.0098). The expression levels of *PtdSnRK1.3* were below the detectable limit in all tissues.

In shoot tips (Figure 3.41), the expression of P*tdAKINβ1.1*, *PtdAKINβ2.1*, and *PtdAKINβ2.2* remained unchanged during short day conditions (p=0.3420, p=0.0628 and p=0.6697, respectively). *PtdAKINβ3.1* and *PtdAKINβ3.2* showed a

trend of decreasing expression as short day conditions continue ($p<0.0007$) while *PtdAKINβ1.2* and *PtdAKINβ4.1* showed a trend of increasing expression after week 4 of short day conditions ($p<0.0001$).

In mature leaves (Figure 3.42), only the expression of *PtdAKINβ1.1* showed any change, decreasing in expression as short days continued ($p=0.0094$).

In secondary phloem (Figure 3.43) and secondary xylem (Figure 3.44), there was a trend of decreased expression of all members of the *PtdAKINβ* gene family after the second week ($p<0.0001$).

In roots (Figure 3.44), the expression levels of *PtdAKINβ1.1*, *PtdAKINβ1.2*, *PtdAKINβ2.1* and *PtdAKINβ3.2* did not change significantly, although there appeared to be a slightly significant decrease in the expression of *PtdAKINβ1.1* ($p=0.0742$). The expression patterns of *PtdAKINβ2.2*, *PtdAKINβ3.1* and *PtdAKINβ4.1* show a trend of decreasing expression at around week 8 of short day conditions ($p<0.0236$).

The expression patterns of the members of the *PtdAKINγ* gene family showed the widest range of variation between the tissues. In shoot tips (Figure 3.46), *PtdAKINγ1.1* showed an increase in expression during the fourth week of short day conditions ($p<0.0001$); this increase was maintained during subsequent weeks. *PtdAKINγ1.2* and *PtdAKINγ1.4* showed a trend of increasing expression beginning during the fourth week ($p<0.0045$). *PtdAKINγ2.3* showed a similar trend beginning on the sixth week ($p<0.0001$). *PtdAKINγ2.1* and *PtdAKINγ2.4* showed a gradual trend of increased expression throughout the experiment

($p<0.0250$).

In mature leaves (Figure 3.47), *PtdAKINγ1.1* showed a weakly significant ($p=0.0518$) trend of decreasing expression during the course of short day conditions. *PtdAKINγ2.4*, *PtdAKINγ2.5*, and *PtdAKINγ2.6* showed the same trend but significantly ($p<0.0323$). The expression levels of *PtdAKINγ1.4* and *PtdAKINγ2.2* were below the detectable limit.

In secondary phloem (Figure 3.48), there was a trend of decreasing expression of *PtdAKINγ1.1*, *PtdAKINγ1.2*, *PtdAKINγ2.5* and *PtdAKINγ2.6* ($p<0.003$). This was coupled, however, with the increased expression of *PtdAKINγ2.1* and *PtdAKINγ2.2* which peaked at week 2 and week 4, respectively, before decreasing ($p<0.0058$). *PtdAKINγ2.3* also showed a trend of increased expression, beginning at week 6 ($p<0.0001$), although the data was not very normal despite log transformation (Shapiro-Wilk test $p=0.039$).

In secondary xylem (Figure 3.49), the expression of most members of the *PtdAKINγ* gene family peaked during the second week of short day conditions before decreasing during subsequent weeks ($p<0.0008$). The exception was the expression of *PtdAKINγ2.3*, which increased throughout short day conditions ($p<0.0001$). The expression of *PtdAKINγ1.2* and *PtdAKINγ2.2* were below the detectable limit.

In roots (Figure 3.50), there was a trend of decreasing expression in *PtdAKINγ2.1* and *PtdAKINγ2.3* ($p<0.0012$). The expression of *PtdAKINγ1.4* was below the detectable limit.

3.3.3.2. Comparison of expression levels within a gene family

Figures 3.51 – 3.53 show the relative level of expression of each gene member in relation to other gene members within the same family in different tissues. In the *PtdSnRK1* family (Figure 3.51), the expression levels of *PtdSnRK1.1* and *PtdSnRK1.2* were generally comparable, showing similar trends and magnitudes, although in roots the expression of *PtdSnRK1.1* appeared to be somewhat greater than *PtdSnRK1.2*.

In the *PtdAKINβ* gene family (Figure 3.52), closely related genes tended to show similar magnitudes of expression, with the exception of *PtdAKINβ1.1* and *PtdAKINβ1.2*. *PtdAKINβ1.1* showed the greatest magnitude of expression during the beginning of short day conditions, though in secondary phloem and secondary xylem the magnitude decreased during the time course until it is comparable to *PtdAKINβ1.2*.

In the *PtdAKINγ* gene family (Figure 3.52), *PtdAKINγ1.1* was the most highly expressed of the gene family members in most tissues throughout the experiment. The exception was in mature leaves, where *PtdAKINγ2.3* was the most highly expressed. Furthermore, the expression of *PtdAKINγ2.3* surpassed the expression of *PtdAKINγ1.1* in secondary phloem and secondary xylem by the eighth week of short day conditions. In general, of the most closely related gene models, there is one gene which is expressed at a higher level than the other.

These were *PtdAKINγ1.1*, *PtdAKINγ2.1, PtdAKINγ2.3* and *PtdAKINγ2.6*.

3.3.3.3. Principal component analysis

A principal component analysis was conducted on the gene expression for SnRK complex gene family members in the different tissues in order to determine which gene expression patterns, if any, drives the variation observed during dormancy acquisition.

In shoot tips (Figure 3.54), the first principal component accounted for 88.66% of the variation and is significantly driven by the expression of *PtdAKINγ1.1*. The PCA showed the separation of the expression patterns of weeks 4, 6 and 8 from day 0 and week 2 along the first component, indicating that the increased expression of *PtdAKINγ1.1* was sufficient to differentiate the expression pattern in early versus late dormancy acquisition. The second principal component accounted for 9.74% of the variation in the data, and appeared to be driven primarily, but not significantly, by *PtdAKINγ2.3*. The second principal component separated the weeks of short day conditions further, especially week 8.

The first principal component for mature leaves (Figure 3.55), which accounted for 98.83% of the variability, showed that the variation in mature leaves was driven significantly by *PtdAKINγ2.3*. The clustering together of week 4, 6 and 8 of short day treatment suggests that the decrease in expression of *PtdAKINγ2.3* is sufficient to differentiate these later time points from earlier time

points and the day 0 control. The second principal component accounted for 0.88% of the variability and appears to be driven primarily by *PtdAKINγ1.1* and *PtdAKINβ1.1*.

The PCA for secondary phloem (Figure 3.56) showed that the first principal component accounted for 95.59% of the variation and was driven significantly by the expression of *PtdAKINγ1.1*. *PtdAKINβ1.1* and the opposing expression pattern of *PtdAKINγ2.3* played a smaller role. The decreasing expression of *PtdAKINγ1.1* and *PtdAKINβ1.1* coupled with the increasing expression of *PtdAKINγ2.3* served to differentiate the earlier time points of dormancy acquisition from later time points. *PtdAKINβ1.1* and *PtdAKINγ2.3* also appear to drive the variation of the second principal component.

The PCA for secondary xylem (Figure 3.57) showed that the first principal component accounted for 95.79% of the variation in the data and was driven significantly by the expression of *PtdAKINγ1.1* as well as, to a lesser extent, *PtdAKINγ2.3* and *PtdAKINγ2.6*. This leads to a separation of the later part of dormancy acquisition from the earlier part. The second principal component accounted for 3.04% of the variation and appeared to be affected primarily by *PtdAKINγ1.1*, *PtdAKINγ2.3*, and *PtdAKINγ2.6*.

The PCA for roots (Figure 3.58) showed that the first principal component accounted for 84.8% of the variation in the data and was driven significantly by the expression of *PtdAKINγ1.1*. The weeks in short day conditions appear to be separated along the axis based on the magnitude of expression of *PtdAKINγ1.1*.

The second principal component accounted for 10.71% of the variation in the data and was driven by the expression of *PtdAKINβ1.1* and *PtdAKINγ2.3*. The second principal component separated the weeks of short day conditions based on the decreasing expression of the two genes.

3.4. Discussion

The main objective of my study was to determine if the genes encoding potential subunits of the SnRK1 protein kinase complex are differentially expressed in poplar in certain tissues, or in response to differential nitrogen availability or during dormancy acquisition. These data would allow me to infer whether the SnRK1 protein complex plays a role in processes associated with the nitrogen response or dormancy acquisition in poplar. A second objective was to use these data to determine which genes are expressed at the highest levels and which genes are expressed at minimal levels. This information could then be used to infer genes which encode subunits which form specific SnRK1 complexes. Together, these findings indicate whether roles for the SnRK1 protein complex in nitrogen response and dormancy acquisition should be further investigated in future studies and identify a subset of *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* genes that can be targeted for future comprehensive functional analyses.

3.4.1. Comparison of expression patterns of putative paralogues

Approximately 92% of the *Populus* genome is believed to have been

affected by a relatively recent duplication event (Tuskan *et al.*, 2006), giving rise to paralogues. Paralogues may have one of four possible fates (Ohno, 1970; Hughes, 1994; Force *et al.*, 1999; Lynch and Conery, 2000; Lynch and Force, 2000; Prince and Pickett, 2002; Gu *et al.*, 2003; Segerman *et al.*, 2007): (1) degeneration leading to nonfunctionalization, (2) division of the ancestral function leading to subfunctionalization, (3) acquisition of a new function (neofunctionalization), or (4) maintenance of redundant function.

The PtdSnRK1 protein complex gene families have members which are hypothesized to be paralogous (refer to Figure 1.1). Differences in expression patterns between putative paralogues could be indicative of subfunctionalization or neofunctionalization while similarities could be indicative of redundancy. For instance, the expression patterns of *PtdSnRK1.1* and *PtdSnRK1.2* show similar trends in the experiments conducted, suggesting functional redundancy. Furthermore, the higher abundance of *PtdSnRK1.1* and *PtdSnRK1.2* in comparison to *PtdSnRK1.3* indicates that *PtdSnRK1.3* may serve a more minor or specialized role. *PtdSnRK1.3* could also be moving towards nonfunctionalization. In contrast, in the nitrogen availability experiment, it was shown that *PtdAKINβ1.1* expression increased under conditions of high nitrogen while the expression of *PtdAKINβ1.2* did not (Figures 3.9, 3.12, 3.15, and 3.18). This implies two alternatives: (1) an ancestral role in response to high nitrogen availability is being fulfilled primarily by *PtdAKINβ1.1* or (2) the response of *PtdAKINβ1.1* to high nitrogen availability is a new development which occurred

after the duplication event.

In general, putative paralogues within the PtdSnRK1 protein complex gene families have similar trends in their expression patterns, although the abundance of the putative paralogues may be different. For instance, in the dormancy experiment, although *PtdAKINγ1.1*and *PtdAKINγ1.2* showed similar expression patterns, *PtdAKINγ1.1* was expressed at a much higher level than *PtdAKINγ1.2* (Figure 3.53). While the similar expression patterns indicate that *PtdAKINγ1.1*and *PtdAKINγ1.2* may be functionally redundant, it is possible that *PtdAKINγ1.1* may be preferentially incorporated into PtdSnRK1 protein complexes, though transcript abundance is not necessarily indicative of protein abundance.

3.4.2. Differential expression of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families across tissues

The role of PtdSnRK1 protein complexes in various tissues is likely complicated and not easily illustrated based solely on transcript abundance. For instance, expression of members of the PtdSnRK1 protein complex subunits appears to generally be lower in shoot tips, young leaves and roots in comparison to other tissues. Shoot tips, young leaves and roots act as sink tissues, and may have different characteristics (reviewed in Kozlowki, 1992). Shoot tips, for instance, contain the apical meristem, where most of the imported carbohydrates are used for metabolism and growth. Roots are reversible sinks with carbohydrate

reserves which can be mobilized as needed. In contrast, secondary xylem and secondary phloem stem tissues, which can also act as reversible sink tissues, can have comparable levels of transcript abundance to source tissues such as mature leaves.

The PCA of the expression patterns of the genes encoding subunits of the SnRK1 protein complex indicates that the expression profiles of the different tissues tend to be similar, with mature leaves and old leaves separated from other tissues as a result of the much greater abundance of *PtdAKINγ2.3* transcripts in these tissues. This suggests that the PtdSnRK1 protein complex in mature and old leaves has *PtdAKINγ2.3* as the predominant AKINγ subunit. Young leaves are primarily sink tissues, although the lamina tip shows net positive photosynthesis before the rest of the leaf (Dickmann, 1971; Larson et al., 1980). Typically, leaves transition into primarily source material by LPI 6 (Larson and Gordon, 1969). Photosynthesis increases as leaves mature but as leaves approach senescence, photosynthesis decreases (Dickmann, 1971). Microarray experiments in *Arabidopsis* indicate that the SnRK1 protein complex is involved in the transcriptional regulation of photosynthetic genes (Baena-González *et al.*, 2007; Baena-González and Sheen, 2008). My data suggest that PtdAKINγ2.3 could form part of a specific SnRK1 complex that plays a role in regulating processes associated with photosynthetic capacity - such as starch synthesis, storage or breakdown – and/or with senescence, such as protein remobilization.

3.4.3. Differential expression of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families under differential nitrogen availability

High nitrogen abundance leads to significant changes to most members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families. In general, the expression of most the genes that were examined was lower under conditions of high nitrogen than under low nitrogen, with the exceptions of *PtdAKINβ1.1* and *PtdAKINγ1.1 PtdAKINγ1.2*. In the case of these three genes, the expression levels were significantly higher in conditions of high nitrogen than low nitrogen in the tissues surveyed, except that *PtdAKINγ1.1* was invariantly expressed in secondary phloem under differing nitrogen conditions. PCA indicated that *PtdAKINγ1.1* accounted for most of the difference in expression patterns of high nitrogen availability compared to low nitrogen availability in all tissues studied. *PtdAKINβ1.1* was also involved, although only significantly in young leaves and roots. The downregulation of other members of the PtdSnRK1 protein complex coupled with the upregulation of *PtdAKINβ1.1* and *PtdAKINγ1.1* may indicate that a PtdSnRK1 protein complex composed of a catalytic subunit, *PtdAKINβ1.1* and *PtdAKINγ1.1* or *PtdAKINγ1.2* is more prevalent in conditions of high nitrogen, and this PtdSnRK1 complex could regulate molecular events that are altered under conditions of high nitrogen.

In young leaves, increased abundance of *PtdAKINβ1.1*, *PtdAKINγ1.1* and *PtdAKINγ1.2* was detected on the fourteenth day after high nitrogen treatment,

although decreased abundance of other transcripts was detected after one day of high nitrogen treatment. This contrasts with other tissues, where increased expression of *PtdAKINβ1.1*, *PtdAKINγ1.1* and *PtdAKINγ1.2* corresponded with decreased expression of other genes. This implies that the response to high nitrogen availability may come in stages in young leaves. Though the response to high nitrogen availability has been investigated in young leaves in poplar, the very early response to high nitrogen availability has not been focused on. For instance, in *P. trichocarpa* × *deltoides*, it has been shown that in shoot tips, which include LPI 1, certain genes are induced after 14 days of high nitrogen availability (50 μM NH_4NO_3), including vegetative storage proteins *pni288* and *win4*, whereas others were induced by low nitrogen availability (0 μM NH_4NO_3), including starch synthase (Cooke *et al.*, 2003). In *Populus deltoides*, the accumulation of *win4* increases in young leaves (LPI 1-5) as nitrogen availability increases when measured after four and eight weeks of treatment (Coleman *et al.*, 1994).

3.4.4. Differential expression of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families during dormancy acquisition

Unlike that observed for conditions of differential nitrogen availability, *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene family members showed diverse expression profiles in the different tissues that were surveyed during dormancy acquisition. This may indicate different roles being played by PtdSnRK1 protein

complex(es) in modulating processes occurring dormancy acquisition within these tissues.

In shoot tips, changes to expression levels of the various subunits of the PtdSnRK1 complex tend to occur midway through dormancy acquisition, approximately during the fourth week. In *Populus tremula x Populus alba*, it was found that this is a dynamic time point in dormancy acquisiton during which many major changes occur (Ruttink *et al.*, 2007). For instance, meristem inactivation begins, bud scale development begins and there is increased expression of starch biosynthetic genes. Furthermore, critical enzymes for the biosynthesis of abscisic acid (ABA) are upregulated at three to four weeks of short day conditions. ABA has been implicated in the regulation of the AKINγ subunit in tomatoes (Bradford *et al.*, 2003) and overexpression of SnRK1 in *Arabidopsis* leads to an ABA hypersensitive response (Jossier *et al.*, 2009), providing a potential point of inquiry for the regulation of the PtdSnRK1 protein complex in shoot tips. Of particular interest is the increased abundance of *PtdAKINγ1.4*, which is normally present in very low abundance in comparison to other gene members of the PtdSnRK1 protein complex. This allows the inference that *PtdAKINγ1.4* may have a specific role during dormancy acquisition in shoot tips. There is also an increase in the abundance of *PtdAKINγ2.3*, which was shown to be expressed in high abundance in mature leaves and old leaves. The PCA indicates that *PtdAKINγ1.1* is significantly involved in differentiating the expression patterns of shoot tips from other tissues during dormancy acquisition,

with *PtdAKINγ2.3* contributing to a lesser extent.

In mature leaves, the expression patterns of most genes do not change during dormancy acquisition. It is important to note that the short day conditions used to invoke bud dormancy do not induce leaf senescence, and photosynthesis continues even under short days. Of the genes that do show altered expression patterns, they generally exhibit a gradual decrease in transcript abundance as short day conditions continue. The PCA indicates that the expression of *PtdAKINγ2.3* continues to be a distinguishing factor in mature leaves, much greater than the expression levels of other genes of the PtdSnRK1 protein complex.

In roots, most changes to expression patterns, although statistically significant, appear to be small, with gradual decreases in abundance over time. The PCA indicates that the expression of *PtdAKINγ1.1* is a significant characteristic of the expression profile of roots even though the expression differences of *PtdAKINγ1.1* was found to be only marginally significant ($p=0.089$). The expression profiles of *PtdAKINβ1.1* and *PtdAKINγ2.3* were also highlighted in the PCA, although only *PtdAKINγ2.3* was shown to exhibit statistically significant changes in expression.

In secondary phloem and secondary xylem, there is an overall decrease in the expression of nearly all subunits of the PtdSnRK1 protein complex, with the exception of *PtdAKINγ2.3*. This general decrease in expression is not seen in other tissues and may be indicative of specific changes occurring in these secondary tissues which do not occur in the other tissues studied, such as

cessation of cambial meristem activity and maturation of cells making up the xylem and phloem. The majority of the cells making up the xylem, in particular, are dead at maturity. Thus, it is possible that SnRK1 complexes regulate processes involved in cell differentiation and maturation, and that expression of the genes making up these complexes declines as these processes conclude.

3.4.5. *PtdAKINβ1.1, PtdAKINγ1.1* and *PtdAKINγ2.3* are expressed at relatively high levels and show the most variation in transcript abundance across the three different experiments

While the ANOVA data indicate that all genes studied show, at some point, statistically significant differential expression (Tables 3.1-3.4), the PCAs indicate that *PtdAKINβ1.1, PtdAKINγ1.1* and *PtdAKINγ2.3* are generally responsible for distinguishing treatments from one another. Table 3.5 consolidates the statistical analyses for genes identified by the PCAs. The analysis reflects not only that these genes are differentially expressed in these three experiments, but are also expressed at relatively high levels. This indicates that *PtdAKINβ1.1*, *PtdAKINγ1.1* and *PtdAKINγ2.3* may be a subset of genes that have primary roles in PtdSnRK1 function in poplar. It is perhaps not surprising that comparatively little variation in transcript abundance was observed for *PtdSnRK1.1* and *PtdSnRK1.2*, the two major genes encoding the α, or catalytic, subunits. Instead, β and γ subunits have been shown to be differentially regulated under various conditions and have

regulatory functions in other organisms.

In *Arabidopsis*, *AKINβ1* has been shown to respond to dark period, with transcripts accumulating rapidly and significantly after 30 minutes of exposure to dark period, while *AKINβ2* does not respond, suggesting subunit specific function (Bouly *et al.*, 1999; Polge *et al.*, 2008). Certain AKINβ isoforms have been shown to interact with nitrate reductase and negatively regulate it (Polge *et al.*, 2008; Li *et al.*, 2009), providing further evidence of substrate specific function. In potato, antisense expression of *StubGAL83* (an *AKINβ*) in leaves leads to stunted roots, delayed tuberisation, and reduced size and number of tubers per plant (Lovas *et al.*, 2003).

In yeast, different β subunits show differential localization in the nucleus, vacuole and cytoplasm, indicating a possible role in directing the localization of the SNF1 complex. Gal83 was shown to direct the nuclear localization of SNF1 in a glucose-regulated manner (Vincent *et al.*, 2001). Gal83 is responsible for mediating the interaction between the SNF1 complex and Sip4, a transcription activator of gluconeogenic genes (Vincent and Carlson, 1999). Deletion of the glycogen binding domain in GAL83 leads to constitutive activity of the SNF1 complex independent of glucose availability, although the deletion of this domain in other β subunits in yeast led to no change in SNF1 activity (Mangat *et al.*, 2010). This provides evidence not only for the regulatory function of the β subunit in yeast but also for isoform specific functions.

In plants, the γ subunit is believed to play a role in seed maturation and

longevity. In tomatoes, *LeSNF4* accumulates during seed development and is low in seeds that have completed germination (Bradford *et al.*, 2003). In *Medicago truncatula*, RNAi silencing of *MtSNF4b* reduces the germination percentage and seedling vigour, as well as decreasing the accumulation of stachyose and increasing the sucrose content (Rosnoblet *et al.*, 2007). Transcriptome analysis of transgenic RNAi *M. truncatula* implicate *MtSNF4b* in the defense response in hydrated, dormant seeds by affecting the expression of genes involved in flavonoid and phenylpropanoid metabolism, WRKY transcription factors and pathogenesis-related proteins (Bolingue *et al.*, 2010).

In mammals, AMPKγ contains Bateman domains which interact with AMP and ATP. Binding of AMP activates the AMPK complex, potentially by leading to a conformational change of AMPKγ. AMPKγ contains a pseudosubstrate recognition site on its N-terminal which is similar to the consensus recognition motif of AMPK substrates but contains residues which cannot be phosphorylated (Scott *et al.* 2007). This site may inhibit kinase activity by interacting with AMPKα and this interaction is hypothesized to be mutually exclusive with binding to AMP. Point mutations which interfere with AMP binding and AMP activation in AMPKγ are associated with heart disease which has as a common feature the elevated storage of glycogen in cardiac myocytes (reviewed in Hardie, 2007).

3.5. Conclusion

Expression profiling of the members of the PtdSnRK1 protein complex gene families was conducted using robust qRT-PCR assays across different poplar tissues, under differential nitrogen availability and during dormancy acquisition. The expression profiles of various members of the PtdSnRK1 protein complex gene families showed differential expression, indicating that the PtdSnRK1 protein complex plays a role in the response to nitrogen availability and during dormancy acquisition. Principal component analysis indicate that *PtdAKINβ1.1*, *PtdAKINγ1.1* and *PtdAKINγ2.3* are commonly responsible for differentiating treatments from each other and are good candidates for further study.

3.6. Tables

Table 3.1. Summary of statististical analyses of expression profiles of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families across various tissues. The ratio of the gene of interest and EF1α-3 was transformed as noted in order to produce the largest p values for the Shapiro-Wilk test of normality and Bartlett's test for homogeneity of variance. P values for the Shapiro-Wilk test and Bartlett's test which were below 0.05 are shown. P values of the one-way ANOVA test are shown as well as if a gene contributes (C) or significantly contributes (SC) to the variation between tissues based on the principal component analysis (PCA).

cDNA	Transformation	Shapiro-Wilk	Bartlett's	ANOVA	PCA
PtdSnRK1.1	log			0.1983	
PtdSnRK1.2	none			0.0188	
PtdSnRK1.3	log			0.0343	
PtdAKINβ1.1	log			0.0598	
PtdAKINβ1.2	none			0.0002	
PtdAKINβ2.1	log			0.0001	
PtdAKINβ2.2	log			<0.0001	
PtdAKINβ3.1	none			0.0172	
PtdAKINβ3.2	log	0.0195		0.3761	
PtdAKINβ4.1	none		0.01	0.0004	C
PtdAKINγ1.1	none		0.04	0.0001	C
PtdAKINγ1.2	log			0.0001	
PtdAKINγ2.1	log			0.0001	
PtdAKINγ2.2	none			0.0001	
PtdAKINγ2.3	log	0.0030		0.0001	SC
PtdAKINγ2.4	log			0.1020	
PtdAKINγ2.5	log	0.0260		<0.0001	
PtdAKINγ2.6	none			<0.0001	

Table 3.2. Summary of statistical analyses of expression profiles of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in various tissues during differential nitrogen availability. The ratio of the gene of interest and *EF1α-1* was transformed (T) as noted in order to produce the largest p values for the Shapiro-Wilk test of normality (S) and Bartlett's test for homogeneity of variance. P values for Bartlett's test were all >0.05. P values below 0.05 for the Shapiro-Wilk test of normality are shown. The first p value listed for an entry is for the overall two-way ANOVA test. The following three p-values listed are the p-values testing the null hypothesis that the predictor does not explain a significant portion of the variance and are for the days of treatment, the amount of nitrogen applied, and the interaction between these two variables. It is also shown if a gene contributes (C) or significantly contributes (SC) to the variation between low an d high nitrogen availability across time based on the principal component analysis (PCA).

cDNA	Young Leaves				Secondary Phloem				Secondary Xylem				Roots			
	T	S	ANOVA	PCA	T	S	ANOVA	PCA	T	S	ANOVA	PCA	T	S	ANOVA	PCA
PtdSnRK1.1	none		<0.0001 0.0510 <0.0001 0.0742		log	0.0012	0.5147 0.9756 0.0233 0.6602		log	0.0133	0.0078 0.3600 0.0293 0.1005		log		<0.0001 0.0299 <0.0001 0.1027	
PtdSnRK1.2	none		<0.0001 0.0005 <0.0001 0.0146		square root	0.0001	0.9807 0.9324 0.1286 0.6992		log		0.4124 0.7468 0.0385 0.2939		log		0.0164 0.0168 0.0044 0.8983	
PtdAKINβ1.1	log		<0.0001 0.0112 0.0326 0.0001	SC	none		0.0008 0.3730 <0.0001 0.3760	C	square root	0.0105	<0.0001 0.0271 <0.0001 0.2199	C	log	0.0346	<0.0001 0.1585 <0.0001 0.0007	SC
PtdAKINβ1.2	none	0.0046	<0.0001 0.0008 <0.0001 0.0028		log		<0.0001 0.5421 <0.0001 0.0660		log	0.0016	0.0220 0.5316 0.0008 0.3548		log	0.0454	0.0322 0.9032 0.0004 0.4004	
PtdAKINβ2.1	square root	0.0162	<0.0001 0.0116		log		<0.0001 0.1449 <0.0001		log	0.0063	0.0005 0.5636 <0.0001		log		<0.0001 0.5257 <0.0001	

			<0.0001 0.4848				0.3071				0.5852				0.2059	
PtdAKINβ2.2	none	0.0234	<0.0001 0.2747 <0.0001 0.1087		log	0.0008	<0.0001 0.1942 <0.0001 0.4085		log	0.0294	<0.0001 0.7061 <0.0001 0.0891		log	0.0104	<0.0001 0.3429 <0.0001 0.2263	
PtdAKINβ3.1	square root		<0.0001 0.0003 <0.0001 0.5554		none		<0.0001 0.8824 <0.0001 0.0805		log	0.0308	0.0061 0.8408 0.0001 0.1839		none		<0.0001 0.2614 <0.0001 0.0043	
PtdAKINβ3.2	log		<0.0001 0.0149 <0.0001 0.0333		log		0.0081 0.8197 <0.0001 0.6722		none		0.0001 0.6399 <0.0001 0.0989		log		<0.0001 0.4708 <0.0001 0.0610	
PtdAKINβ4.1	none	0.0062	<0.0001 0.0025 <0.0001 0.0422		none		<0.0001 0.2080 <0.0001 0.1264		none	0.0000	<0.0001 0.8661 <0.0001 0.5712		log	0.0001	<0.0001 0.1847 <0.0001 0.0037	
PtdAKINγ1.1	log	0.0007	<0.0001 0.0002 0.0005 <0.0001	SC	log	0.0401	0.3291 0.4335 0.3178 0.1847	SC	log		<0.0001 0.1642 <0.0001 0.0534	SC	log		<0.0001 0.3883 <0.0001 0.0009	SC
PtdAKINγ1.2	log	0.0029	<0.0001 0.0029 0.0005 0.0019		log		<0.0001 0.9861 <0.0001 0.2390		square root	0.0493	<0.0001 0.6447 <0.0001 0.2280		log	<0.0001	<0.0001 0.1982 <0.0001 0.0001	C
PtdAKINγ1.4					none		0.0105 0.0091 0.7912 0.0188		none		0.0015 0.0047 0.0263 0.0590					
PtdAKINγ2.1	log	0.0322	<0.0001 0.3264 <0.0001		square root	0.0473	<0.0001 0.2712 <0.0001		none		0.1408 0.4888 0.0095		log		0.0002 0.9492 <0.0001	

			0.1451				0.0371			0.7075			0.0239
PtdAKINγ2.2	none		0.0149 0.0021 0.2209 0.3507		none		0.0108 0.0209 0.2182 0.0341				log		0.3619 0.9387 0.0741 0.1340
PtdAKINγ2.3	log		0.0041 0.0037 0.3544 0.0425	SC	log		<0.0001 0.1604 0.0145 <0.0001	none		<0.0001 0.3560 0.0005 <0.0001	log		0.0494 0.2052 0.2102 0.0305
PtdAKINγ2.4	log		0.3440 0.1965 0.8759 0.3530		log		0.0006 0.8707 0.0011 0.0019	none		0.0980 0.1696 0.1650 0.3659	log	<0.0001	0.4015 0.1803 0.7205 0.4422
PtdAKINγ2.5	square root		<0.0001 0.5566 <0.0001 0.2198		log	0.0039	0.0001 0.9969 <0.0001 0.9568	log		0.0061 0.2925 <0.0001 0.9127	log		0.0002 0.1963 <0.0001 0.9572
PtdAKINγ2.6	none	0.0474	<0.0001 0.0138 <0.0001 0.0018		log	0.0044	0.0039 0.5429 <0.0001 0.8265	none		<0.0001 0.2617 <0.0001 0.1479	log	0.0004	0.2249 0.1864 0.0586 0.9142

Table 3.3. Summary of statistical analyses of expression profiles of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in shoot tips, mature leaves and roots during dormancy acquisition.The ratio of the gene of interest and VHA-A was transformed (T) as noted in order to produce the largest p values for the Shapiro-Wilk test of normality (S) and Bartlett's test for homogeneity of variance (B). P values below 0.05 for the Shapiro-Wilk test and Bartlett's test are shown. P values of the one-way ANOVA test are shown as well as if a gene contributes (C) or significantly contributes (SC) to the variation between low and high nitrogen availability across time based on the principal component analysis (PCA).

cDNA	Shoot Tips					Mature Leaves					Roots				
	T	S	B	ANOVA	PCA	T	S	B	ANOVA	PCA	T	S	B	ANOVA	PCA
PtdSnRK1.1	none	0.0090		0.9293		none			0.6458		none			0.0098	
PtdSnRK1.2	log		0.0270	<0.0001		none			0.2876		none			0.4757	
PtdAKINβ1.1	log			0.3520		log			0.0094	C	none			0.0742	C
PtdAKINβ1.2	log			<0.0001		none			0.1935		none			0.1765	
PtdAKINβ2.1	log			0.0628		none			0.3382		none			0.0527	
PtdAKINβ2.2	none			0.6697		square root			0.4885		none			0.0213	
PtdAKINβ3.1	none			<0.0001		log			0.3701		none			0.0236	
PtdAKINβ3.2	none			0.0007		log			0.0672		log			0.3336	
PtdAKINβ4.1	log			0.0001		log			0.4793		none			0.0042	
PtdAKINγ1.1	log			<0.0001	SC	log			0.0518	C	none			0.0890	SC
PtdAKINγ1.2	log			0.0045		log			0.9944		log			0.0067	
PtdAKINγ1.4	none			0.0003							log			0.0012	

PtdAKINγ2.1	log	0.0183		none	0.4776						
PtdAKINγ2.2	none	0.3243					log			0.2646	
PtdAKINγ2.3	log	<0.0001	C	log	0.2417	SC	log			<0.0001	C
PtdAKINγ2.4	none	0.0250		log	0.0085		log	0.0001		0.0319	
PtdAKINγ2.5	none	0.8854		none	0.0323		log	0.0043		0.3692	
PtdAKINγ2.6	none	0.7441		none	0.0004		log		0.0360	0.7810	

Table 3.4. Summary of statistical analyses of expression profiles of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in secondary phloem and secondary xylem during dormancy acquisition. The ratio of the gene of interest and VHA-A was transformed (T) as noted in order to produce the largest p values for the Shapiro-Wilk test of normality (S) and Bartlett's test for homogeneity of variance. P values for Bartlett's test were all >0.05. P values below 0.05 for the Shapiro-Wilk test are shown. P values of the one-way ANOVA test are shown as well as if a gene contributes (C) or significantly contributes (SC) to the variation between low and high nitrogen availability across time based on the principal component analysis (PCA).

cDNA	Secondary Phloem				Secondary Xylem			
	T	S	ANOVA	PCA	T	S	ANOVA	PCA
PtdSnRK1.1	log		<0.0001		square root	0.0443	<0.0001	
PtdSnRK1.2	log		<0.0001		log		<0.0001	
PtdAKINβ1.1	log		<0.0001	C	log		<0.0001	
PtdAKINβ1.2	log		<0.0001		square root		<0.0001	
PtdAKINβ2.1	log		<0.0001		log		<0.0001	
PtdAKINβ2.2	log		<0.0001		log		<0.0001	
PtdAKINβ3.1	log		<0.0001		log		<0.0001	
PtdAKINβ3.2	log		<0.0001		square root	0.0472	<0.0001	
PtdAKINβ4.1	log		<0.0001		log		<0.0001	
PtdAKINγ1.1	log	0.0499	<0.0001	SC	log		<0.0001	SC
PtdAKINγ1.2	log		0.0030					
PtdAKINγ1.4	none		0.1037		none		0.0008	

PtdAKINγ2.1	log		<0.0001		log		0.1845	
PtdAKINγ2.2	square root		0.01					
PtdAKINγ2.3	log	0.0390	<0.0001	C	log		<0.0001	C
PtdAKINγ2.4	none		0.1159					
PtdAKINγ2.5	log		<0.0001		none		<0.0001	
PtdAKINγ2.6	log		<0.0001		square root	0.0092	<0.0001	C

Table 3.5. Overall summary of PCA and ANOVA results for *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene family members identified by PCA as contributors to the variation between different tissues, nitrogen treatments or days of dormancy acquisition explained by principal components. In the tissue survey experiment, the tissues surveyed from poplar were shoot tips, young leaves, mature leaves, old leaves, secondary phloem, secondary xylem and roots. In the nitrogen availability experiment, poplars were fertilized with high or low nitrogen for up to 14 days. In the dormancy acquisition experiment, poplars were grown in short day conditions for up to eight weeks. Overall ANOVA p values are denoted with * ($p<0.05$) or ** ($p<0.005$). SC = significant contributor to the variation of the principal component; C = contributor to the principal component.

Experiment	Tissue	*PtdAKINβ1.1*	*PtdAKINγ1.1*	*PtdAKINγ2.3*	Other
tissue survey	all			SC**	
nitrogen availability	young leaves	SC**	SC**	SC**	
	secondary phloem	C**	SC		
	secondary xylem	C**	SC**		
	roots	SC**	SC**		*PtdAKINγ1.2* C**
dormancy acquisition	shoot tips		SC**	C**	
	mature leaves	C*	C	SC*	
	secondary phloem	C**	SC**	C**	
	secondary xylem		SC**	C**	*PtdAKINγ2.6* C**
	roots	C	SC	C**	

3.7. Figures

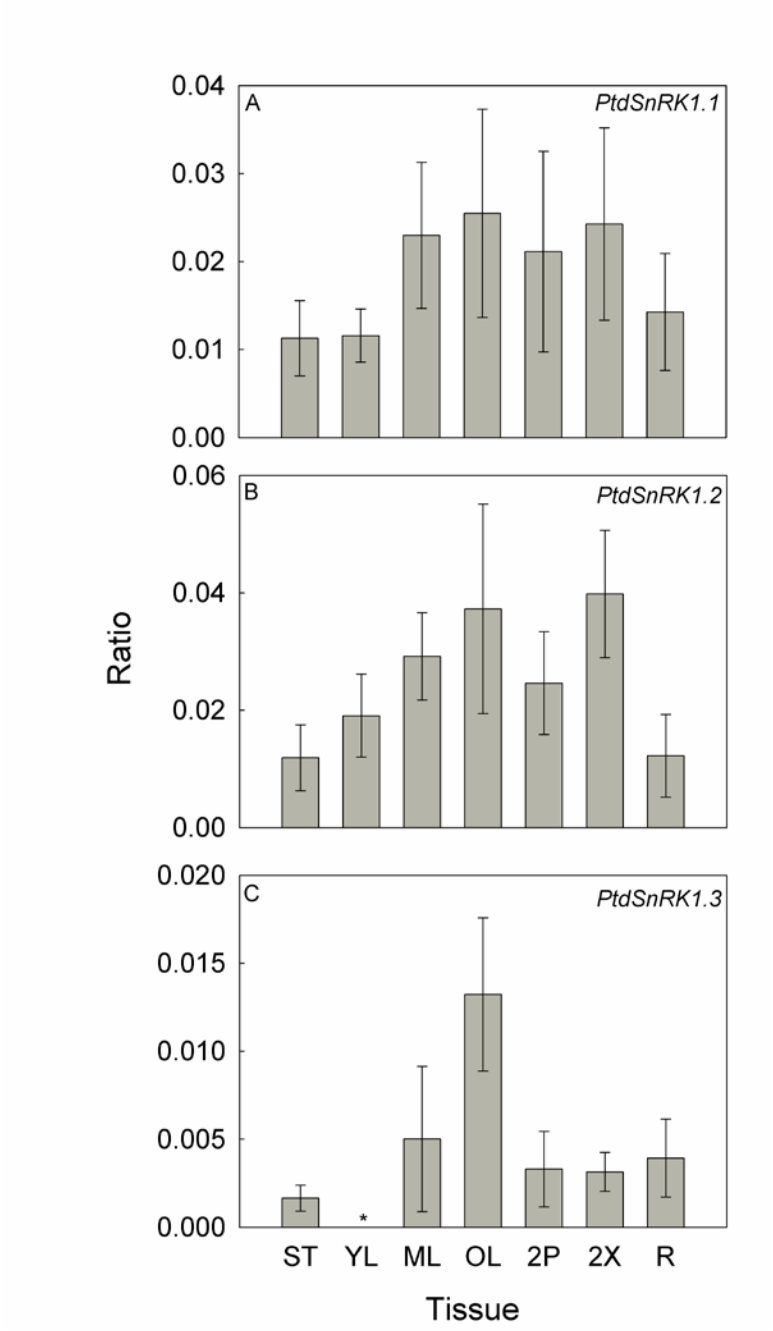

Figure 3.1. Expression of *PtdSnRK1* gene family members in different tissues of poplar. The ratio of the expression of *PtdSnRK1* to *EF1α-3* is shown in shoot tip (ST), young foliage (YF), mature foliage (MF), old foliage (OF), secondary phloem (2P), secondary xylem (2X) and roots (R). Expression below the detectable limit is denoted with *. Error bars show standard deviation. N = 3

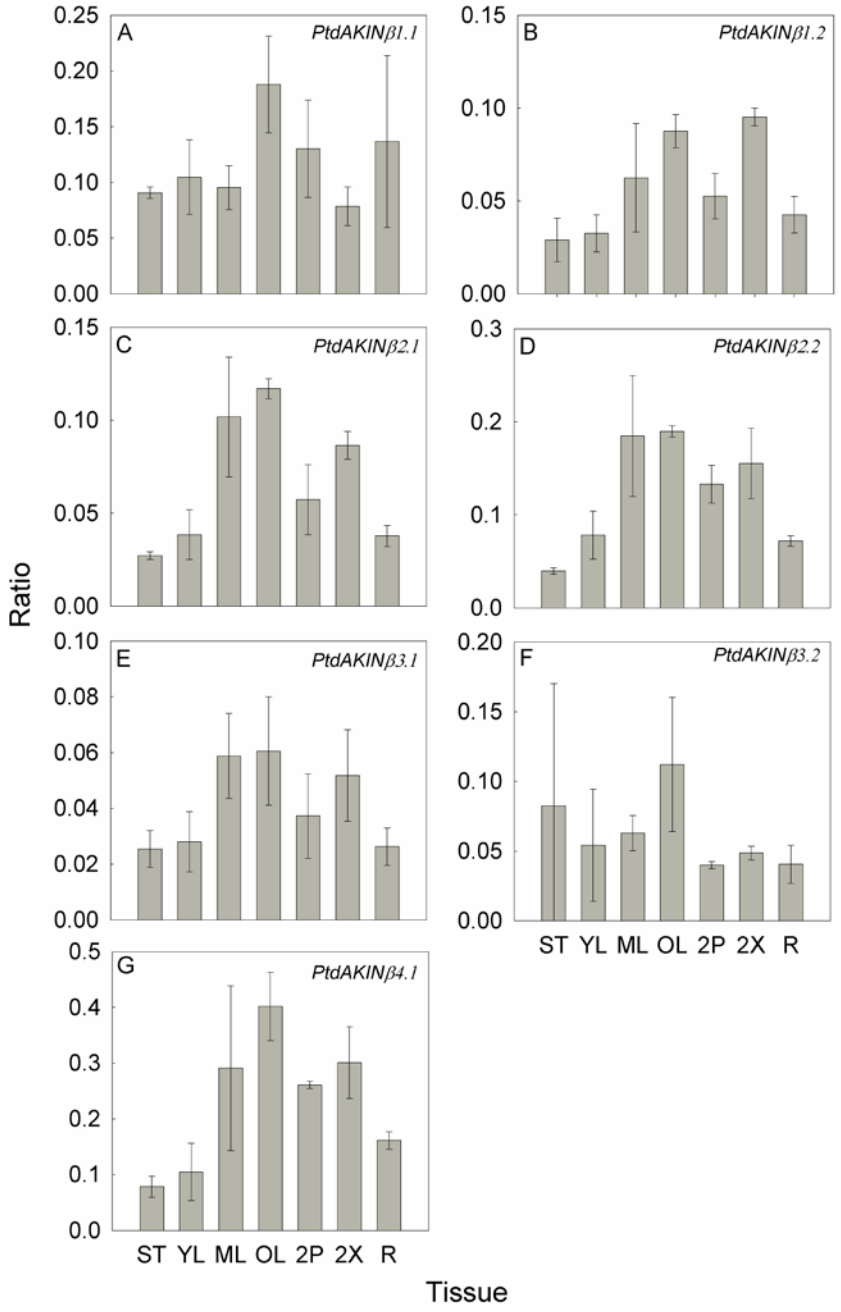

Figure 3.2. Expression of *PtdAKINβ* gene family members in different tissues of poplar. The ratio of the expression of *PtdAKINβ* to *EF1α-3* is shown in shoot tip (ST), young foliage (YF), mature foliage (MF), old foliage (OF), secondary phloem (2P), secondary xylem (2X) and roots (R). Error bars show standard deviation. Data was obtained from Fedosejevs (2008). N = 3

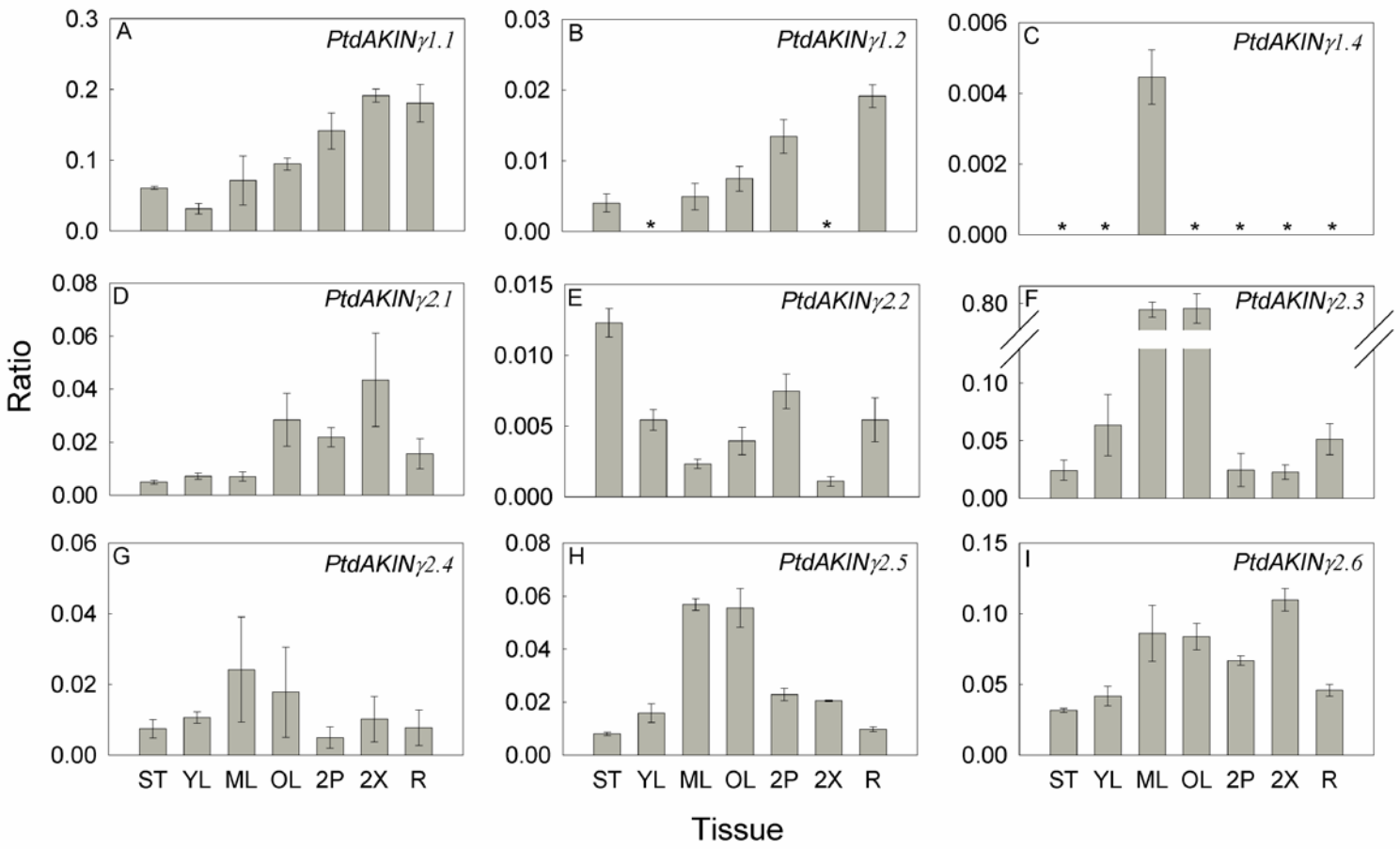

Figure 3.3. Expression of *PtdAKINγ* gene family members in different tissues of poplar. The ratio of the expression of *PtdAKINγ* to *EF1α-3* is shown in shoot tip (ST), young foliage (YF), mature foliage (MF), old foliage (OF), secondary phloem (2P), secondary xylem (2X) and roots (R). Expression below the detectable limit is denoted with *. Error bars show standard deviation. N = 3

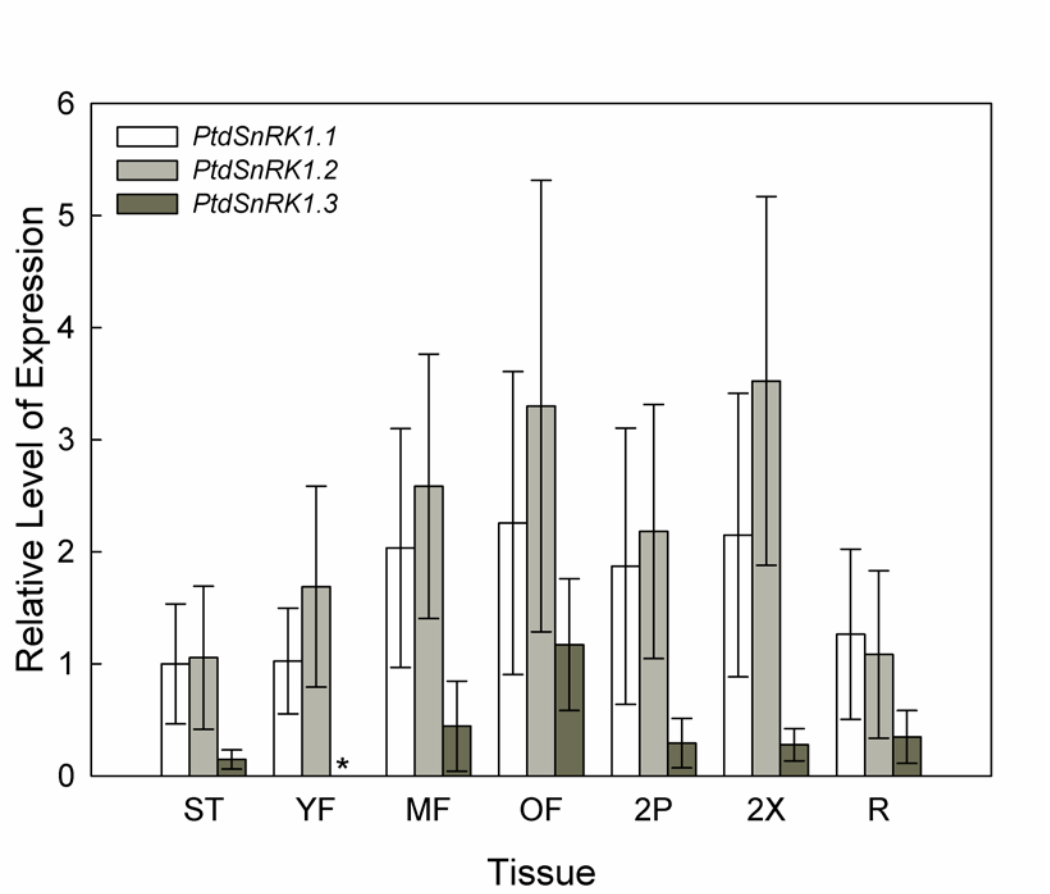

Figure 3.4. Relative level of expression of members of the *PtdSnRK1* gene family. The relative level of expression was calculated by setting the expression of *PtdSnRK1.1* in shoot tips to 1 and normalizing the expression of other members of the *PtdSnRK1* gene family to *PtdSnRK1.1* in shoot tips. Relative level of expression is shown in shoot tip (ST), young foliage (YF), mature foliage (MF), old foliage (OF), secondary phloem (2P), secondary xylem (2X) and roots (R). Expression below the detectable limit is denoted with *. Error bars show standard deviation.

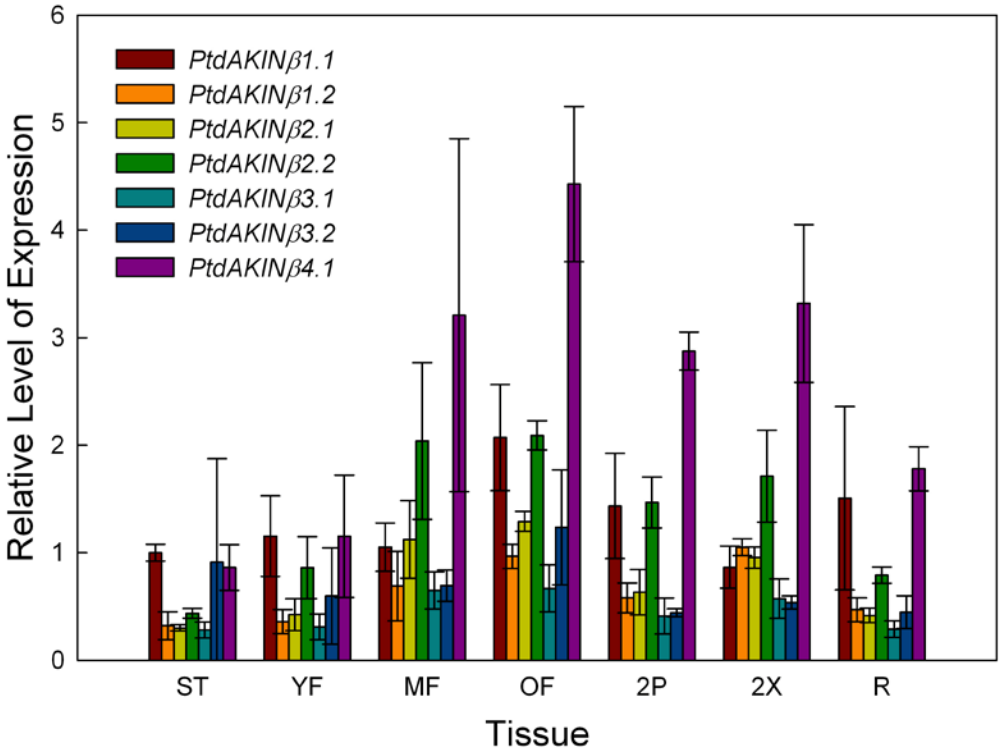

Figure 3.5. Relative level of expression of members of the *PtdAKINβ* gene family. The relative level of expression was calculated by setting the expression of *PtdAKINβ 1.1* in shoot tips to 1 and normalizing the expression of other members of the *PtdAKINβ* gene family to *PtdAKINβ* in shoot tips. Relative level of expression is shown in shoot tip (ST), young foliage (YF), mature foliage (MF), old foliage (OF), secondary phloem (2P), secondary xylem (2X) and roots (R). Error bars show standard deviation.

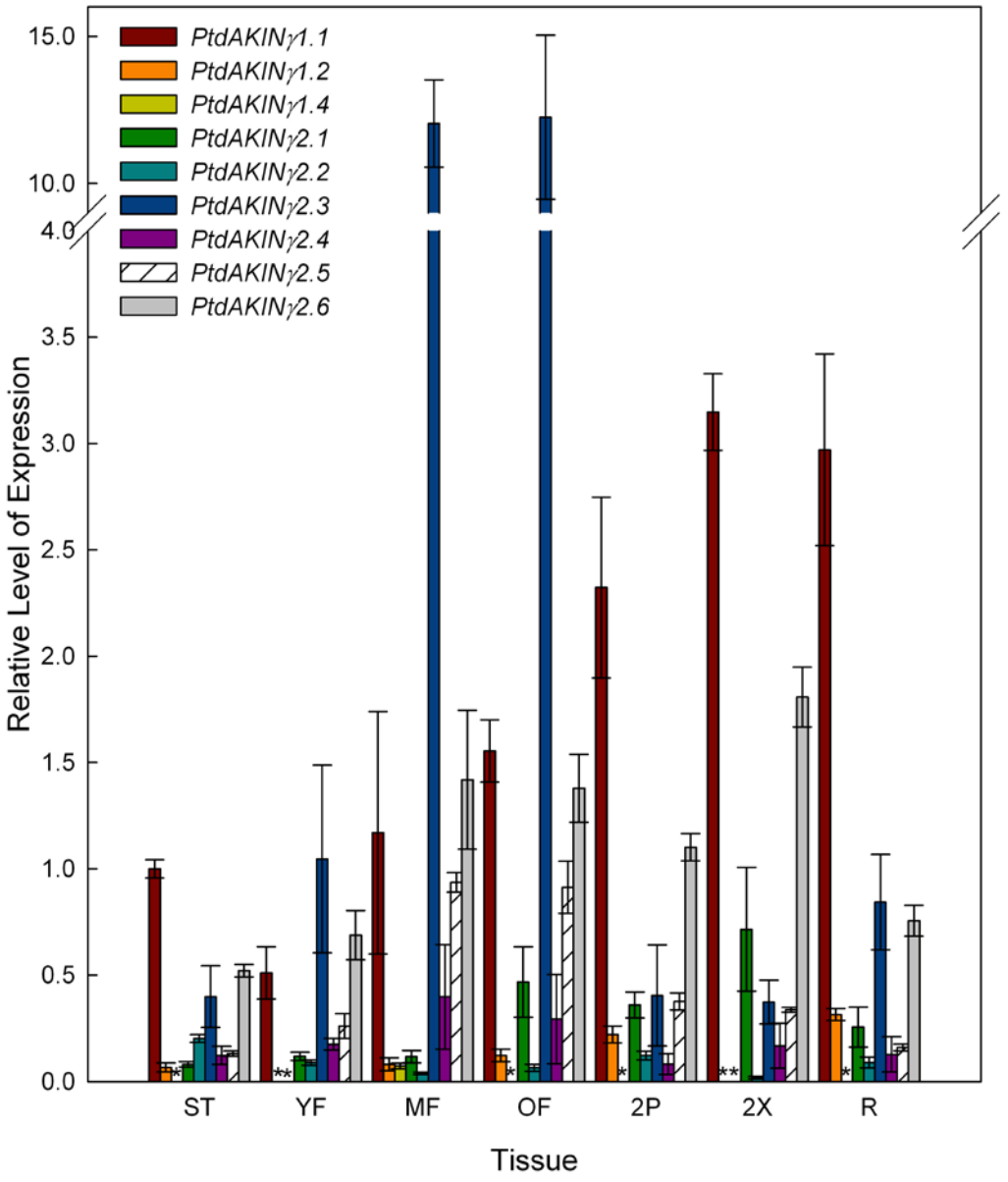

Figure 3.6. Relative level of expression of members of the *PtdAKINγ* gene family. The relative level of expression was calculated by setting the expression of *PtdAKINγ1.1* in shoot tips to 1 and normalizing the expression of other members of the *PtdAKINγ* gene family to *PtdAKINγ* in shoot tips. Relative level of expression is shown in shoot tip (ST), young foliage (YF), mature foliage (MF), old foliage (OF), secondary phloem (2P), secondary xylem (2X) and roots (R). Expression below the detectable limit is denoted with *. Error bars show standard deviation.

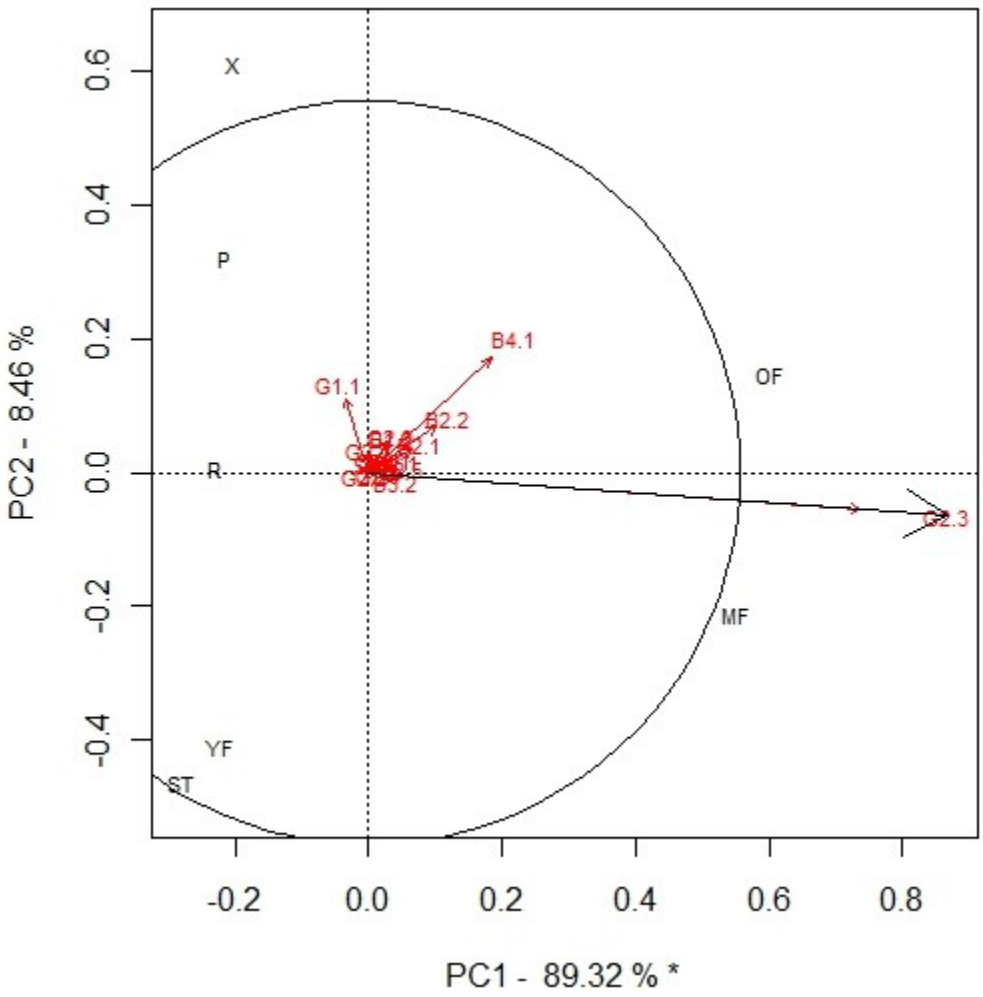

Figure 3.7. Principal component analysis of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in various tissues. Principal components which are significant based on the broken stick distribution are denoted with *. Magnitude of the contribution of a particular gene to the variation of the principle components is denoted by the length and direction of the arrow. Arrows which exceed the equilibrium the circle significantly contribute to the variation of the principal components. *PtdSnRK1.3*, *PtdAKINγ1.2* and *PtdAKINγ1.4* were not included as PCA does not accommodate null data entries. Members of the *PtdSnRK1* gene family are denoted with "S" followed by the gene member number. Members of the *PtdAKINβ* gene family are denoted with "B" followed by the gene member number. Members of the *PtdAKINγ* gene family are denoted with "G" followed by the gene member number. Tissues surveyed are shoot tip (ST), young foliage (YF), mature foliage (MF), old foliage (OF), secondary phloem (2P), secondary xylem (2X) and roots (R).

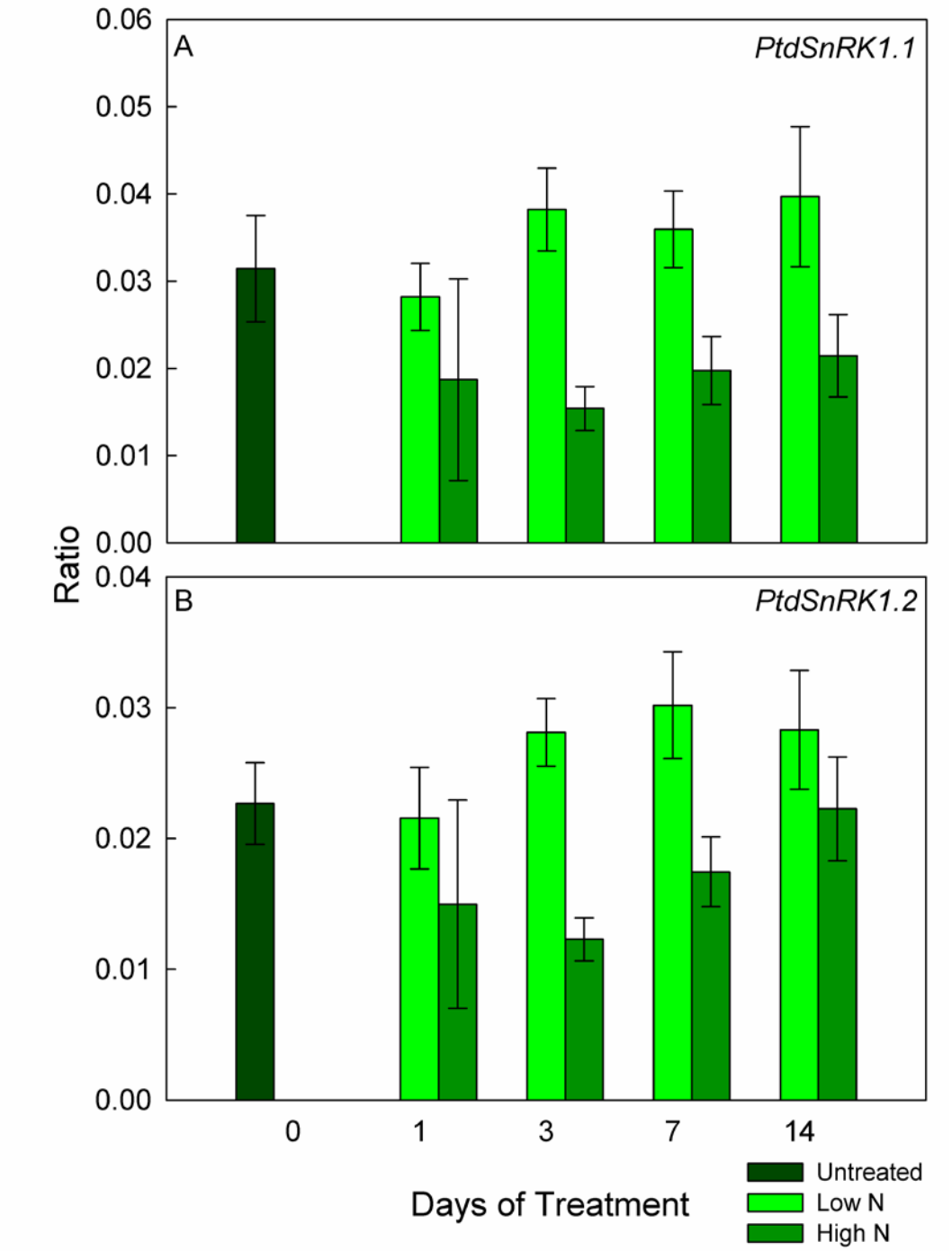

Figure 3.8. Expression profiles of *PtdSnRK1* family members in young leaves of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Expression is shown as the ratio of the expression of *PtdSnRK1* members to *EF1α-1*. Error bars show standard deviation. N=6

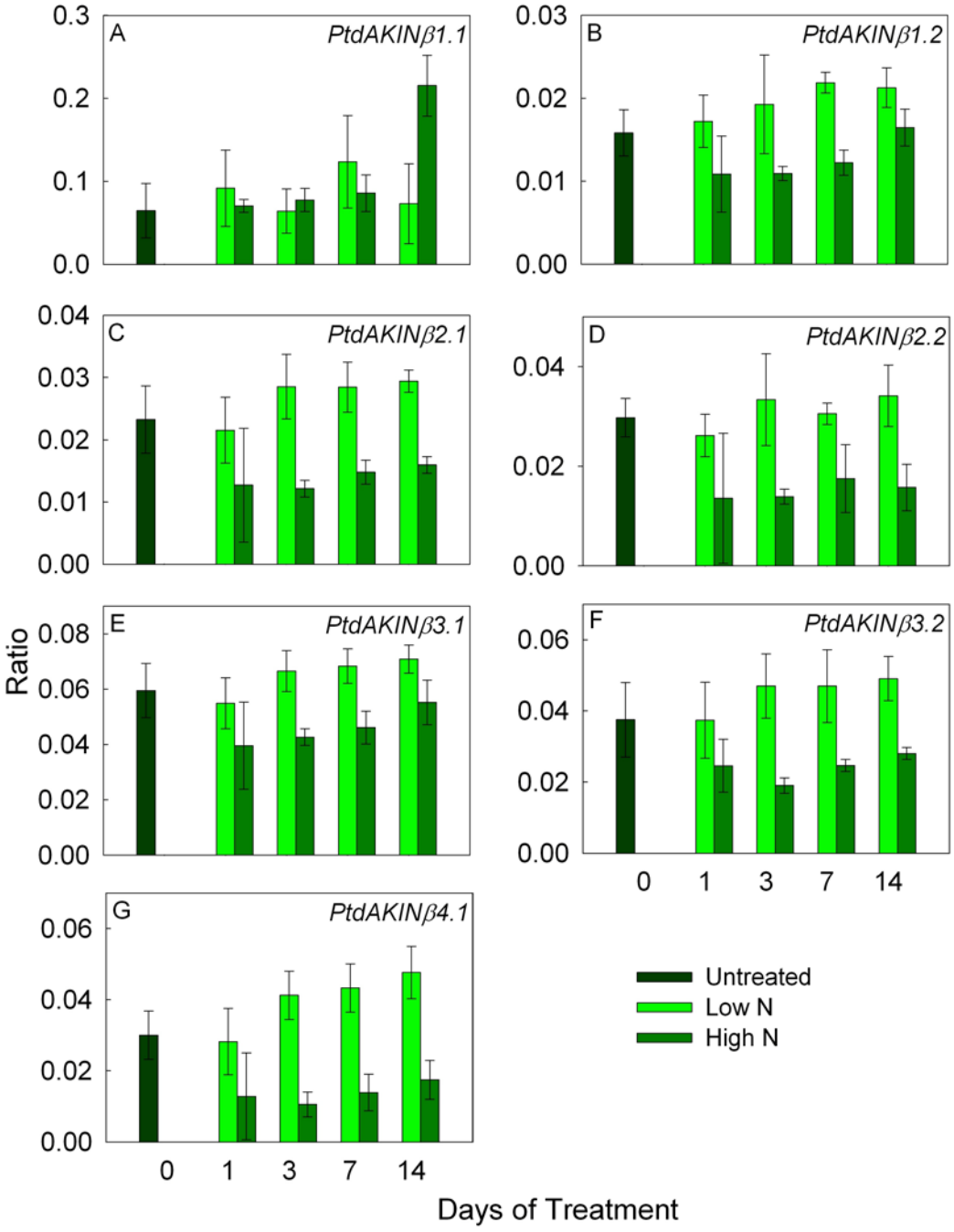

Figure 3.9. Expression profiles of *PtdAKINβ* family members in young leaves of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Expression is shown as the ratio of the expression of *PtdAKINβ* members to *EF1α-1*. Error bars show standard deviation. N=6

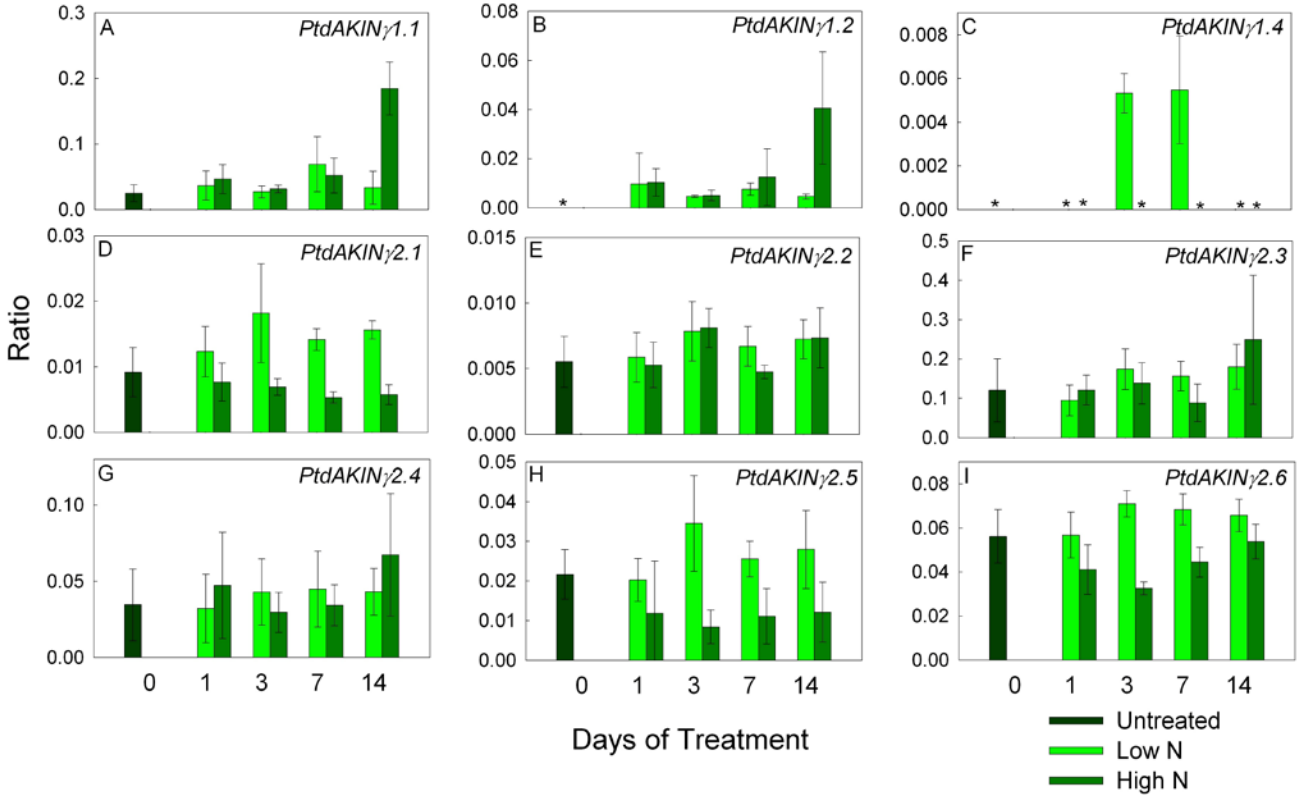

Figure 3.10. Expression profiles of *PtdAKINγ* family members in young leaves of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Expression is shown as the ratio of the expression of *PtdAKINγ* members to *EF1α-1*. Error bars show standard deviation. N=6

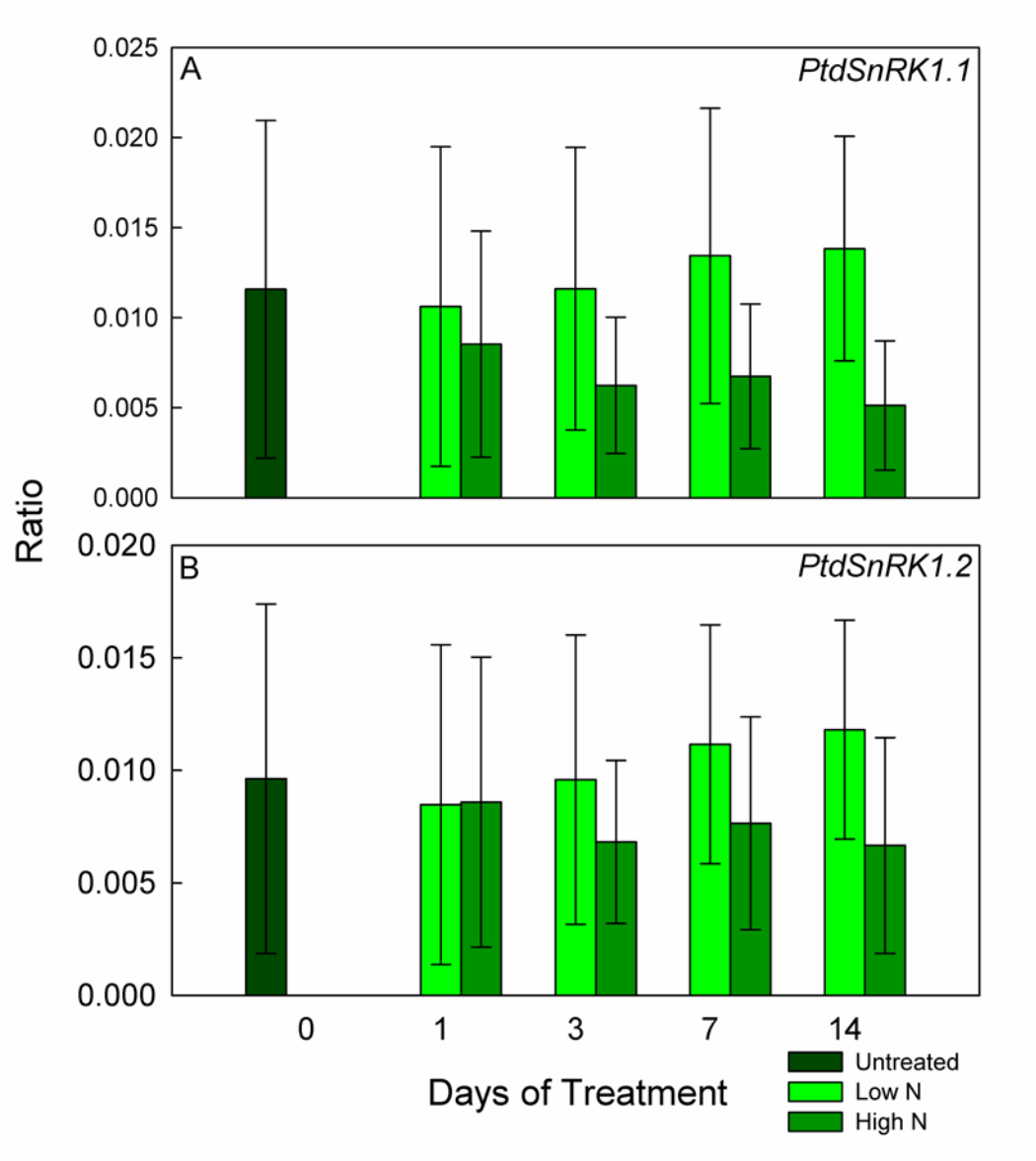

Figure 3.11. Expression profiles of *PtdSnRK1* family members in secondary phloem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Expression is shown as the ratio of the expression of *PtdSnRK1* members to *EF1α-1*. Error bars show standard deviation. N=6

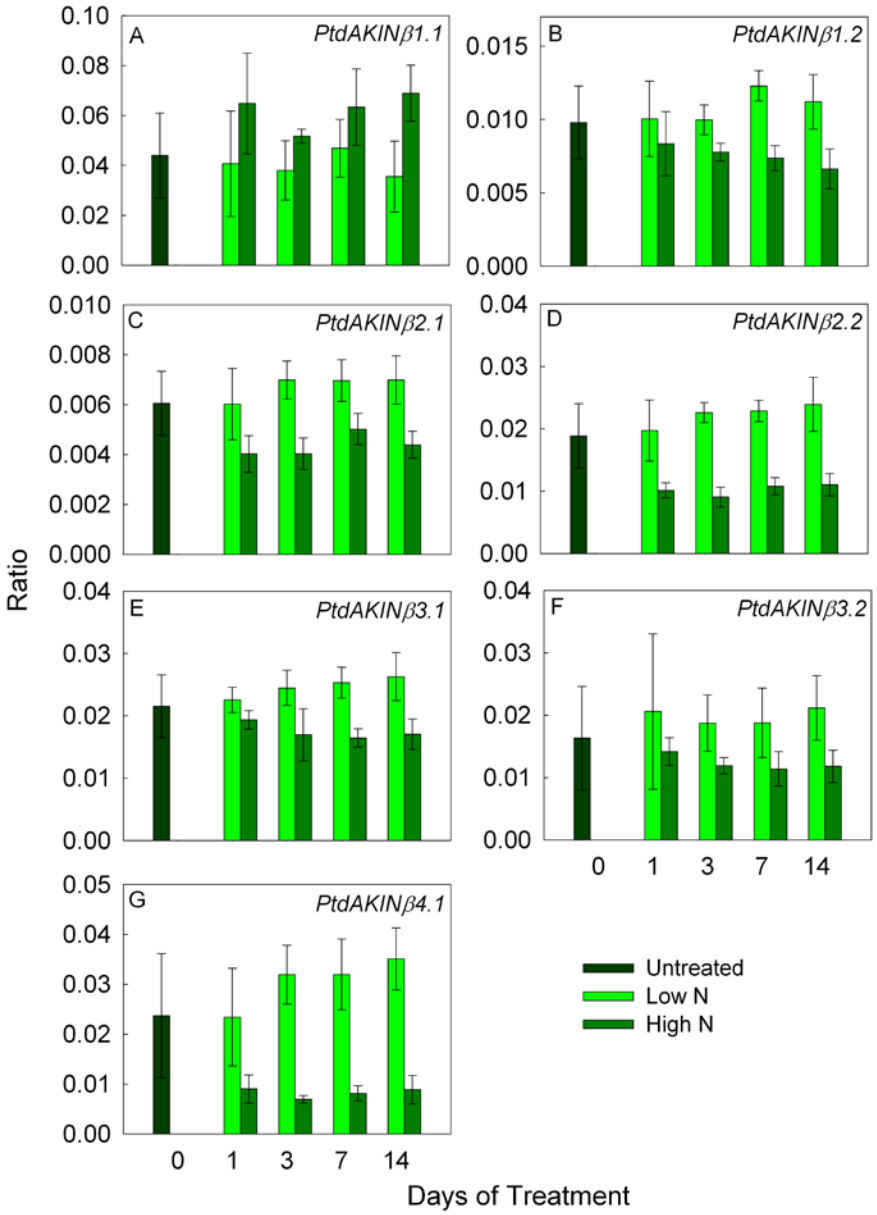

Figure 3.12. Expression profiles of *PtdAKINβ* family members in secondary phloem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Expression is shown as the ratio of the expression of *PtdAKINβ* members to *EF1α-1*. Error bars show standard deviation. N=6

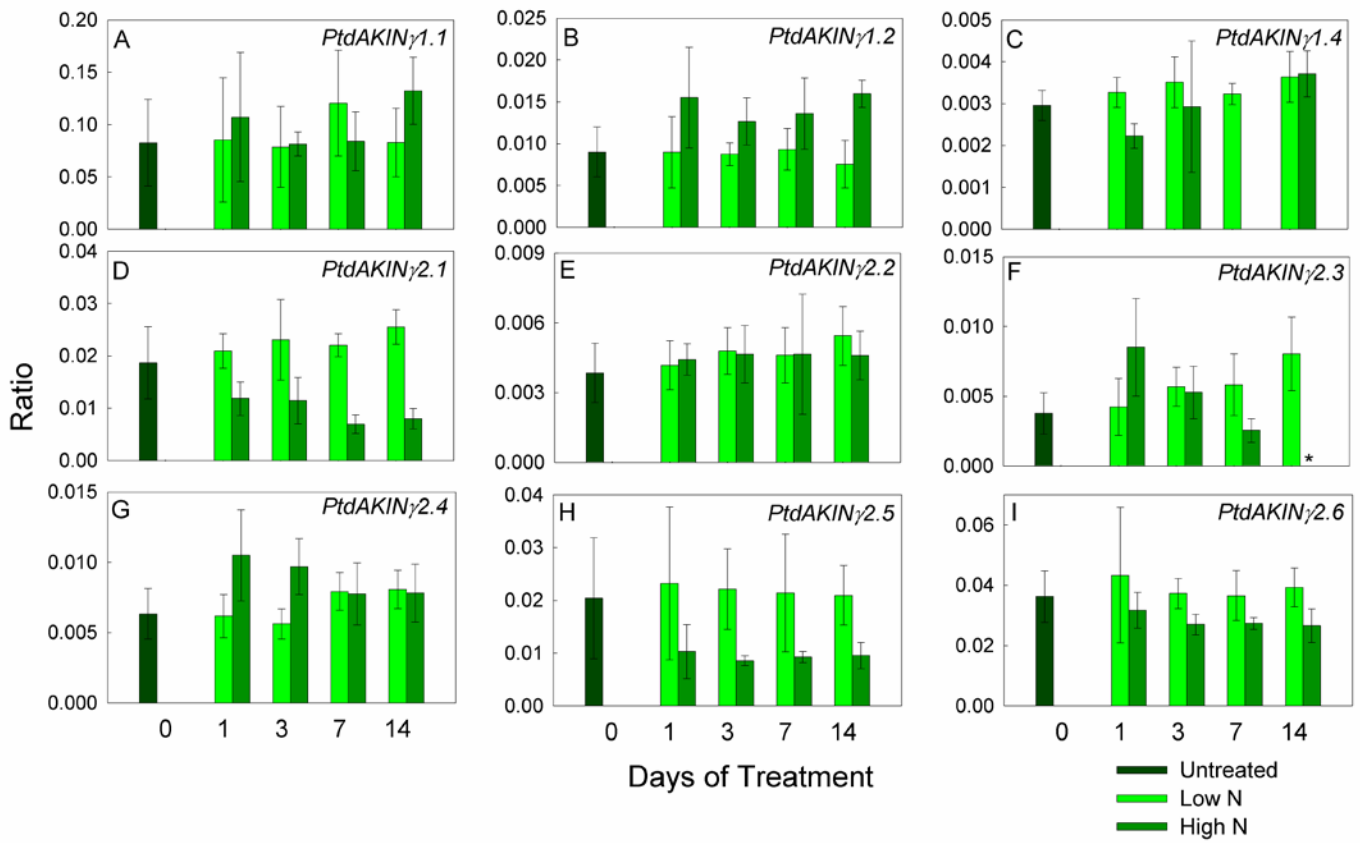

Figure 3.13. Expression profiles of *PtdAKINγ* family members in secondary phloem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Expression is shown as the ratio of the expression of *PtdAKINγ* members to *EF1α-1*. Error bars show standard deviation. N=6

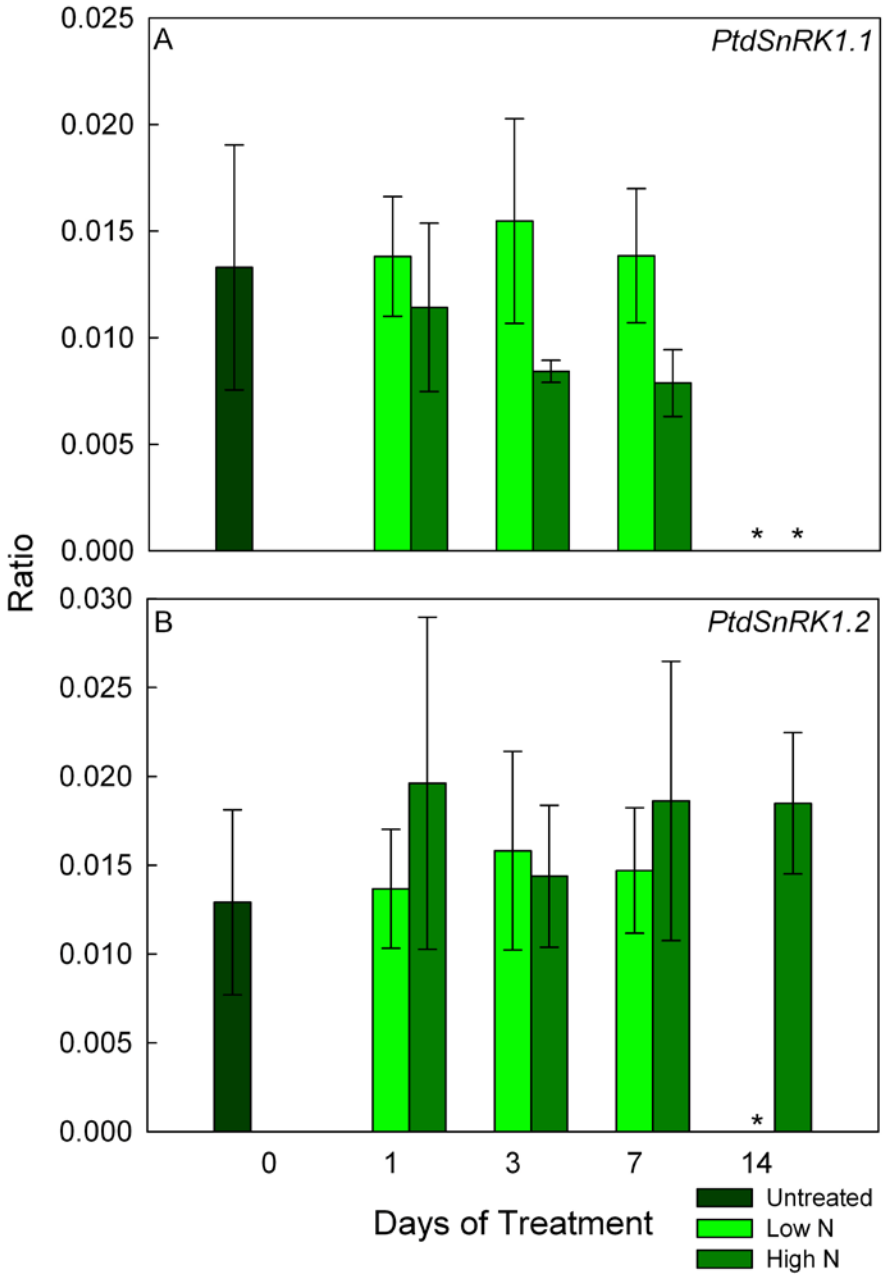

Figure 3.14. Expression profiles of *PtdSnRK1* family members in secondary xylem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Expression is shown as the ratio of the expression of *PtdSnRK1* members to *EF1α-1*. Error bars show standard deviation. N=6

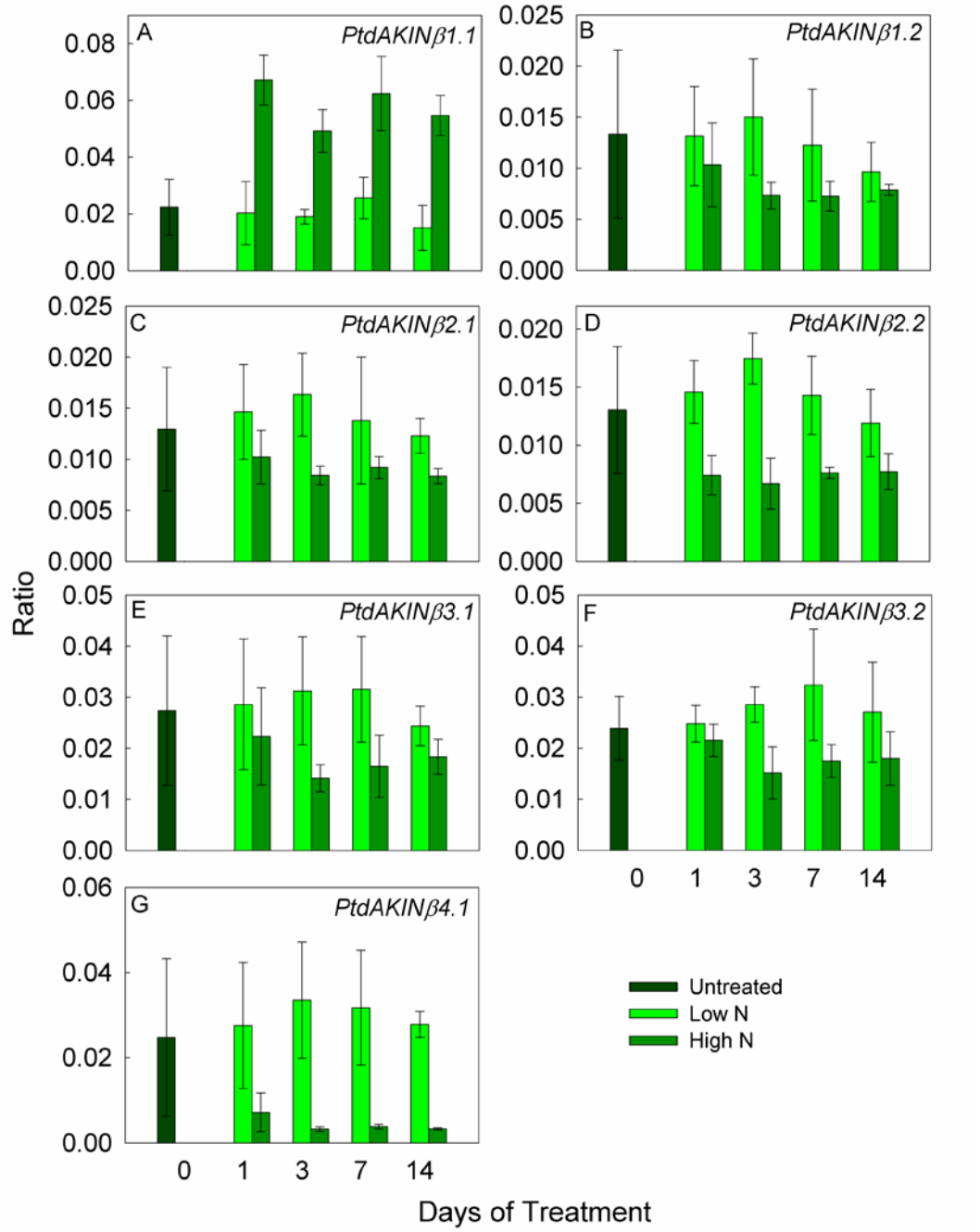

Figure 3.15. Expression profiles of *PtdAKINβ* family members in secondary xylem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Expression is shown as the ratio of the expression of *PtdAKINβ* members to *EF1α-1*. Error bars show standard deviation. N=6

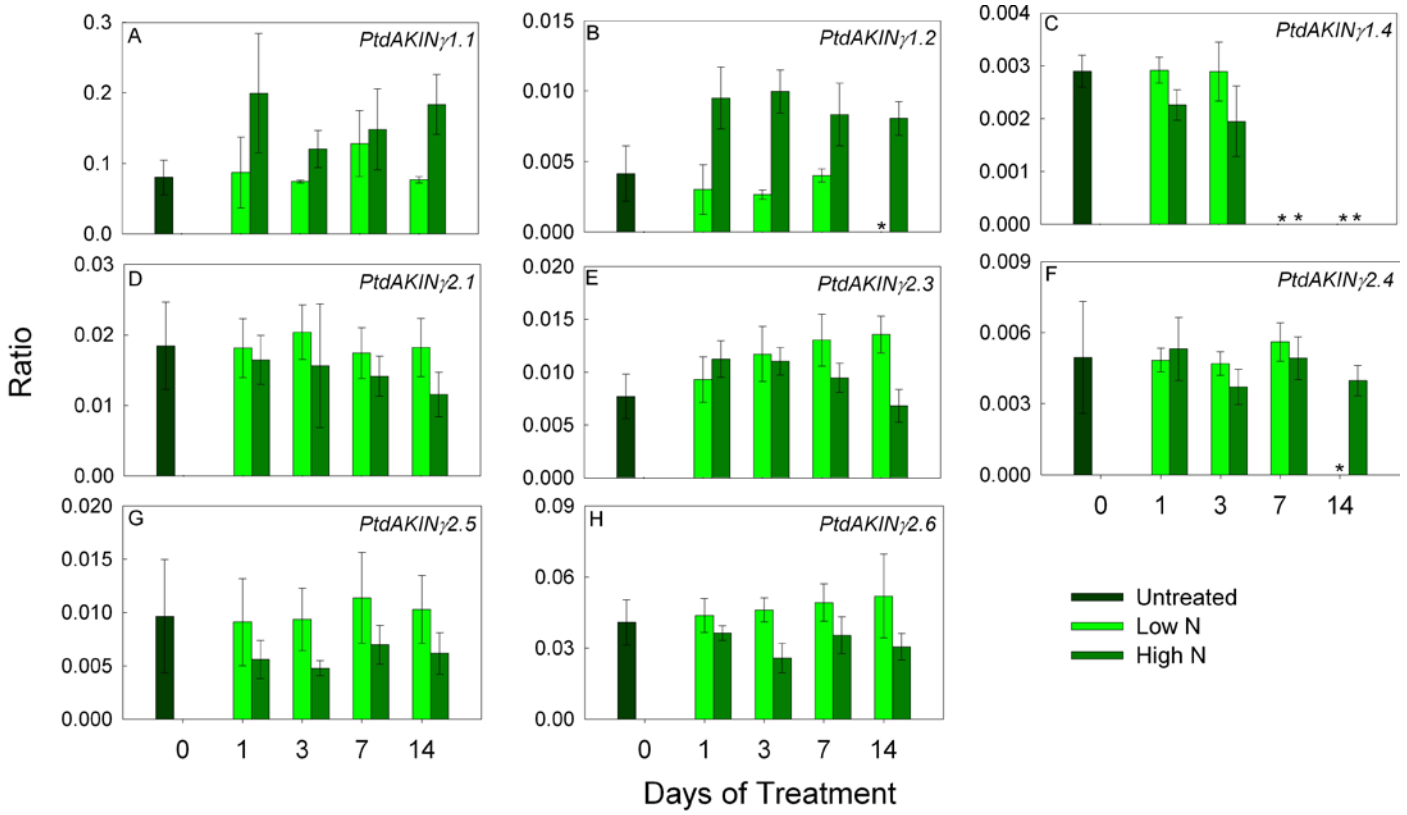

Figure 3.16. Expression profiles of *PtdAKINγ* family members in secondary xylem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Expression is shown as the ratio of the expression of *PtdAKINγ* members to *EF1α-1*. Error bars show standard deviation. N=6

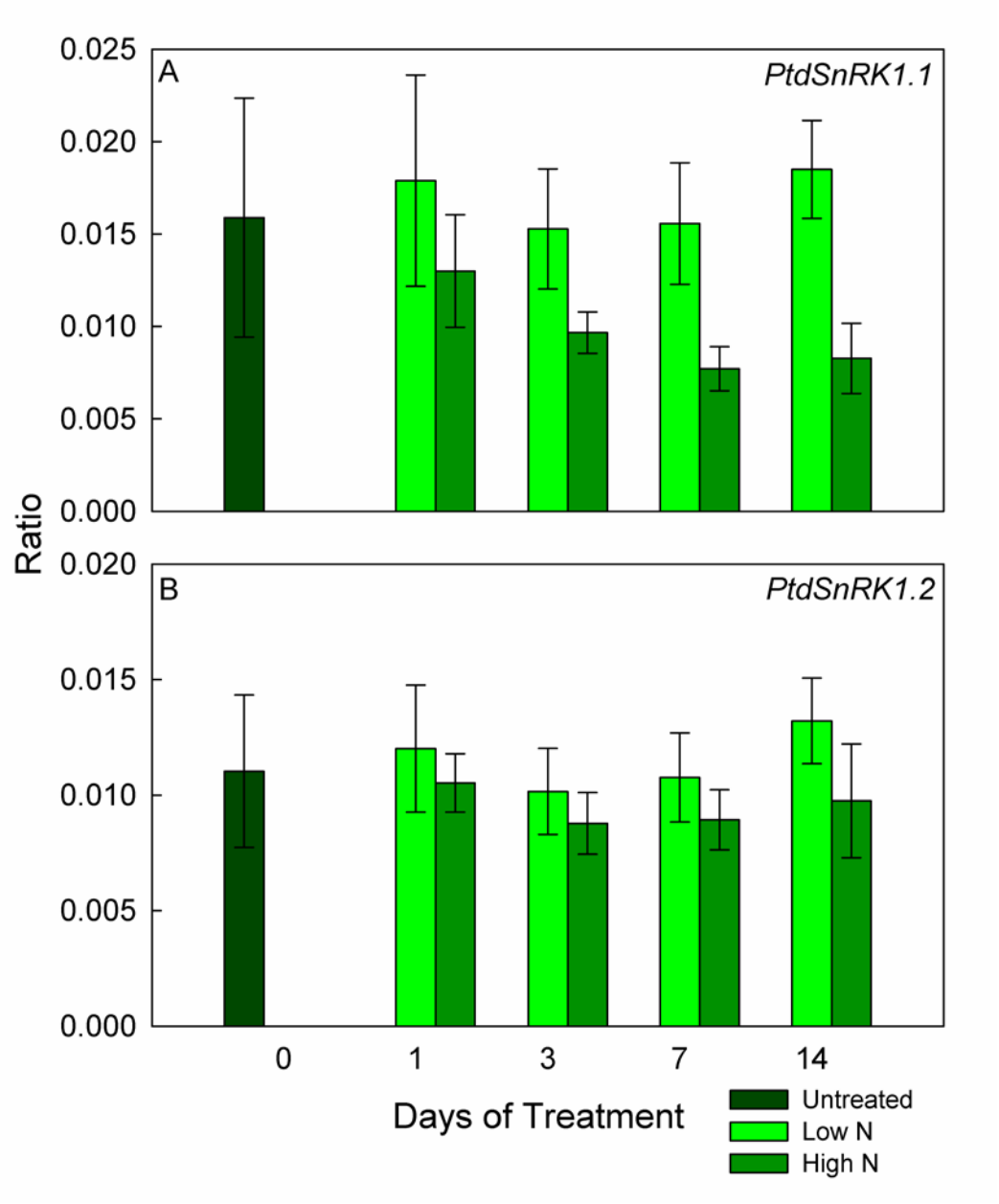

Figure 3.17. Expression profiles of *PtdSnRK1* family members in roots of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Expression is shown as the ratio of the expression of *PtdSnRK1* members to *EF1α-1*. Error bars show standard deviation. N=6

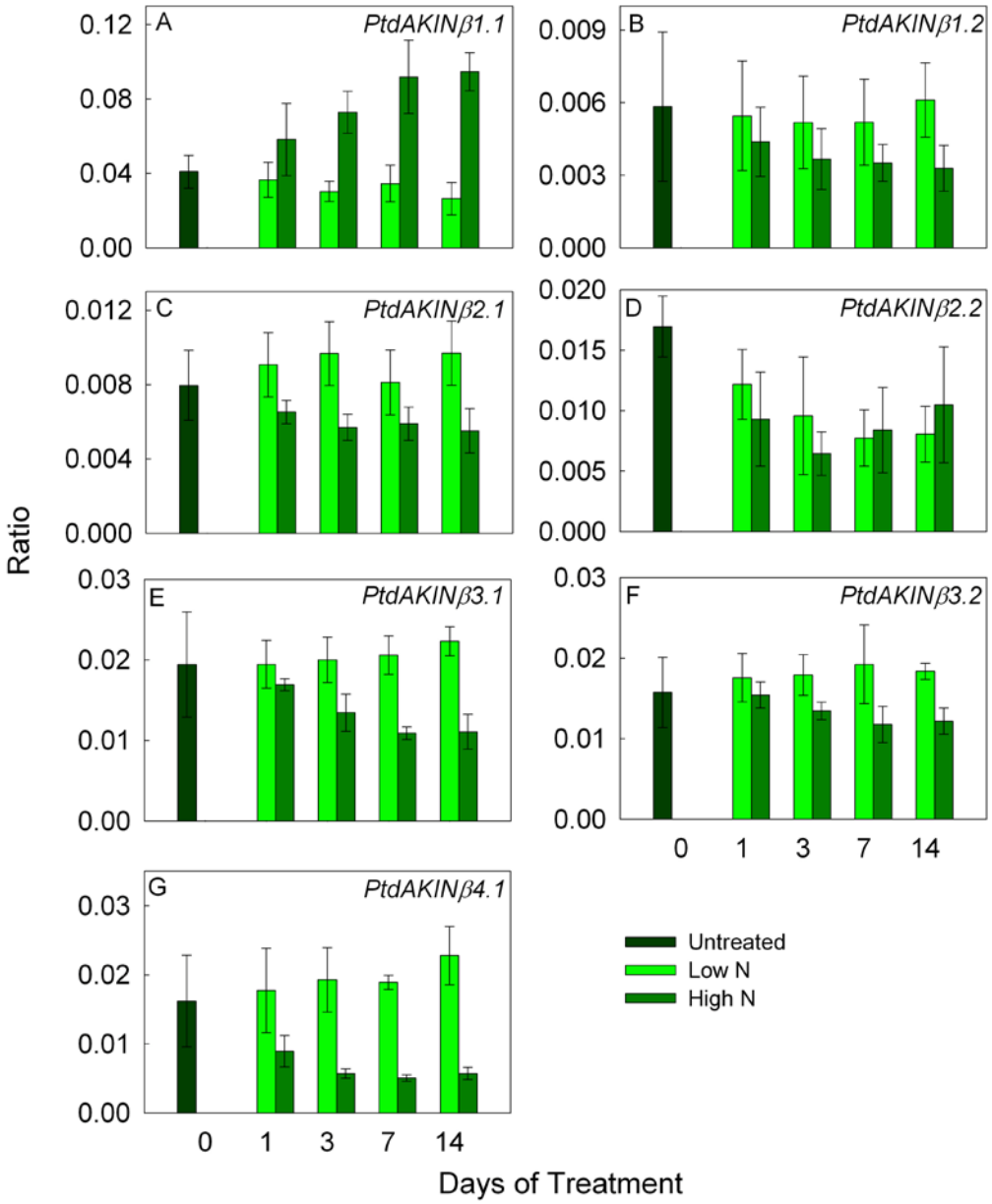

Figure 3.18. Expression profiles of *PtdAKINβ* family members in roots of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Expression is shown as the ratio of the expression of *PtdAKINβ* members to *EF1α-1*. Error bars show standard deviation. N=6

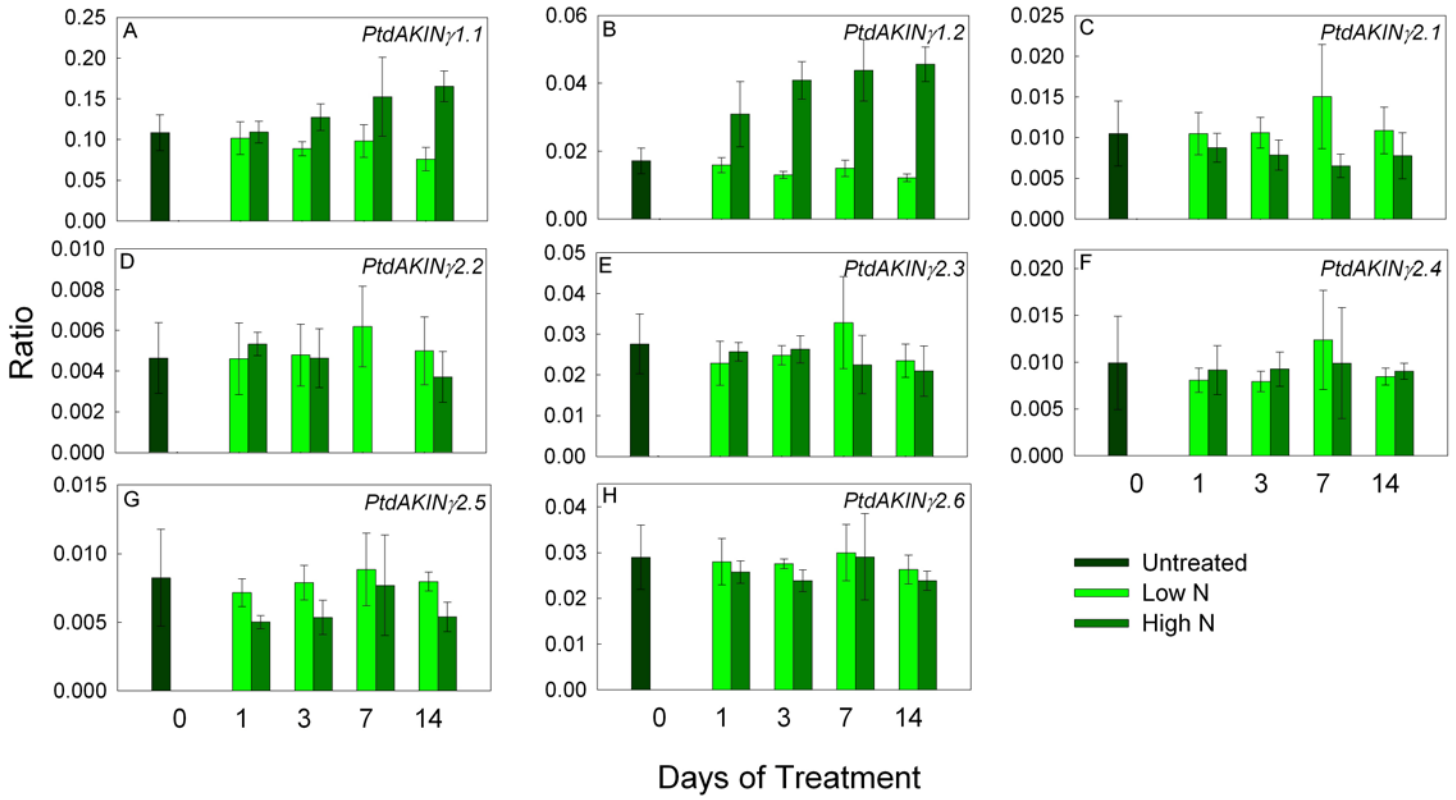

Figure 3.19. Expression profiles of *PtdAKINγ* family members in roots of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Expression is shown as the ratio of the expression of *PtdAKINγ* members to *EF1α-1*. Error bars show standard deviation. N=6

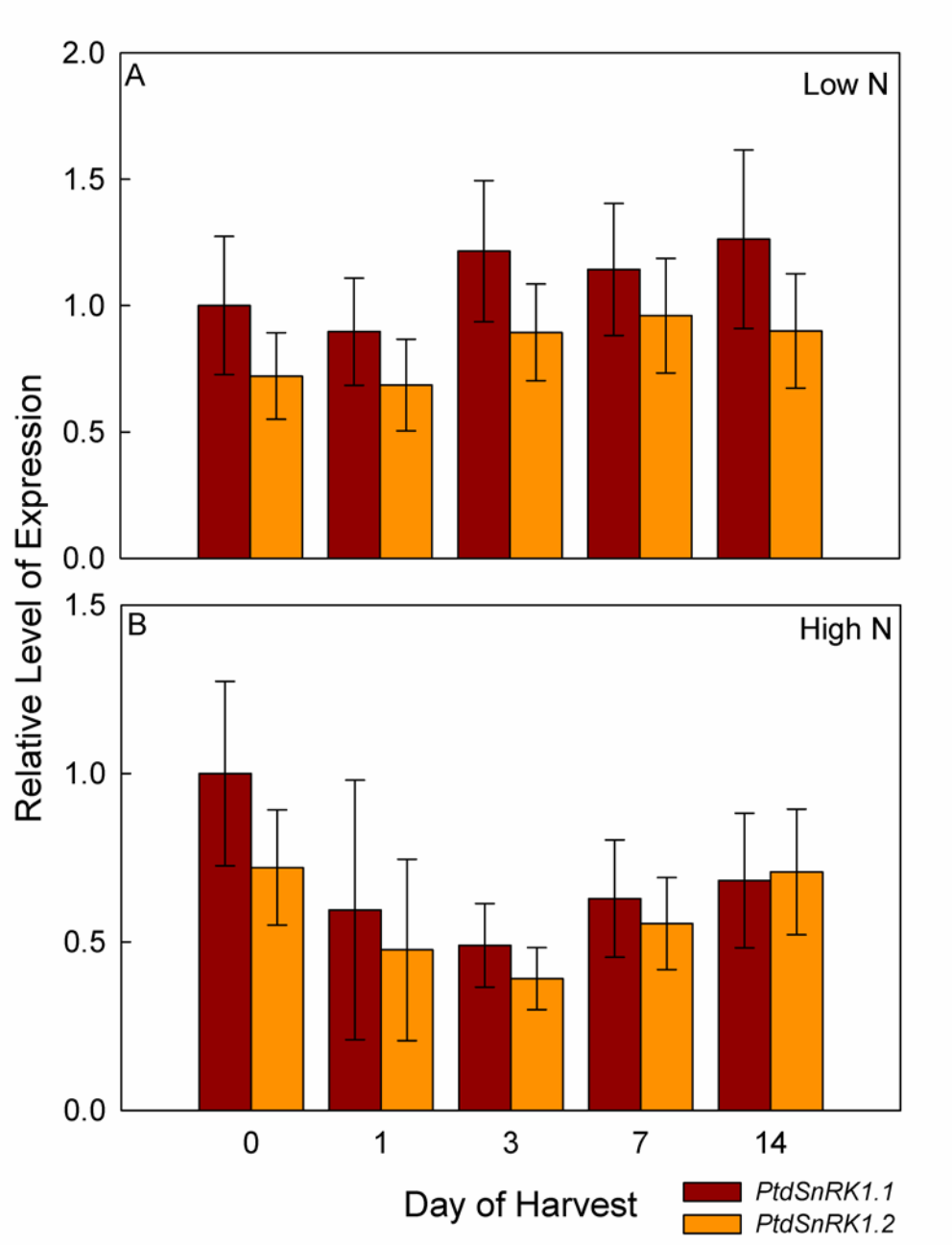

Figure 3.20. Relative level of expression of members of the *PtdSnRK1* gene family in young leaves of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. The relative level of expression was calculated by setting the expression of *PtdSnRK1.1* on day 0 to 1 and normalizing the expression of other members of the *PtdSnRK1* gene family to *PtdSnRK1.1*. Error bars show standard deviation.

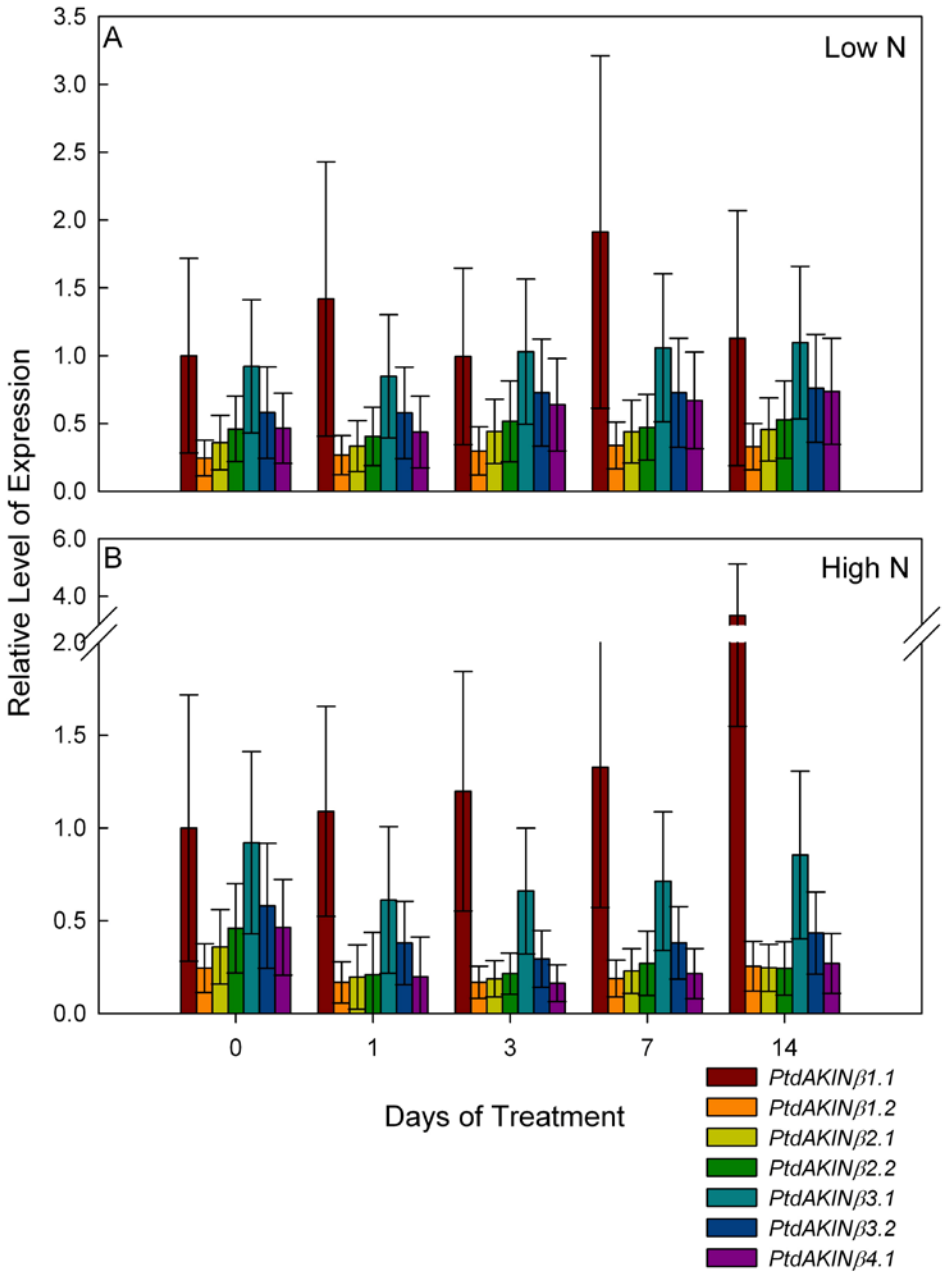

Figure 3.21. Relative level of expression of members of the *PtdAKINβ* gene family in young leaves of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. The relative level of expression was calculated by setting the expression of *PtdAKINβ1.1* on day 0 to 1 and normalizing the expression of other members of the *PtdAKINβ1* gene family to *PtdAKINβ1.1*. Error bars show standard deviation.

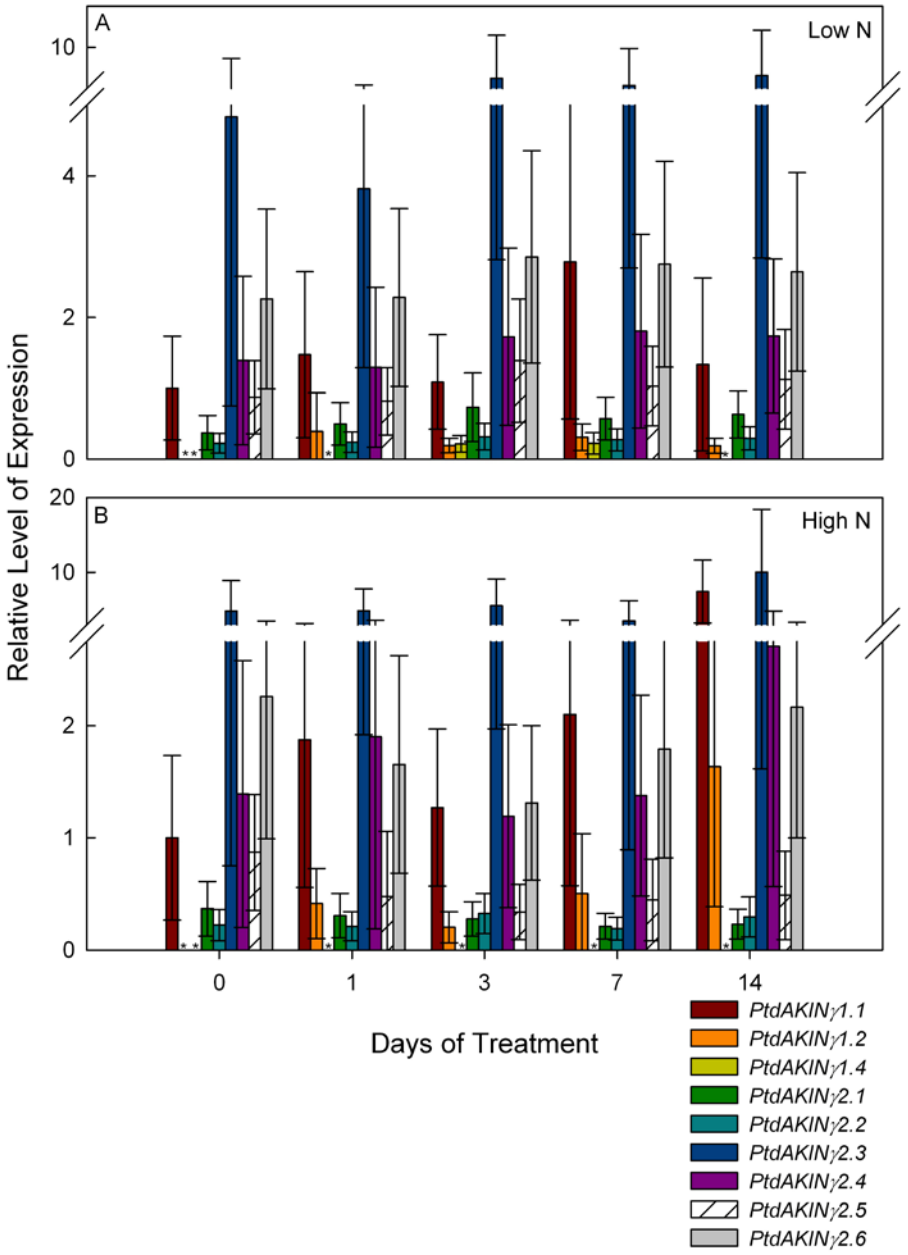

Figure 3.22. Relative level of expression of members of the *PtdAKINγ* gene family in young leaves of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. The relative level of expression was calculated by setting the expression of *PtdAKINγ1.1* on day 0 to 1 and normalizing the expression of other members of the *PtdAKINγ1* gene family to *PtdAKINγ1.1*. Error bars show standard deviation.

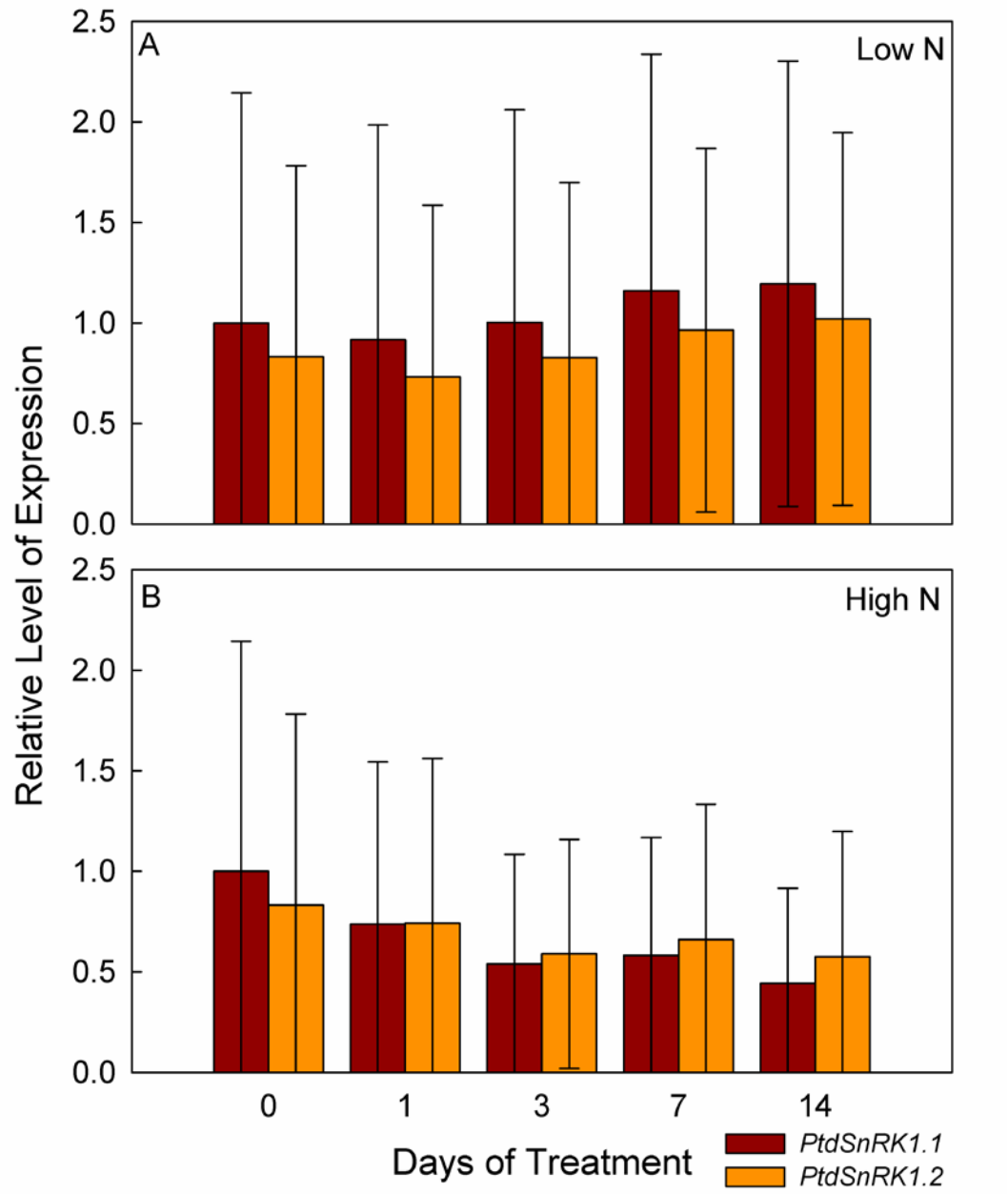

Figure 3.23. Relative level of expression of members of the *PtdSnRK1* gene family in secondary phloem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. The relative level of expression was calculated by setting the expression of *PtdSnRK1.1* on day 0 to 1 and normalizing the expression of other members of the *PtdSnRK1* gene family to *PtdSnRK1.1*. Error bars show standard deviation.

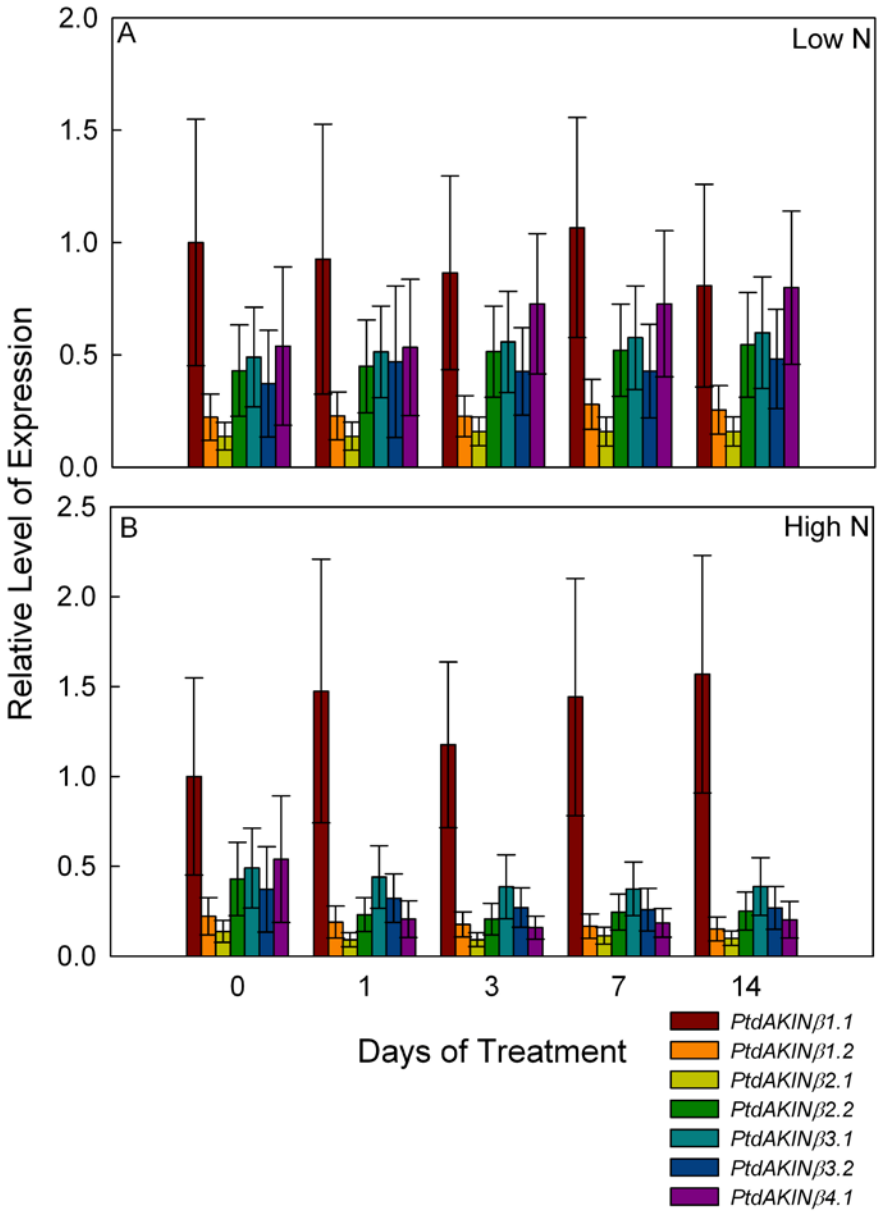

Figure 3.24. Relative level of expression of members of the *PtdAKINβ* gene family in secondary phloem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. The relative level of expression was calculated by setting the expression of *PtdAKINβ1.1* on day 0 to 1 and normalizing the expression of other members of the *PtdAKINβ1* gene family to *PtdAKINβ1.1*. Error bars show standard deviation.

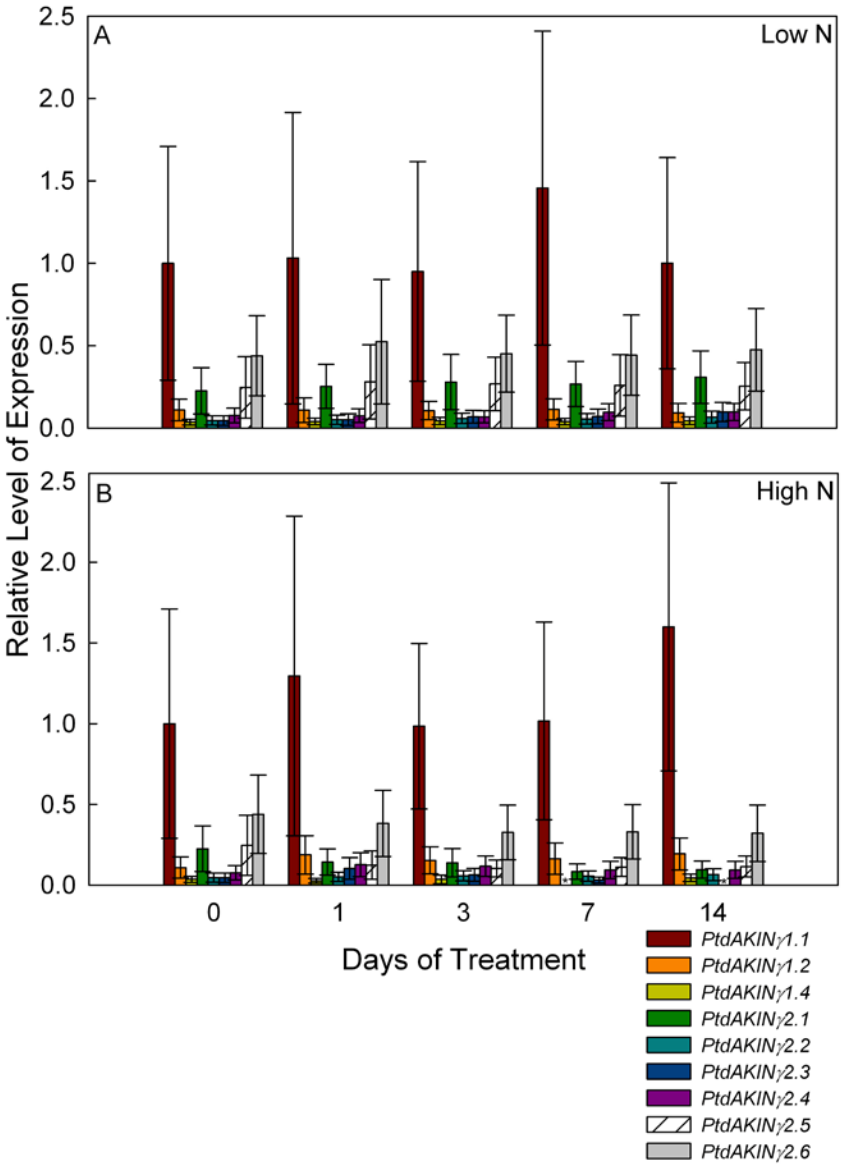

Figure 3.25. Relative level of expression of members of the *PtdAKINγ* gene family in secondary phloem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. The relative level of expression was calculated by setting the expression of *PtdAKINγ1.1* on day 0 to 1 and normalizing the expression of other members of the *PtdAKINγ1* gene family to *PtdAKINγ1.1*. Error bars show standard deviation.

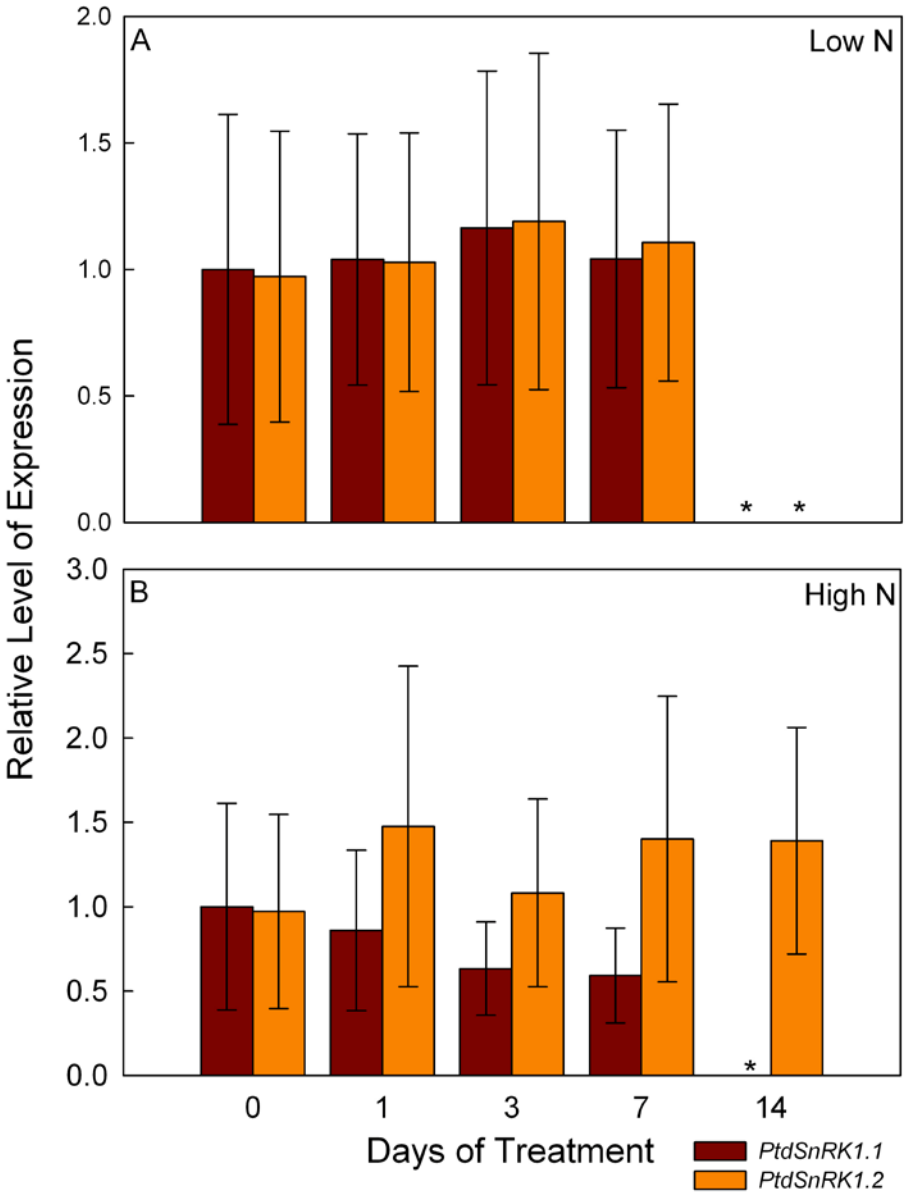

Figure 3.26. Relative level of expression of members of the *PtdSnRK1* gene family in secondary xylem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. The relative level of expression was calculated by setting the expression of *PtdSnRK1.1* on day 0 to 1 and normalizing the expression of other members of the *PtdSnRK1* gene family to *PtdSnRK1.1*. Error bars show standard deviation.

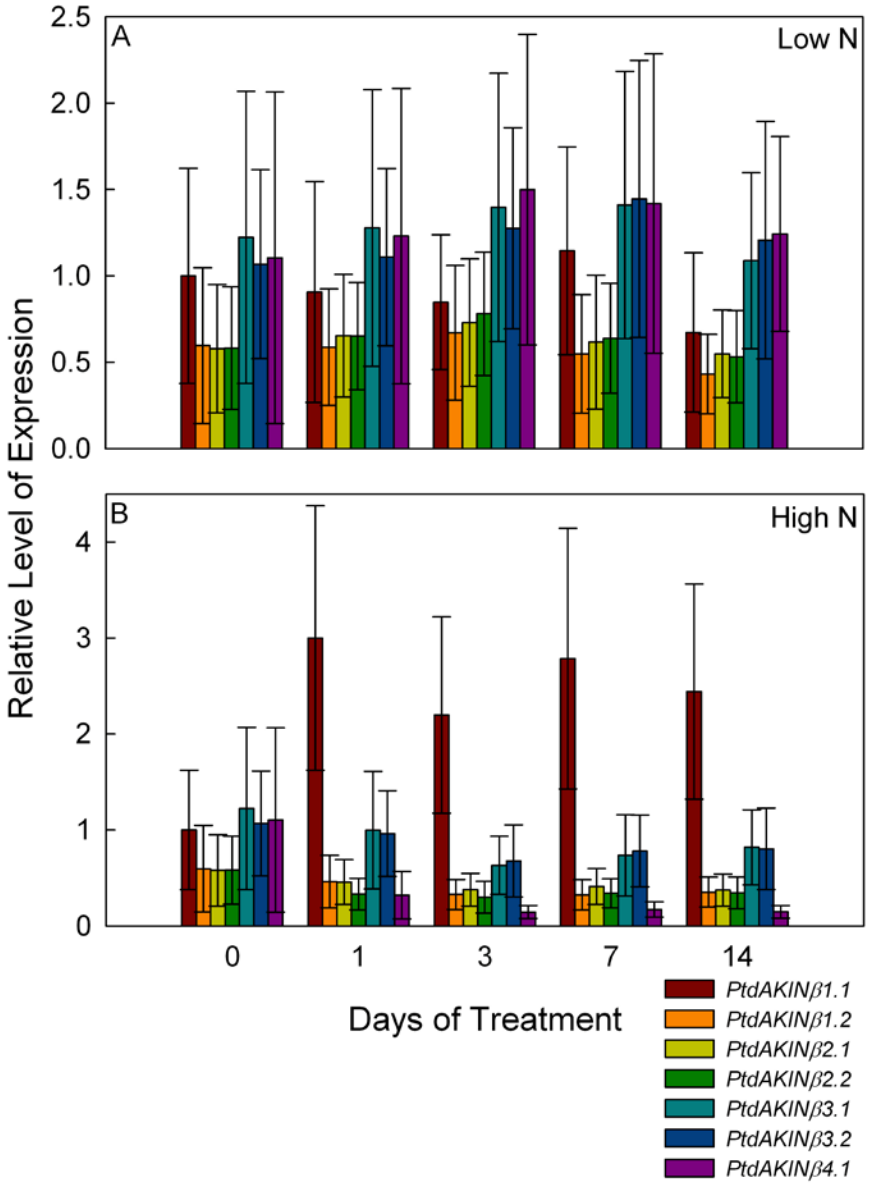

Figure 3.27. Relative level of expression of members of the *PtdAKINβ* gene family in secondary xylem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. The relative level of expression was calculated by setting the expression of *PtdAKINβ1.1* on day 0 to 1 and normalizing the expression of other members of the *PtdAKINβ1* gene family to *PtdAKINβ1.1*. Error bars show standard deviation.

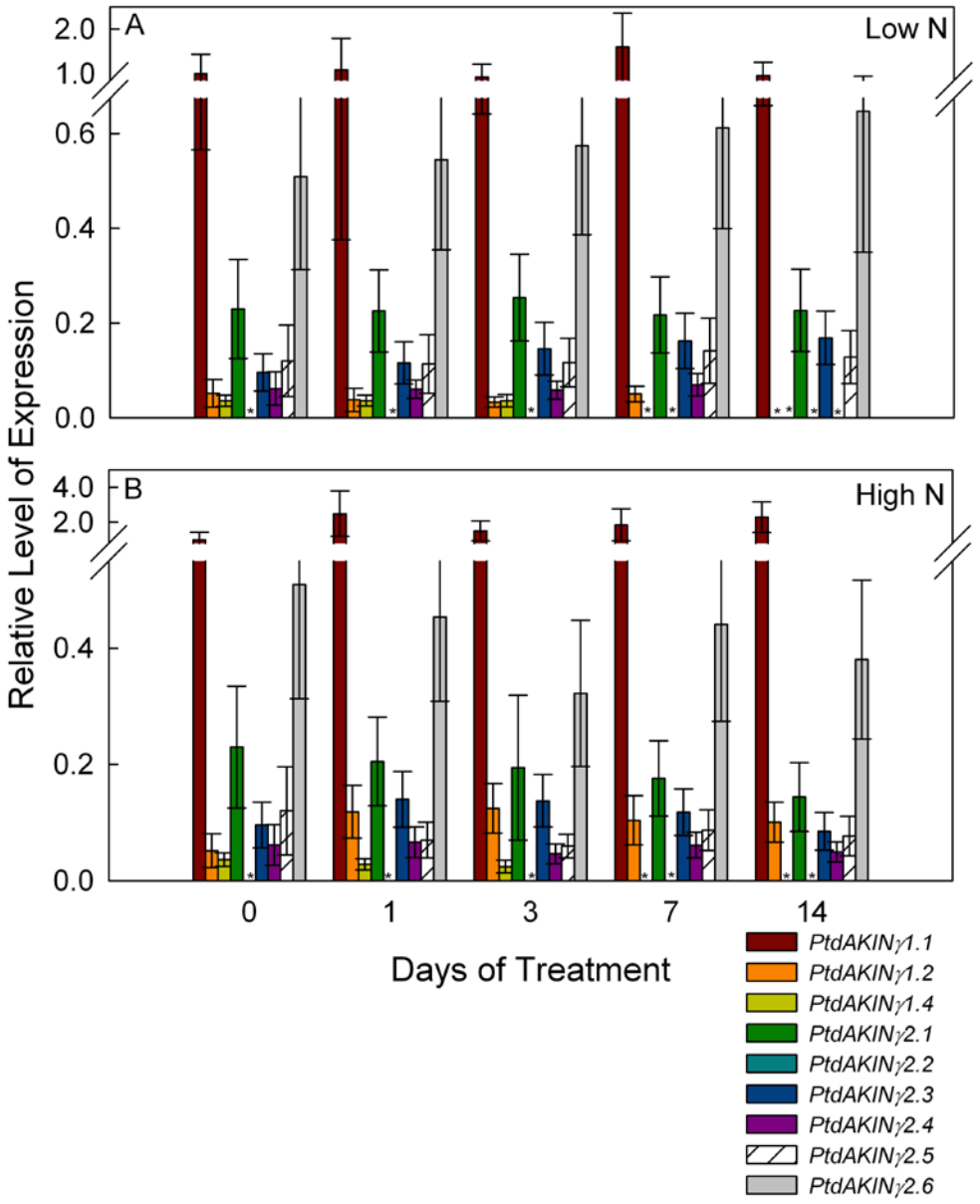

Figure 3.28. Relative level of expression of members of the *PtdAKINγ* gene family in secondary xylem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. The relative level of expression was calculated by setting the expression of *PtdAKINγ1.1* on day 0 to 1 and normalizing the expression of other members of the *PtdAKINγ1* gene family to *PtdAKINγ1.1*. Error bars show standard deviation.

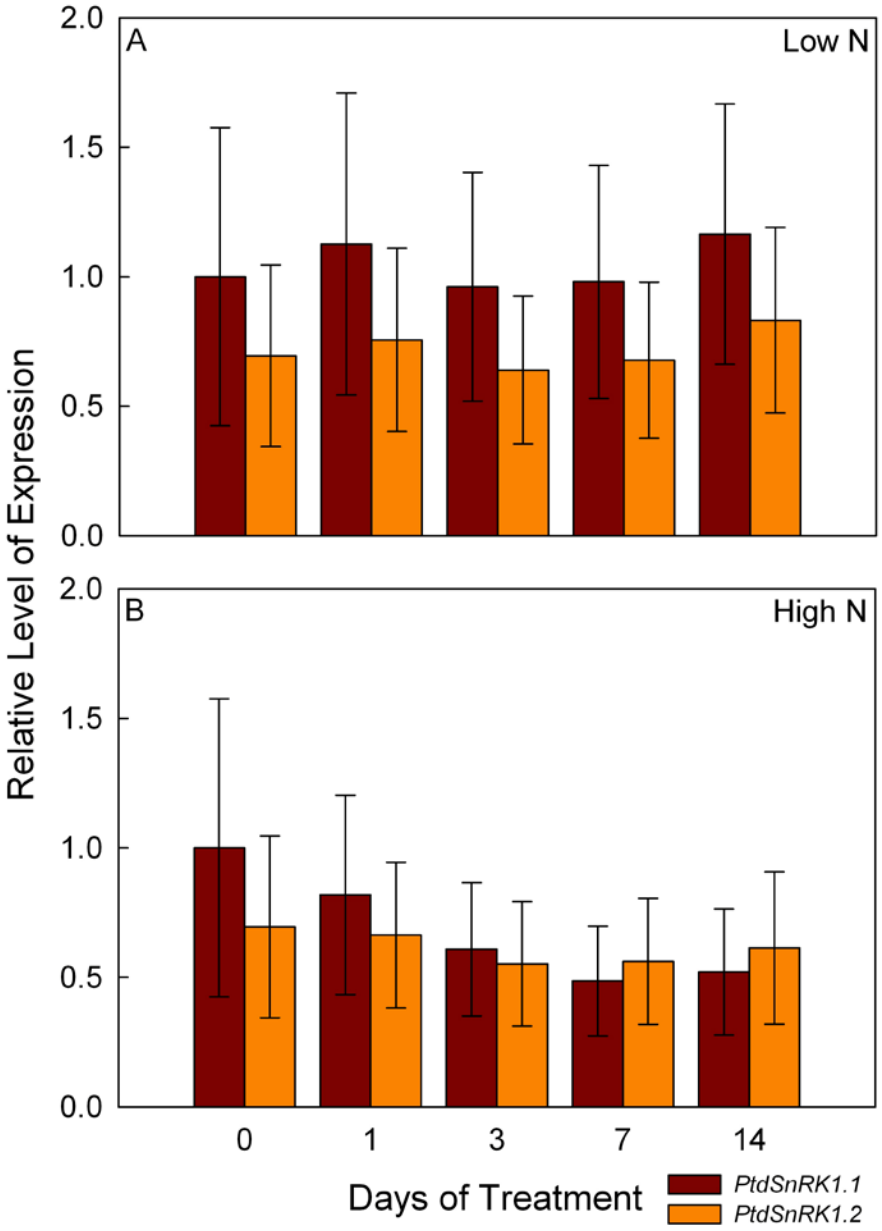

Figure 3.29. Relative level of expression of members of the *PtdSnRK1* gene family in roots of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. The relative level of expression was calculated by setting the expression of *PtdSnRK1.1* on day 0 to 1 and normalizing the expression of other members of the *PtdSnRK1* gene family to *PtdSnRK1.1*. Error bars show standard deviation.

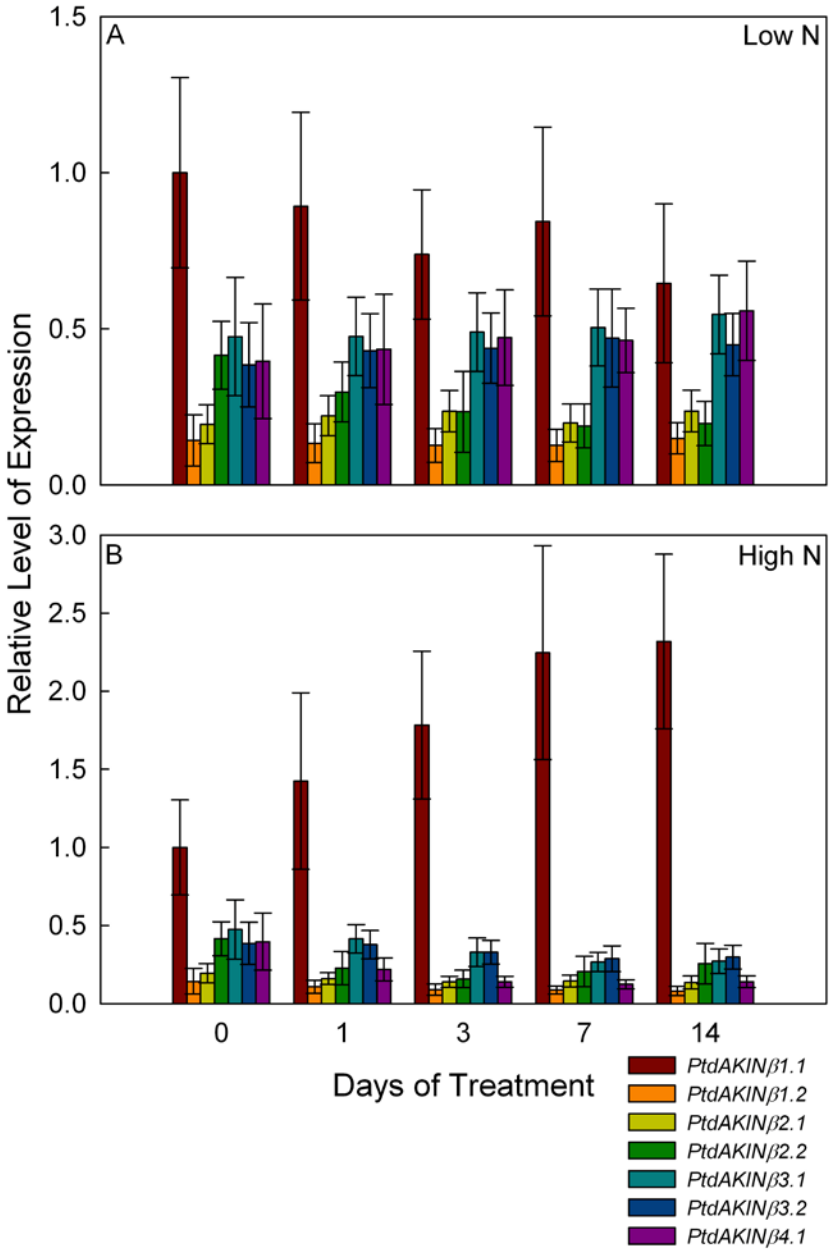

Figure 3.30. Relative level of expression of members of the *PtdAKINβ* gene family in roots of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. The relative level of expression was calculated by setting the expression of *PtdAKINβ1.1* on day 0 to 1 and normalizing the expression of other members of the *PtdAKINβ1* gene family to *PtdAKINβ1.1*. Error bars show standard deviation.

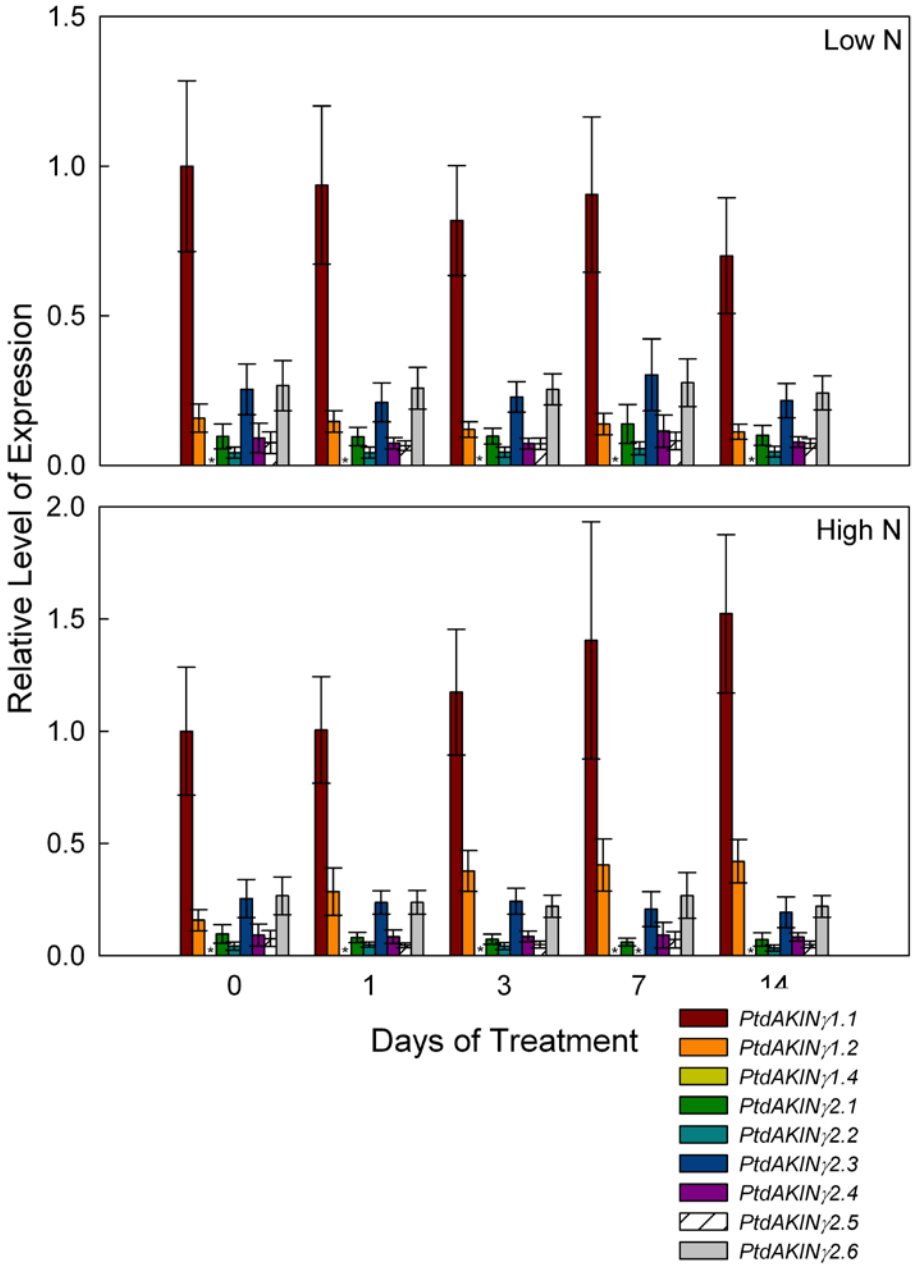

Figure 3.31. Relative level of expression of members of the *PtdAKINγ* gene family in roots of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. The relative level of expression was calculated by setting the expression of *PtdAKINγ1.1* on day 0 to 1 and normalizing the expression of other members of the *PtdAKINγ1* gene family to *PtdAKINγ1.1*. Error bars show standard deviation.

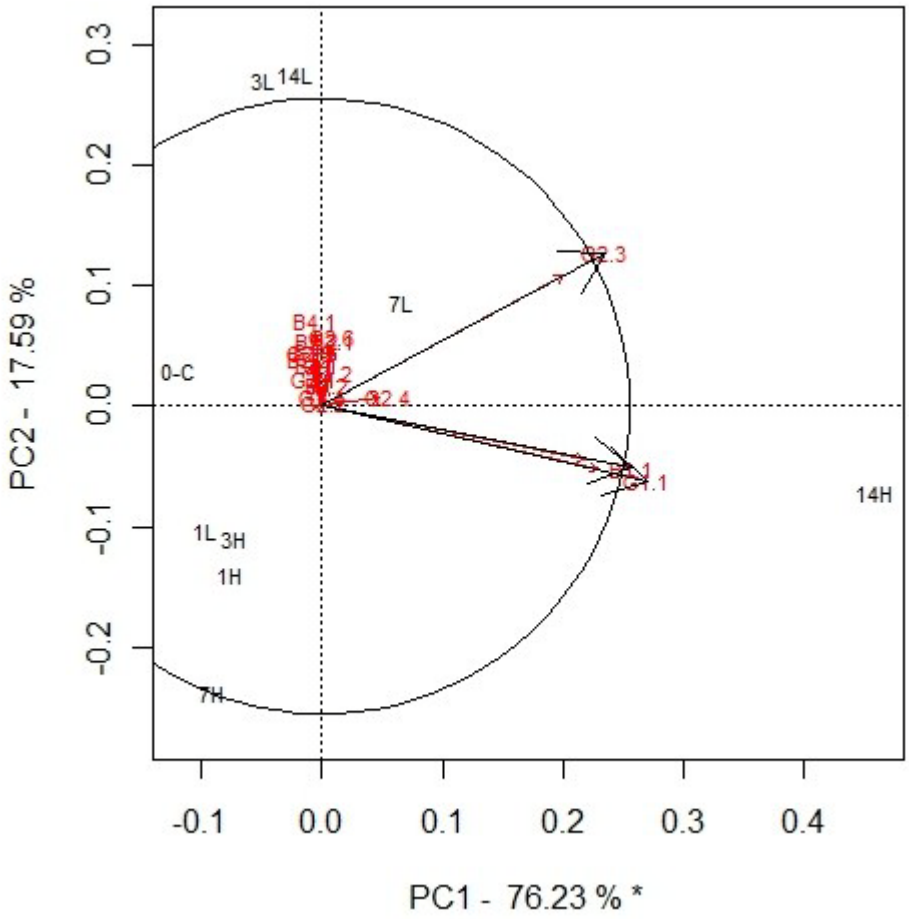

Figure 3.32. Principal component analysis of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in young leaves of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Principal components which are significant based on the broken stick distribution are denoted with *. Magnitude of the contribution of a particular gene to the variation of the principle components is denoted by the length and direction of the arrow. Arrows which exceed the equilibrium the circle significantly contribute to the variation of the principal components. *PtdAKINγ1.2* was not included as PCA does not accommodate null data entries. Members of the *PtdSnRK1* gene family are denoted with "S" followed by the gene member number. Members of the *PtdAKINβ* gene family are denoted with "B" followed by the gene member number. Members of the *PtdAKINγ* gene family are denoted with "G" followed by the gene member number. Low nitrogen availability is denoted with L, high nitrogen availability is denoted with H, the time point is denoted with 1, 3, 7 or 14, and the day 0 control is denoted with 0-C.

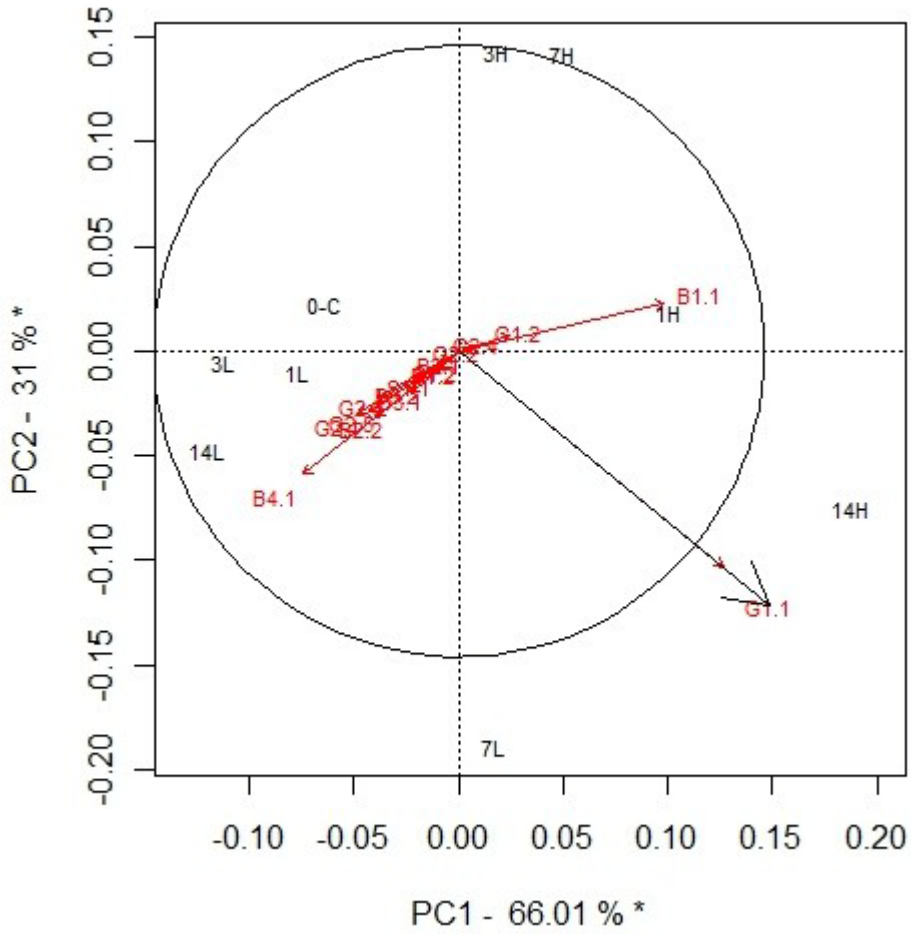

Figure 3.33. Principal component analysis of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in secondary phloem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Principal components which are significant based on the broken stick distribution are denoted with *. Magnitude of the contribution of a particular gene to the variation of the principle components is denoted by the length and direction of the arrow. Arrows which exceed the equilibrium the circle significantly contribute to the variation of the principal components. *PtdAKINγ1.4* and *PtdAKINγ2.3* were not included as PCA does not accommodate null data entries. Members of the *PtdSnRK1* gene family are denoted with "S" followed by the gene member number. Members of the *PtdAKINβ* gene family are denoted with "B" followed by the gene member number. Members of the *PtdAKINγ* gene family are denoted with "G" followed by the gene member number. Low nitrogen availability is denoted with L, high nitrogen availability is denoted with H, the time point is denoted with 1, 3, 7 or 14, and the day 0 control is denoted with 0-C.

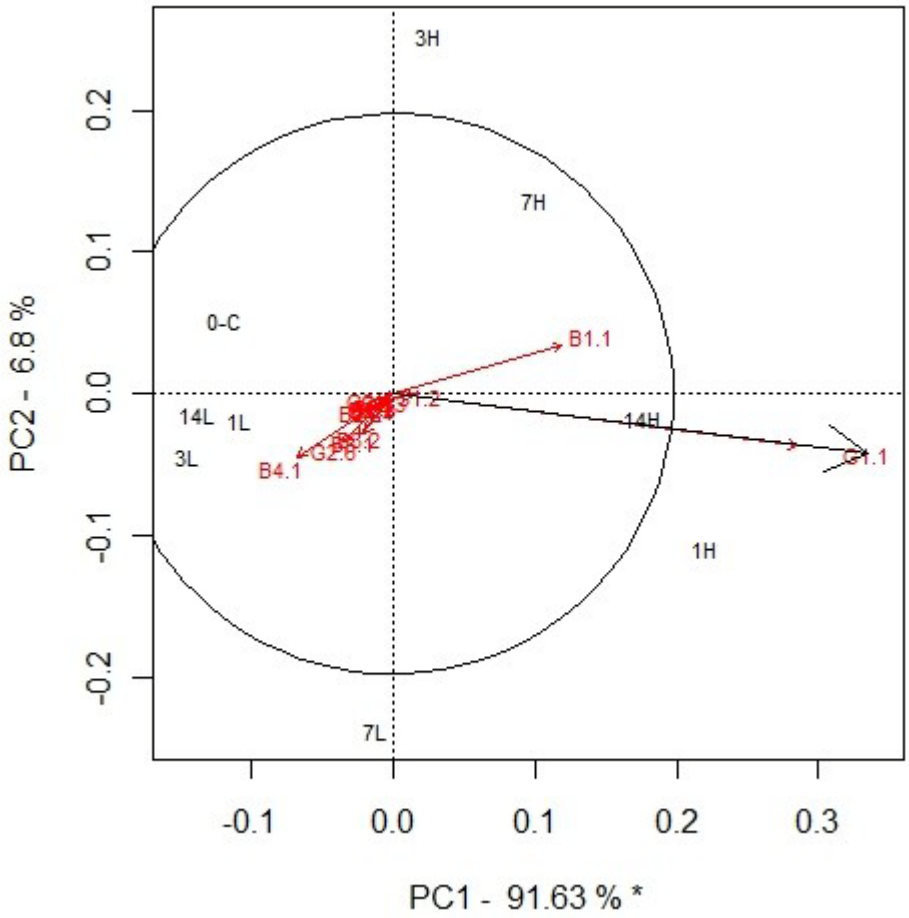

Figure 3.34. Principal component analysis of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in secondary xylem of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Principal components which are significant based on the broken stick distribution are denoted with *. Magnitude of the contribution of a particular gene to the variation of the principle components is denoted by the length and direction of the arrow. Arrows which exceed the equilibrium the circle significantly contribute to the variation of the principal components. *PtdAKINγ1.4* and *PtdAKINγ2.4* were not included as PCA does not accommodate null data entries. Members of the *PtdSnRK1* gene family are denoted with "S" followed by the gene member number. Members of the *PtdAKINβ* gene family are denoted with "B" followed by the gene member number. Members of the *PtdAKINγ* gene family are denoted with "G" followed by the gene member number. Low nitrogen availability is denoted with L, high nitrogen availability is denoted with H, the time point is denoted with 1, 3, 7 or 14, and the day 0 control is denoted with 0-C.

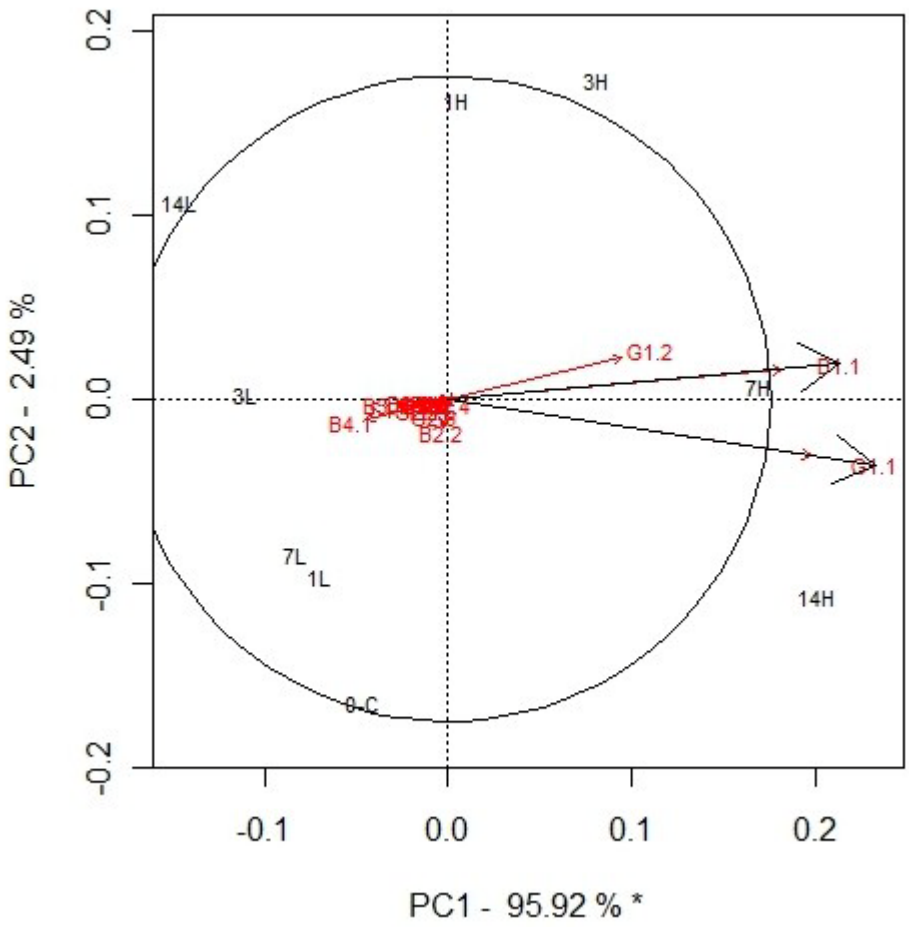

Figure 3.35. Principal component analysis of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in roots of poplars treated with low versus high nitrogen for up to 14 days. Plants were given adequate levels of nitrogen fertilization until day 0 of the experiment. Following harvest of the day 0 (control) samples, plants were fertilized daily with either 0 mM NH_4NO_3 or 10 mM NH_4NO_3 in a complete nutrient solution. Principal components which are significant based on the broken stick distribution are denoted with *. Magnitude of the contribution of a particular gene to the variation of the principle components is denoted by the length and direction of the arrow. Arrows which exceed the equilibrium the circle significantly contribute to the variation of the principal components. *PtdAKINγ2.2* was not included as PCA does not accommodate null data entries. Members of the *PtdSnRK1* gene family are denoted with "S" followed by the gene member number. Members of the *PtdAKINβ* gene family are denoted with "B" followed by the gene member number. Members of the *PtdAKINγ* gene family are denoted with "G" followed by the gene member number. Low nitrogen availability is denoted with L, high nitrogen availability is denoted with H, the time point is denoted with 1, 3, 7 or 14, and the day 0 control is denoted with 0-C.

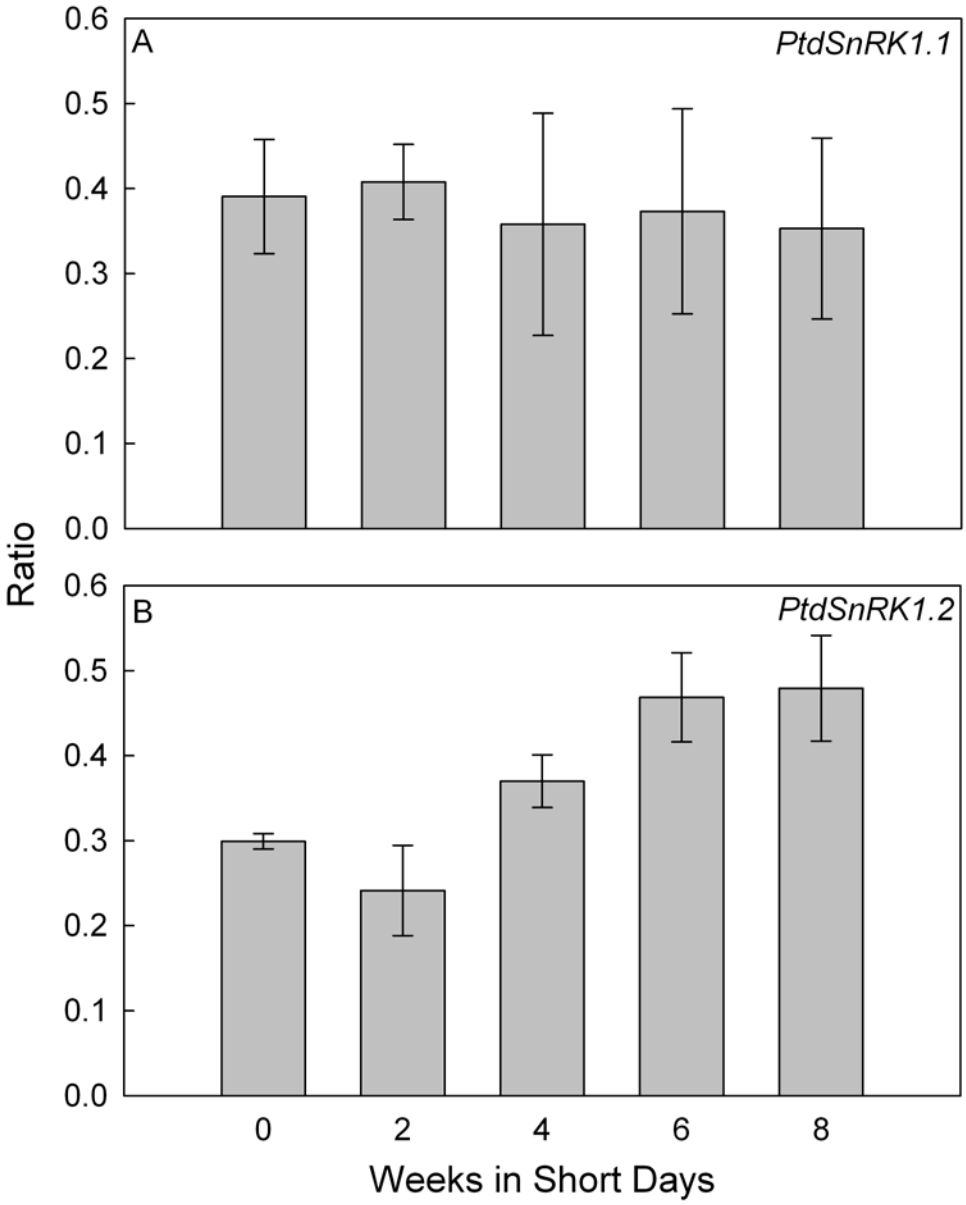

Figure 3.36. Expression of members of the *PtdSnRK1* gene family in shoot tips of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *SnRK1* to *VHA-A*. Error bars show standard deviation. N=6

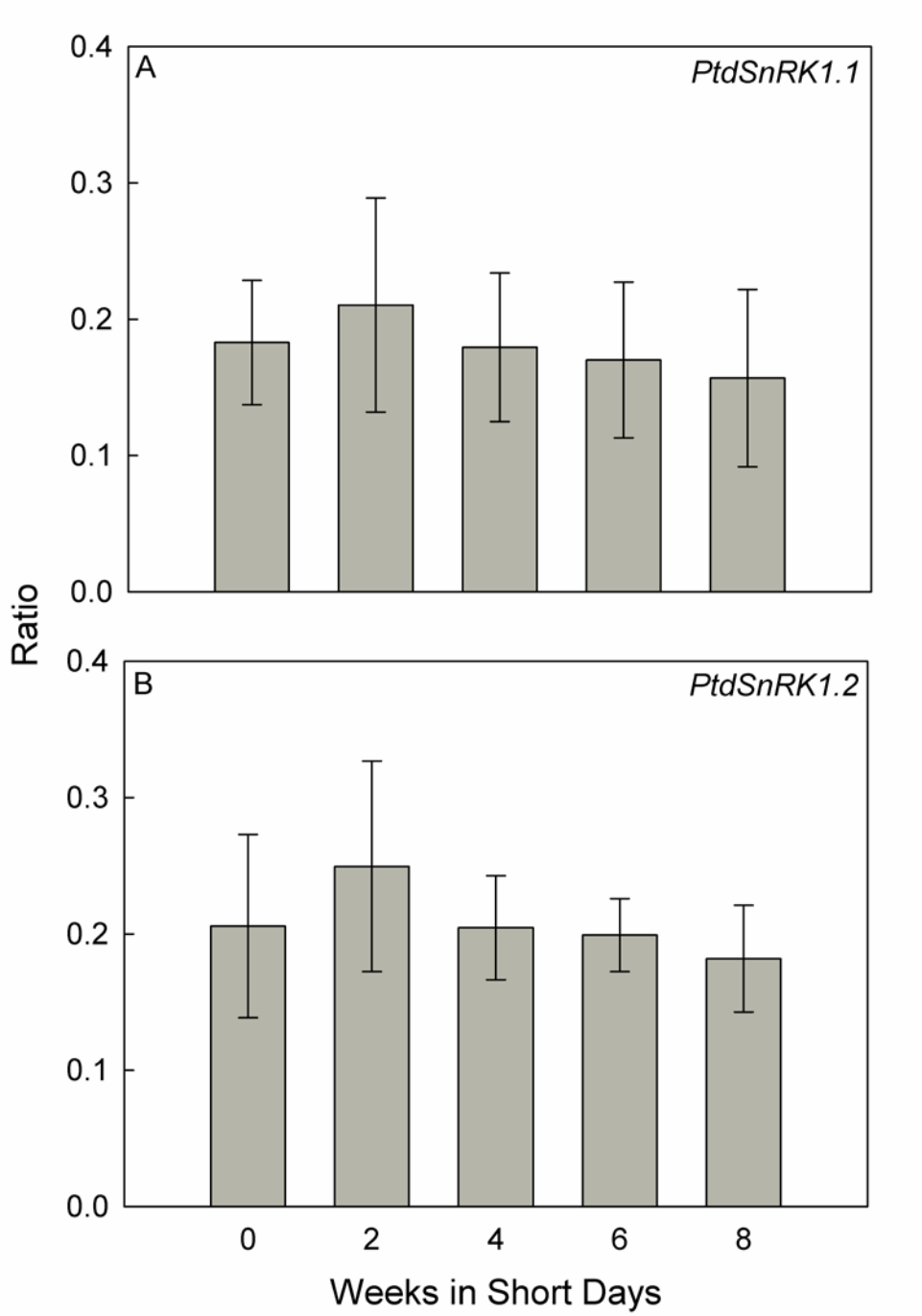

Figure 3.37. Expression of members of the *PtdSnRK1* gene family in mature leaves of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *SnRK1* to *VHA-A.* Error bars show standard deviation. N=6

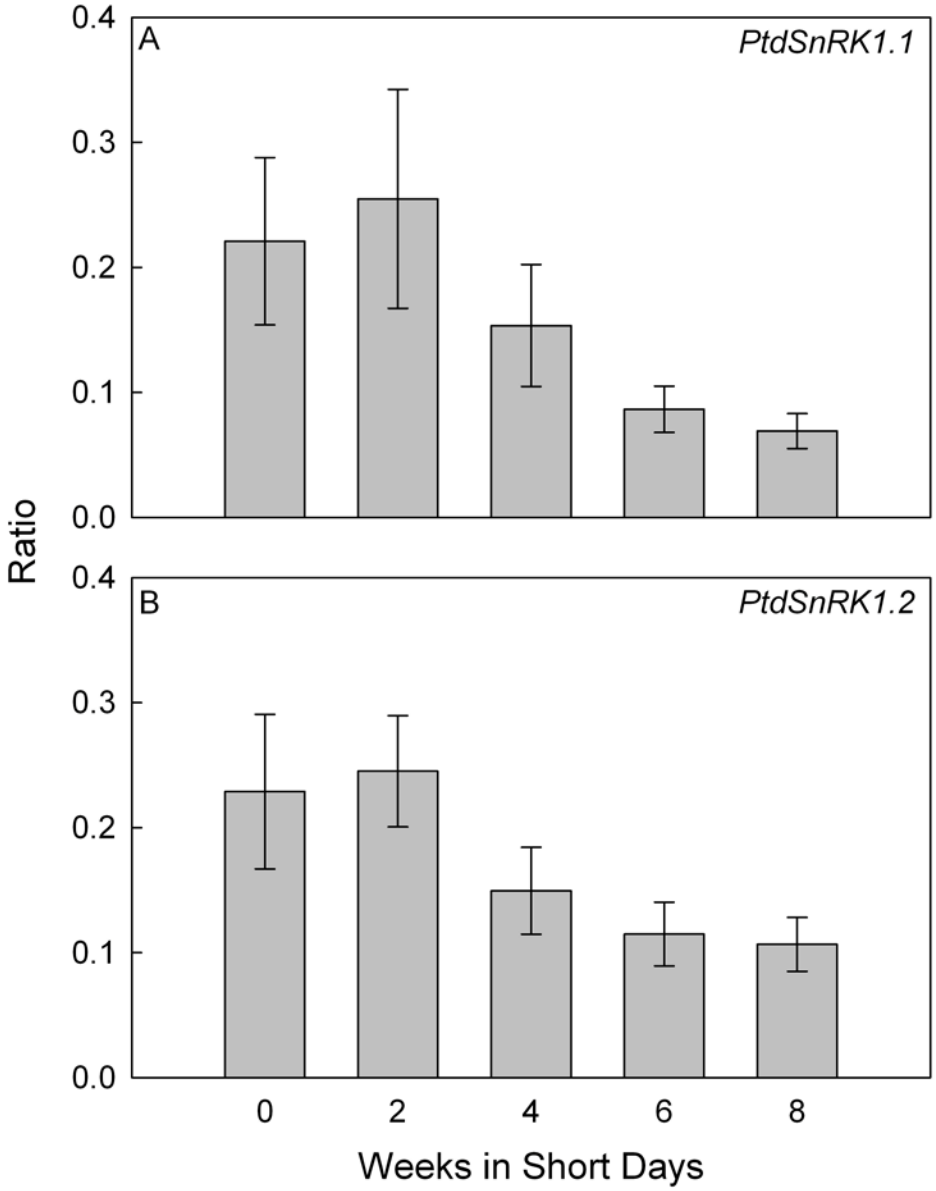

Figure 3.38. Expression of members of the *PtdSnRK1* gene family in secondary phloem of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *SnRK1* to the geometric mean of *VHA-A* and *phosphorylase.* Error bars show standard deviation. N=6

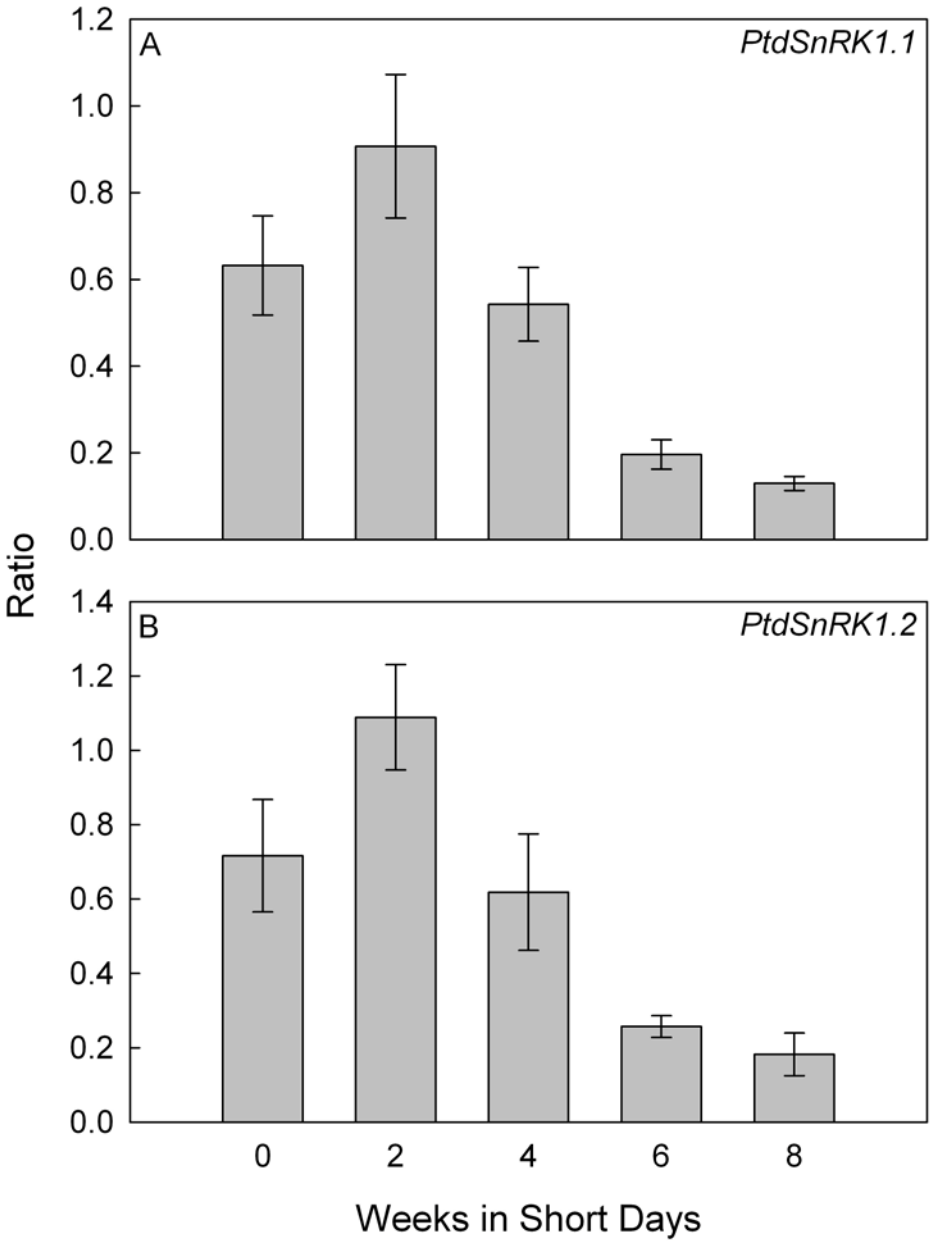

Figure 3.39. Expression of members of the *PtdSnRK1* gene family in secondary xylem of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *SnRK1* to geometric mean of *VHA-A* and *phosphorylase.* Error bars show standard deviation. N=6

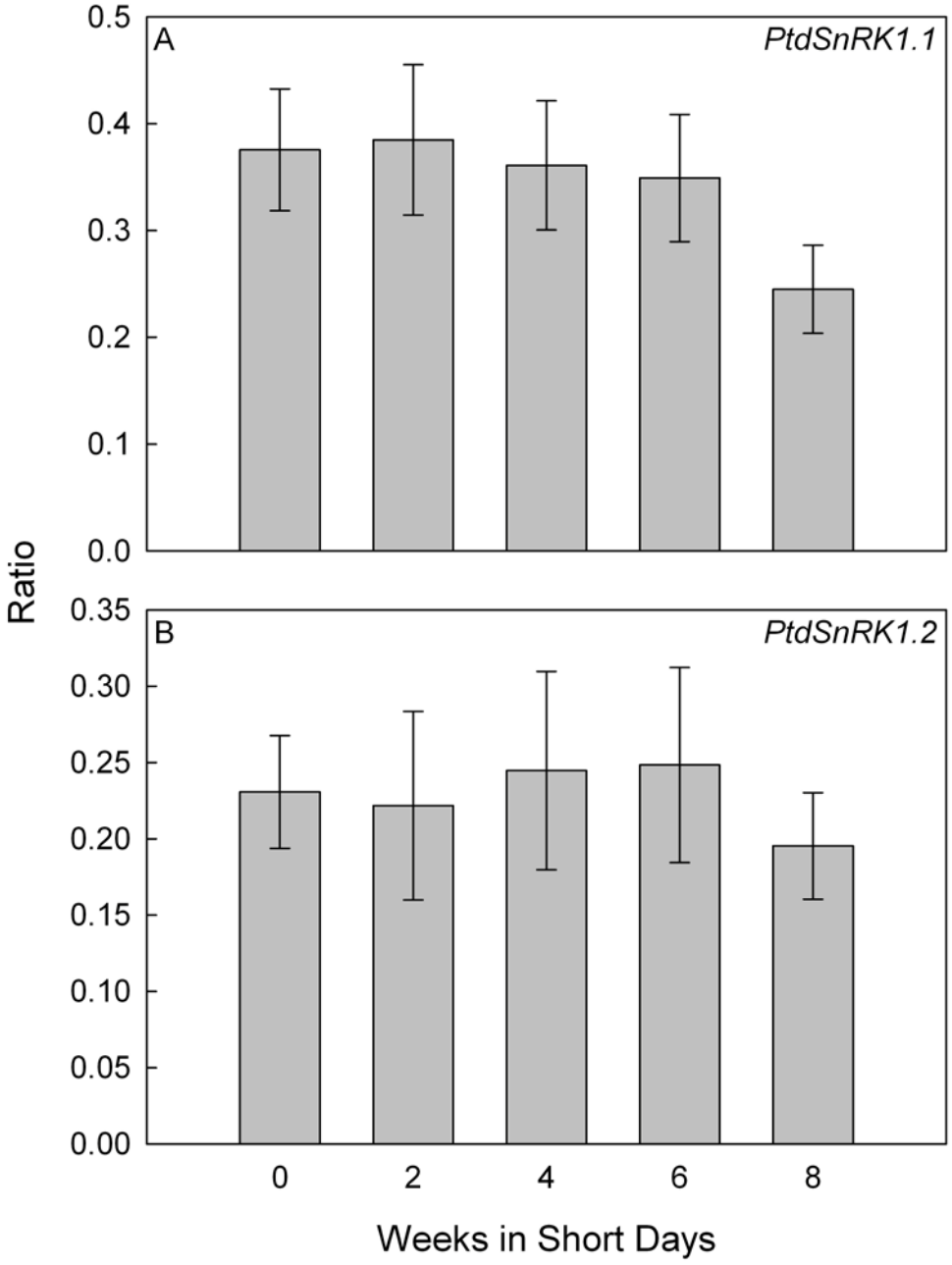

Figure 3.40. Expression of members of the *PtdSnRK1* gene family in roots of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *SnRK1* to *VHA-A.* Error bars show standard deviation. N=6

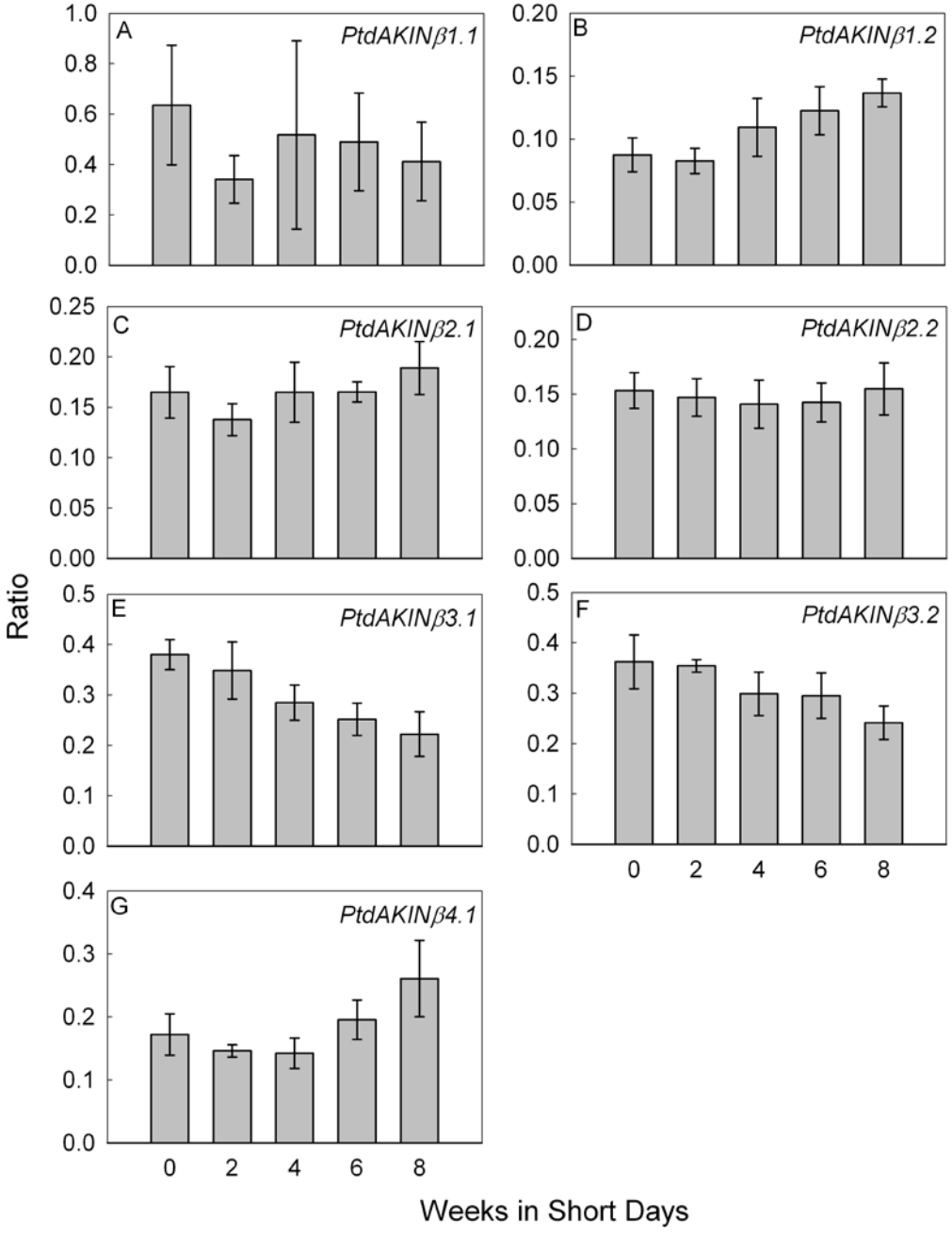

Figure 3.41. Expression of members of the *PtdAKINβ* gene family in shoot tips of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *AKINβ* to *VHA-A.* Error bars show standard deviation. N=6

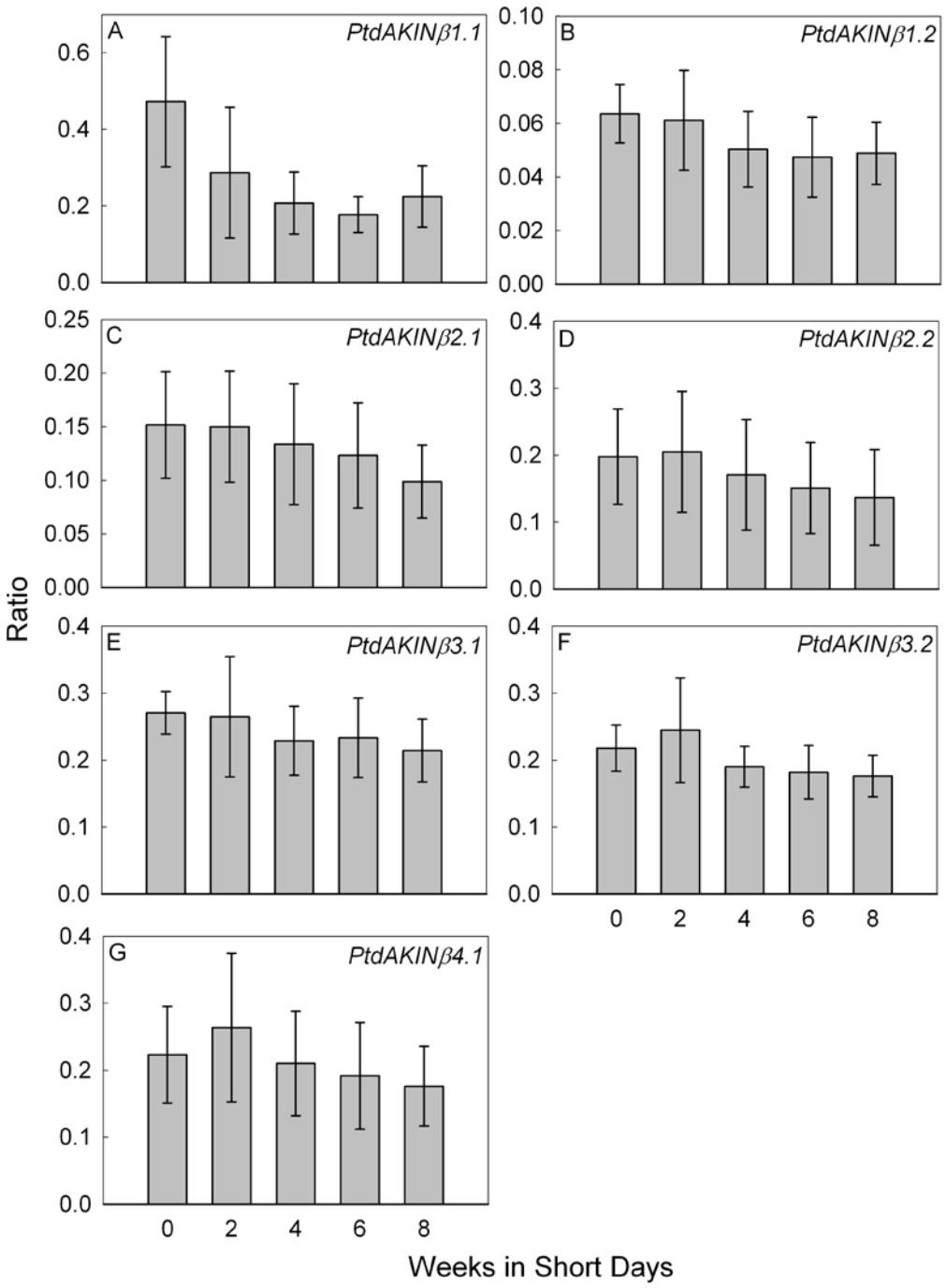

Figure 3.42. Expression of members of the *PtdAKINβ* gene family in mature leaves of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *AKINβ* to *VHA-A.* Error bars show standard deviation. N=6

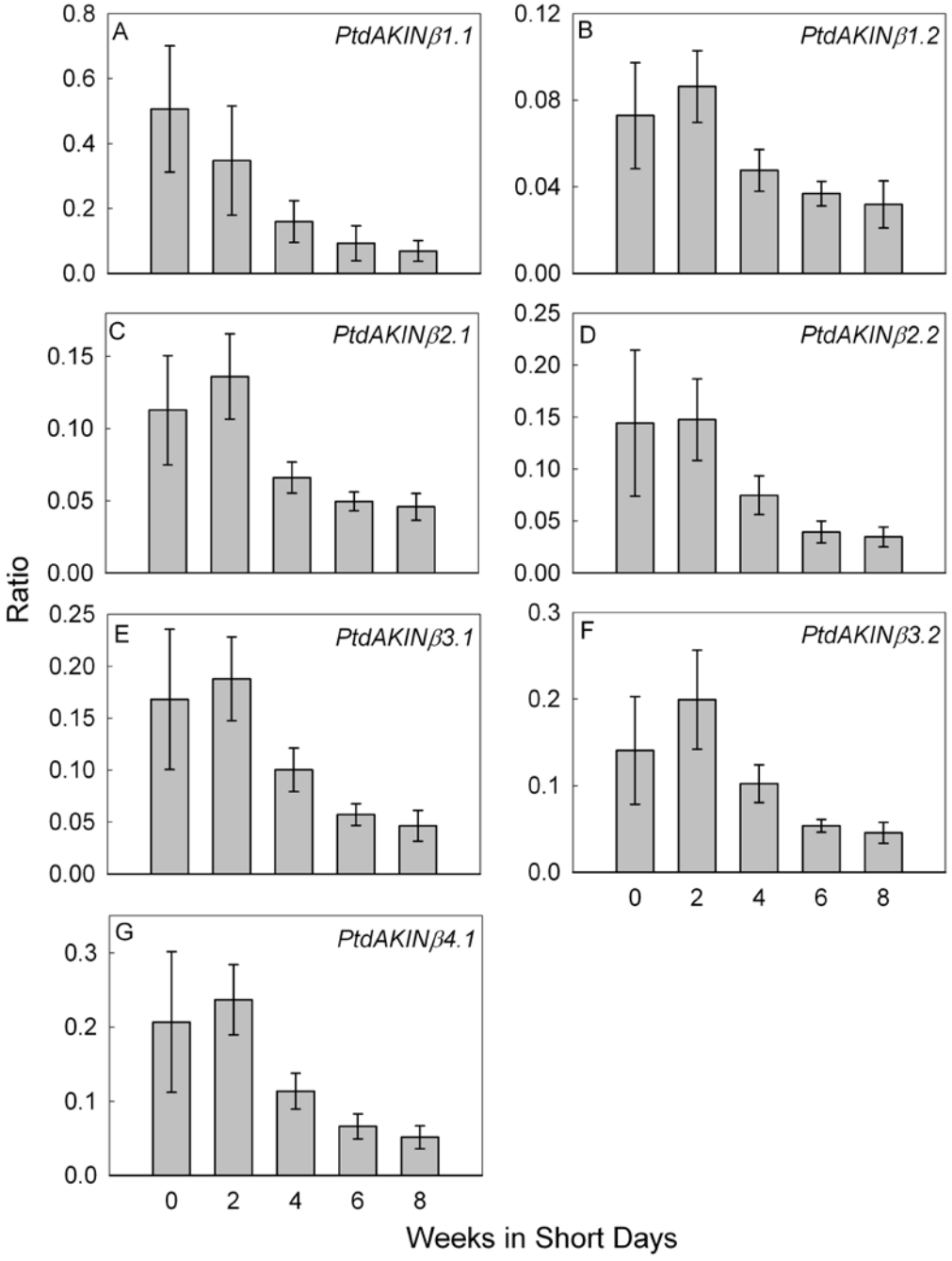

Figure 3.43. Expression of members of the *PtdAKINβ* gene family in secondary phloem of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *AKINβ* to the geometric mean of *VHA-A* and *phosphorylase.* Error bars show standard deviation. N=6

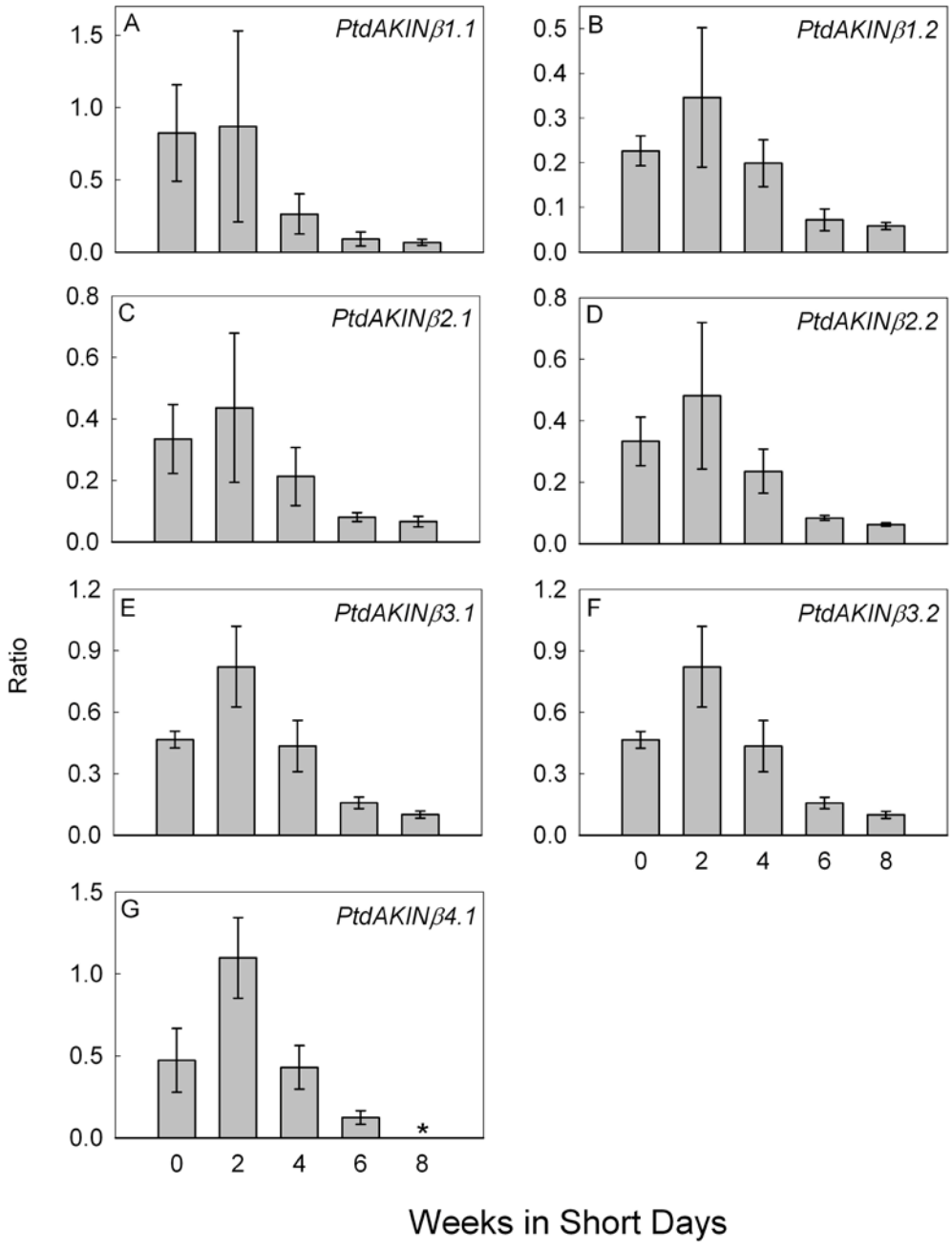

Figure 3.44. Expression of members of the *PtdAKINβ* gene family in secondary xylem of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *AKINβ* to geometric mean of *VHA-A* and *phosphorylase.* Expression below the detectable limit is denoted with *. Error bars show standard deviation. N=6

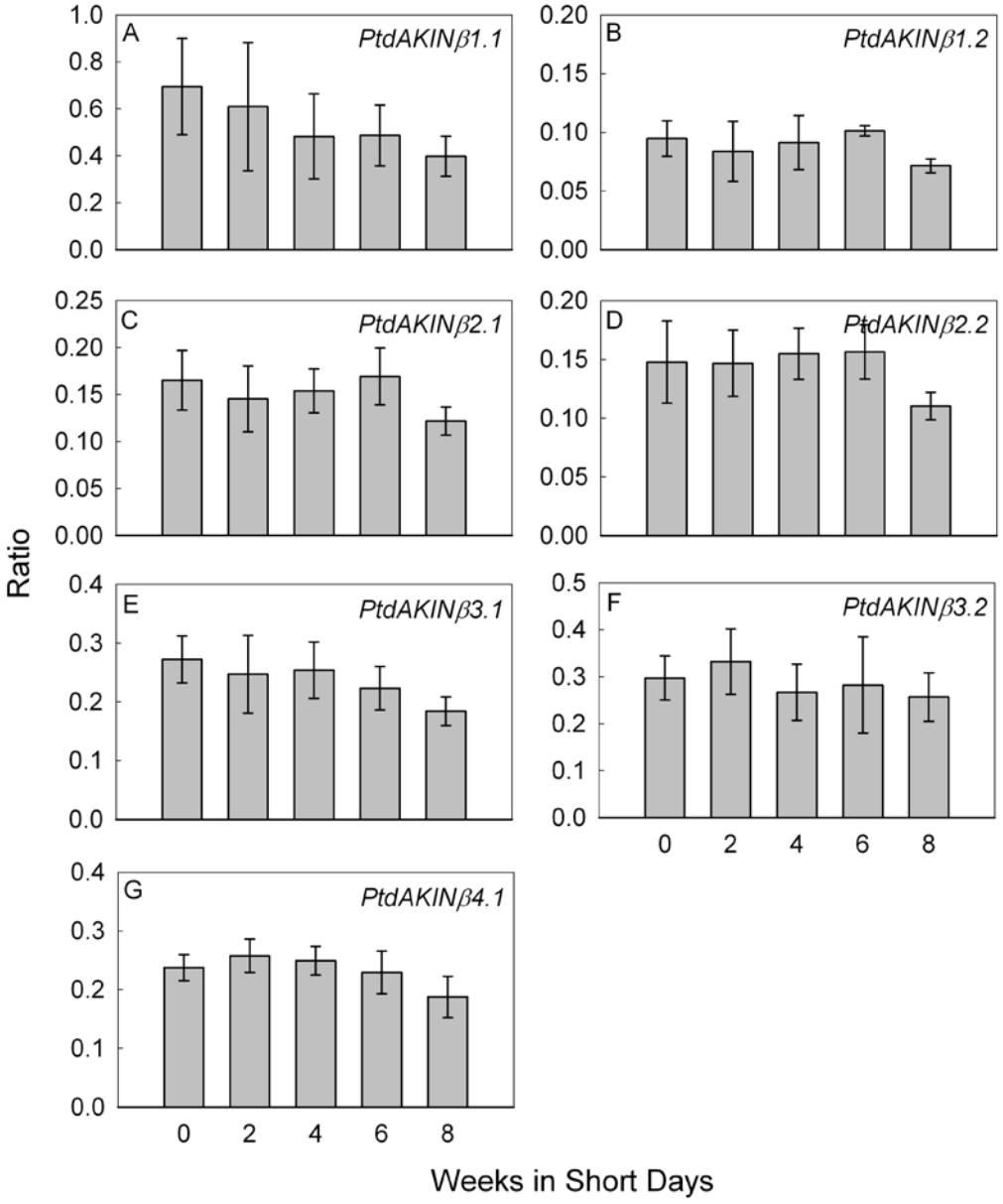

Figure 3.45. Expression of members of the *PtdAKINβ* gene family in roots of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *AKINβ* to *VHA-A.* Error bars show standard deviation. N=6

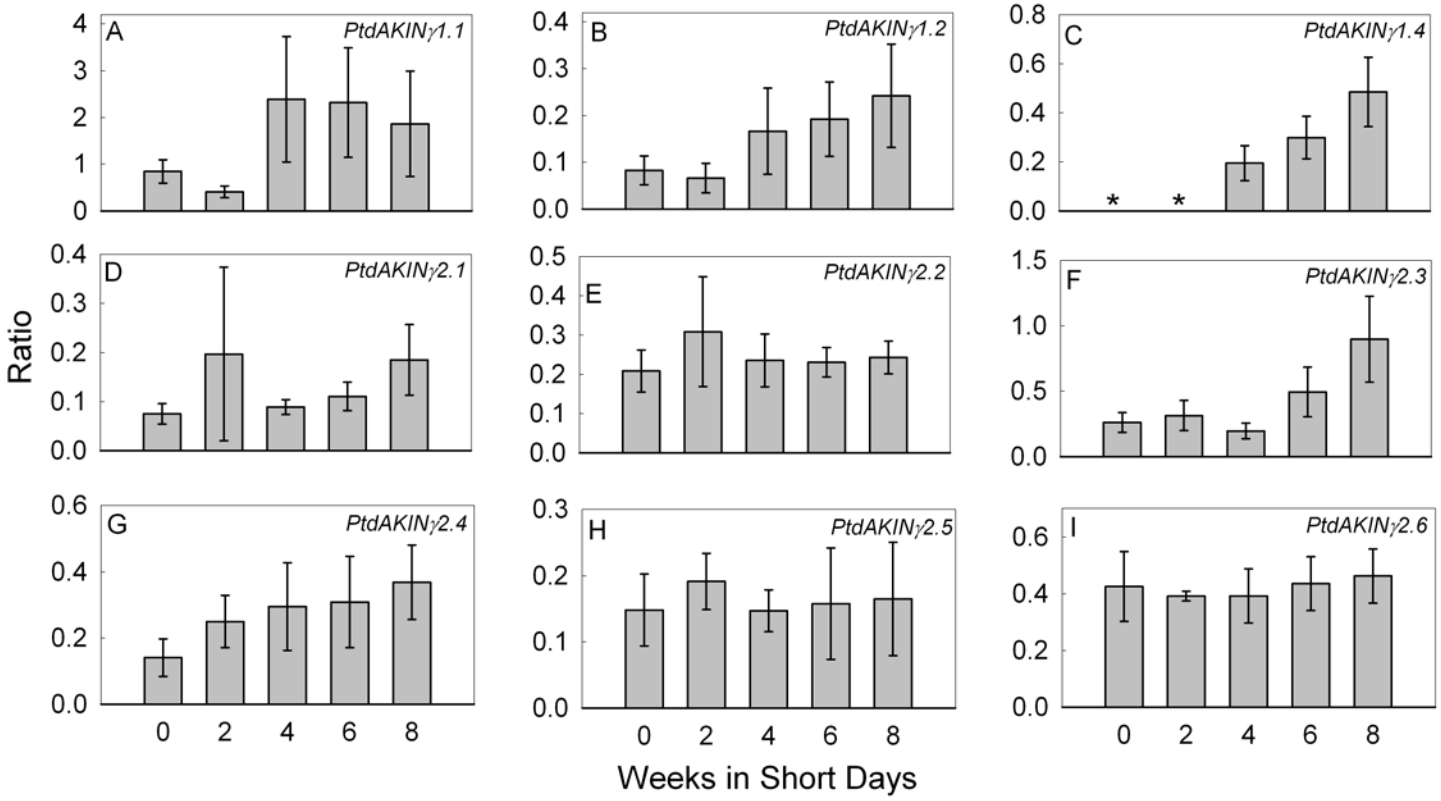

Figure 3.46. Expression of members of the *PtdAKINγ* gene family in shoot tips of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *AKINγ* to *VHA-A.* Error bars show standard deviation. N=6

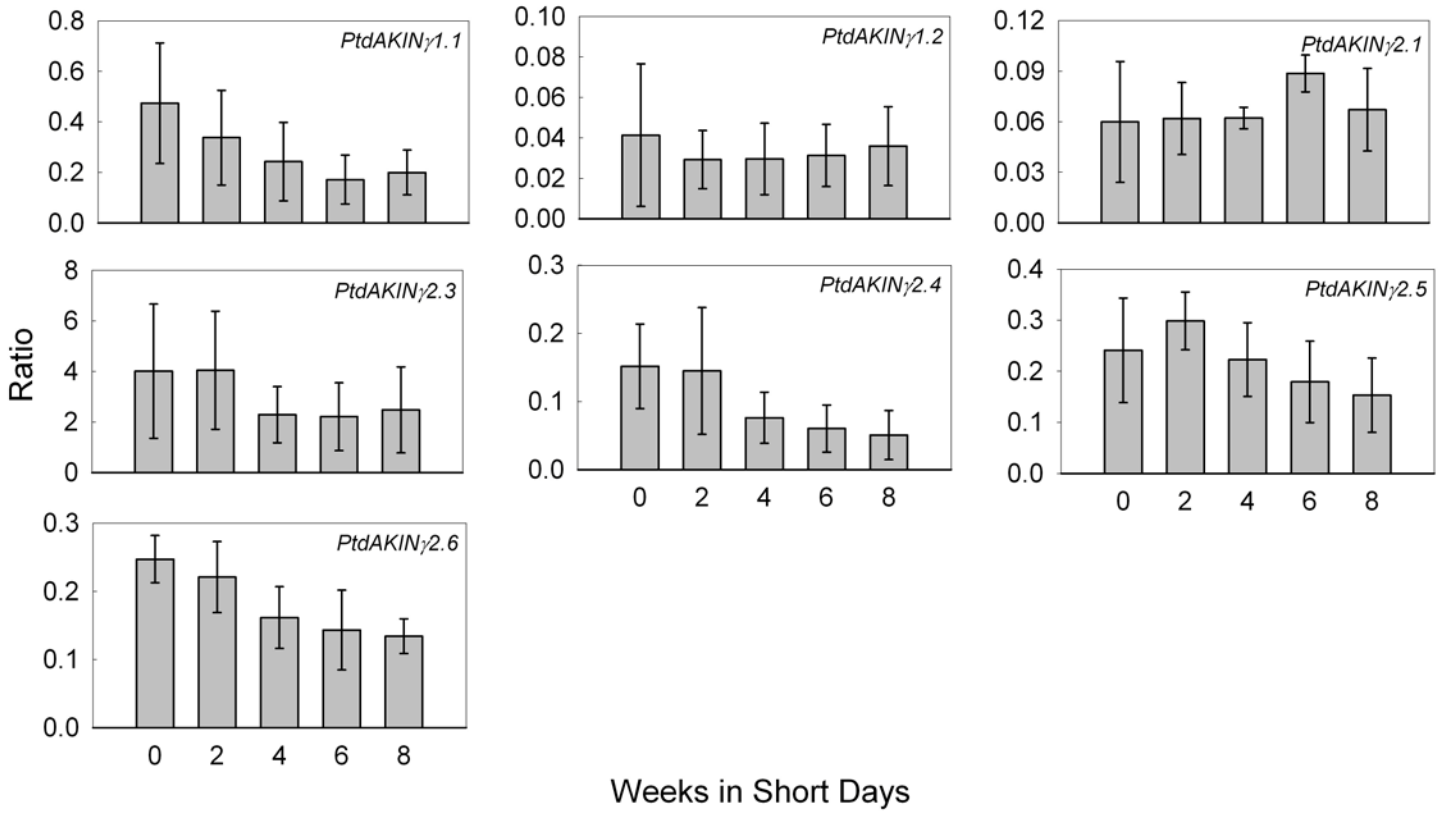

Figure 3.47. Expression of members of the *PtdAKINγ* gene family in mature leaves of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *AKINγ* to *VHA-A.* Error bars show standard deviation. N=6

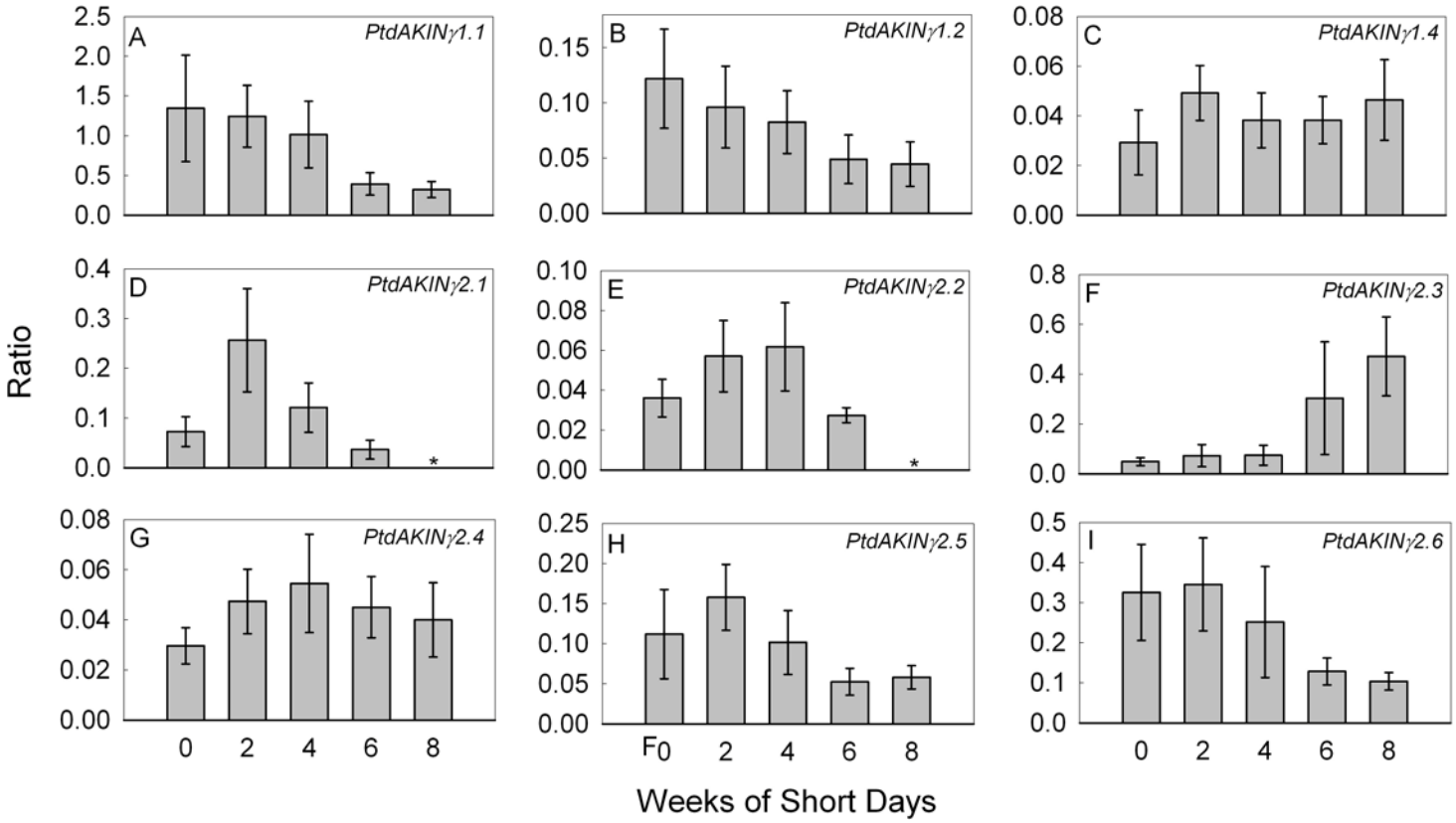

Figure 3.48. Expression of members of the *PtdAKINγ* gene family in secondary phloem of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *AKINγ* to the geometric mean of *VHA-A* and *phosphorylase.* Expression below the detectable limit is denoted with *. Error bars show standard deviation. N=6

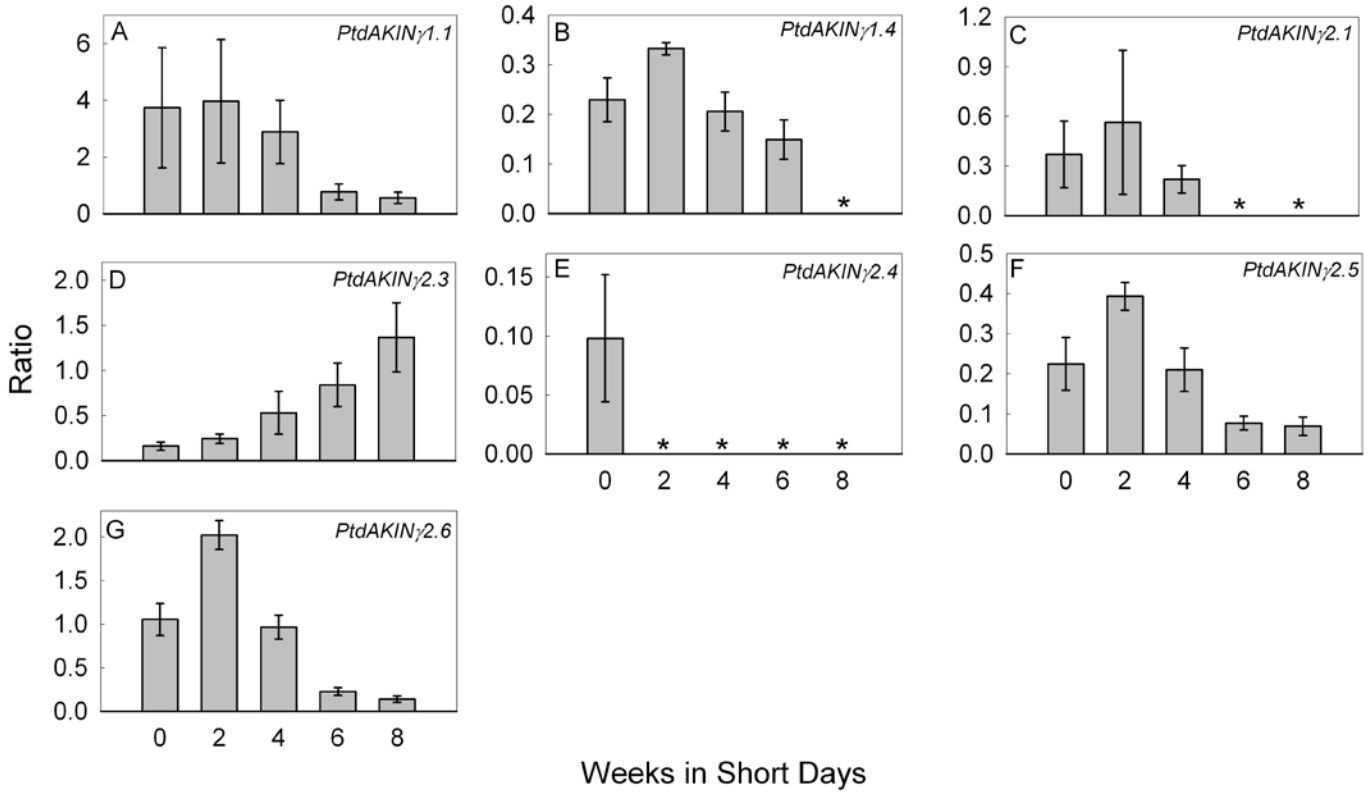

Figure 3.49. Expression of members of the *PtdAKINγ* gene family in secondary xylem of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *AKINγ* to geometric mean of *VHA-A* and *phosphorylase.* Expression below the detectable limit is denoted with *. Error bars show standard deviation. N=6

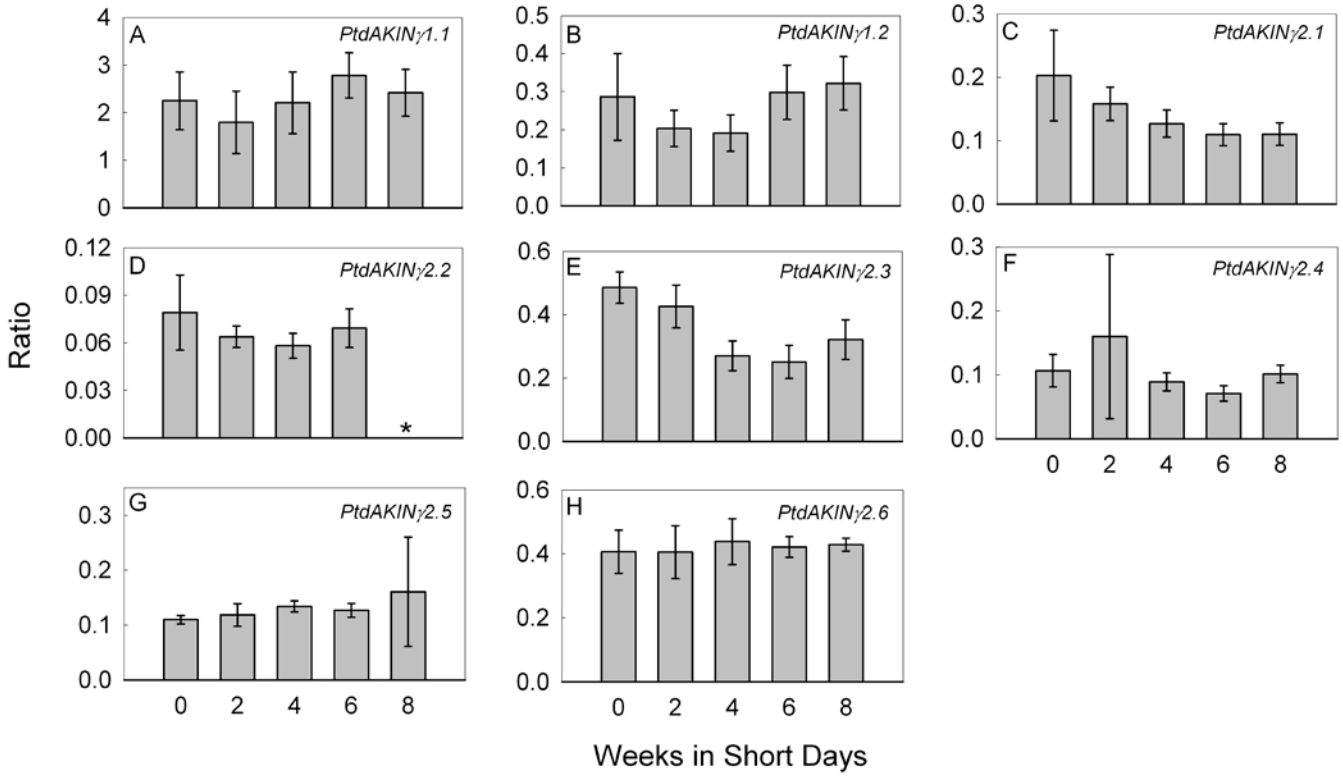

Figure 3.50. Expression of members of the *PtdAKINγ* gene family in roots of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Expression is shown as the ratio of the expression of the *AKINγ* to *VHA-A.* Expression below the detectable limit is denoted with *. Error bars show standard deviation. N=6

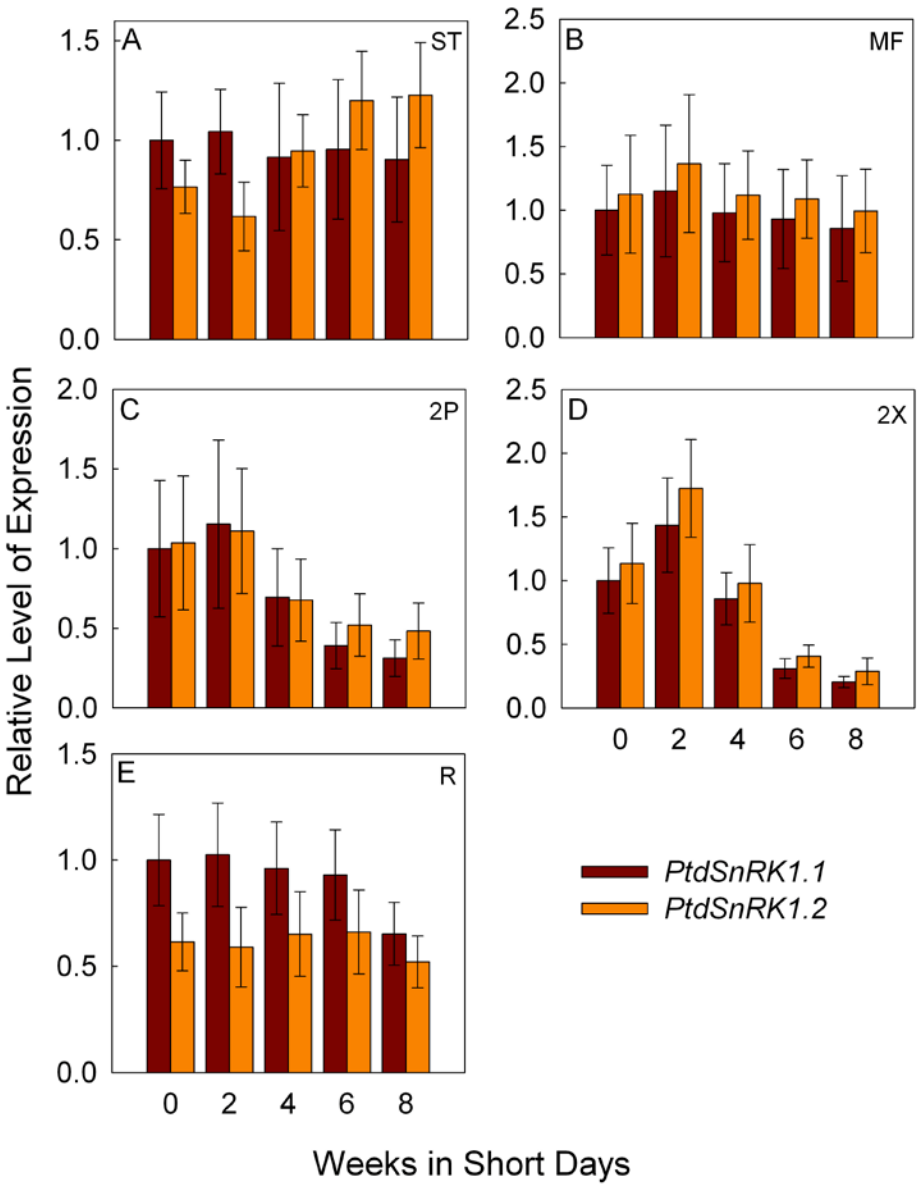

Figure 3.51. Relative level of expression of members of the *PtdSnRK1* gene family in various tissues of poplars undergoing short day-induced dormancy. The relative level of expression was calculated by setting the expression of *PtdSnRK1.1* to 1 in each tissue and normalizing the expression of other members of the *PtdSnRK1* gene family to *PtdSnRK1.1*. (A) Shoot tips (ST). (B) Mature leaves. (MF) (C). Secondary phloem (2P). (D) Secondary xylem (2X). (E) Roots (R). Error bars show standard deviation. N=6

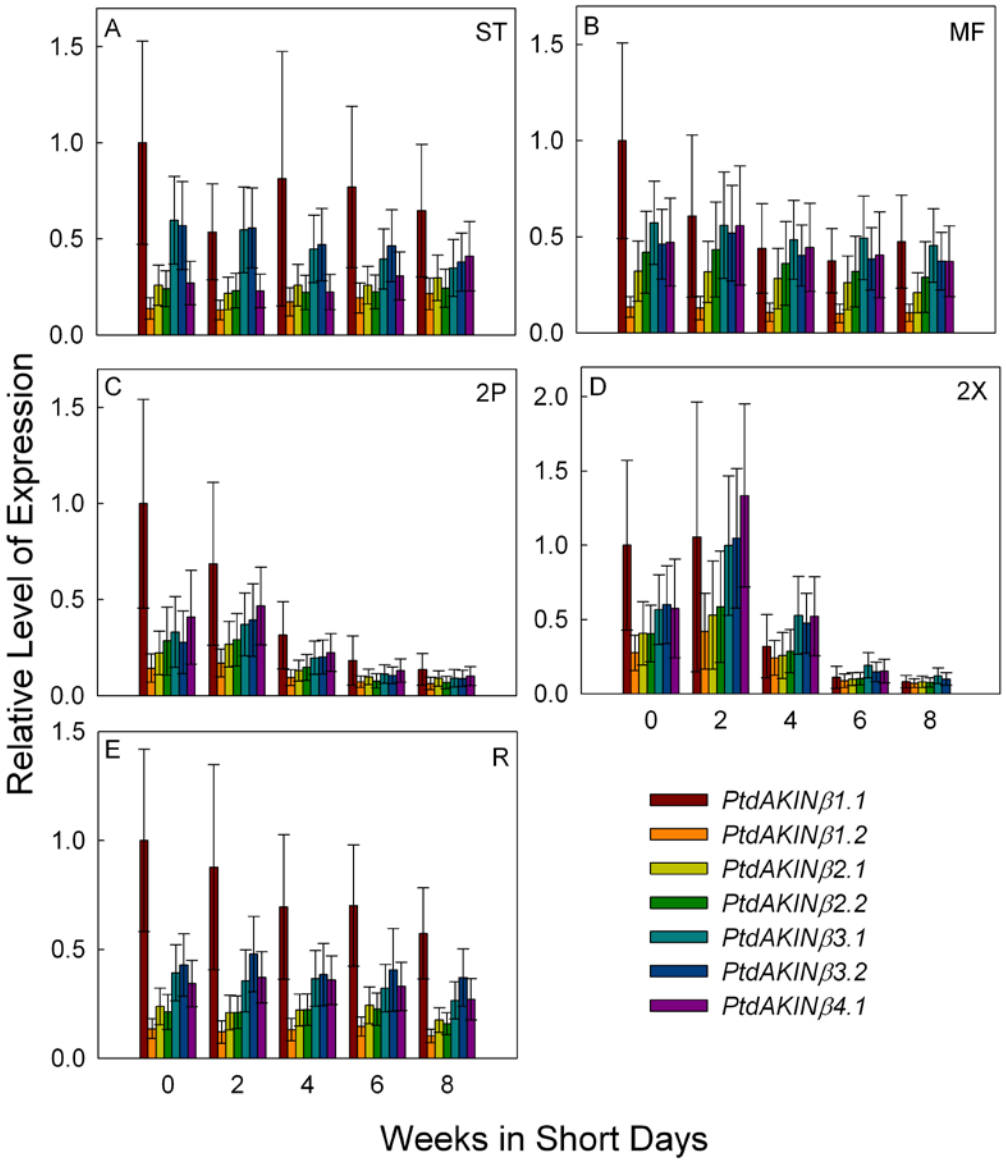

Figure 3.52. Relative level of expression of members of the *PtdAKINβ* gene family in various tissues of poplars undergoing short day-induced dormancy. The relative level of expression was calculated by setting the expression of *PtdAKINβ1.1* to 1 in each tissue and normalizing the expression of other members of the *PtdAKINβ* gene family to *PtdAKINβ1.1*. (A) Shoot tips (ST). (B) Mature leaves. (MF) (C). Secondary phloem (2P). (D) Secondary xylem (2X). (E) Roots (R). Error bars show standard deviation. N=6

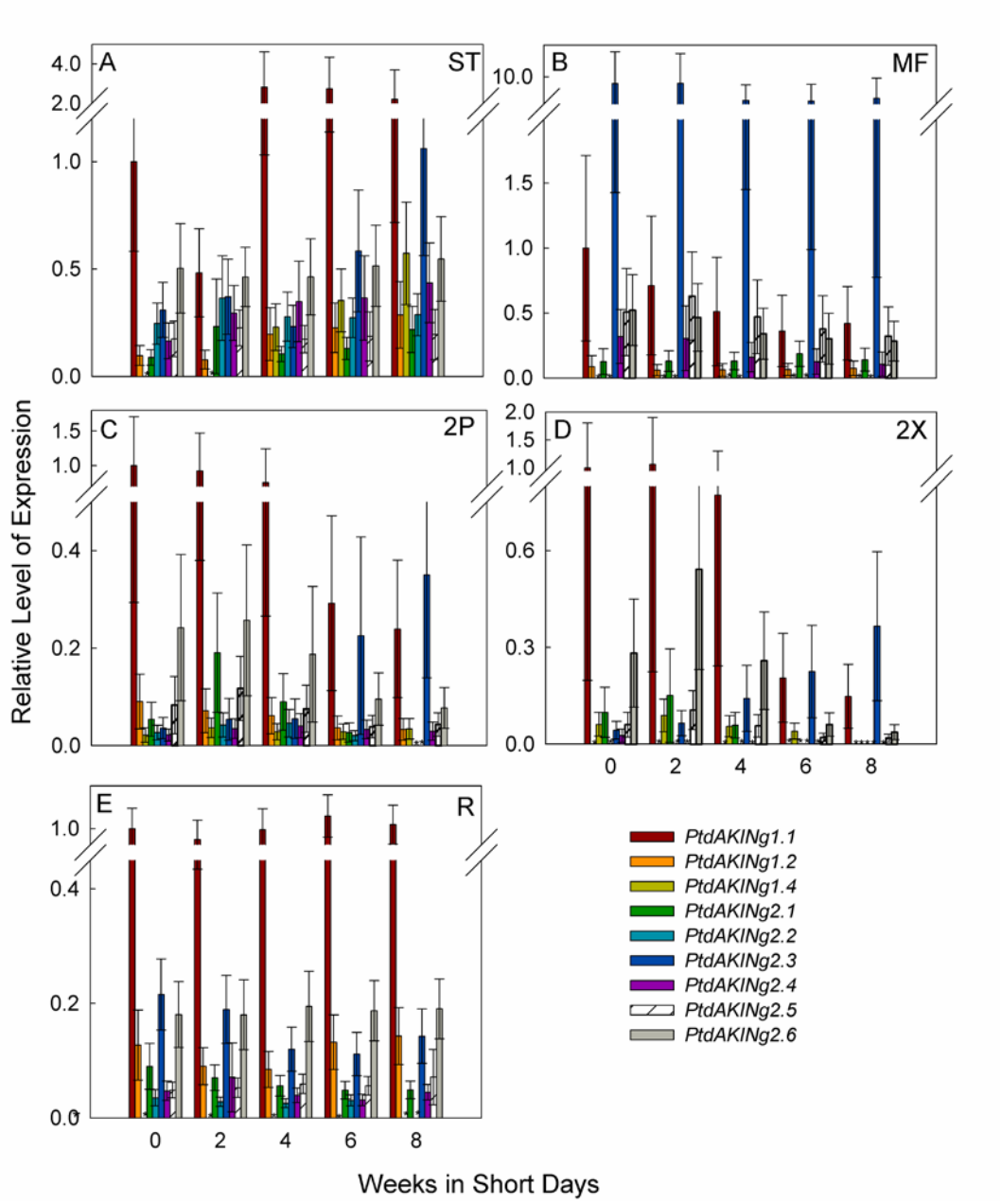

Figure 3.53. Relative level of expression of members of the *PtdAKINγ* gene family in various tissues of poplars undergoing short day-induced dormancy. The relative level of expression was calculated by setting the expression of *PtdAKINγ1.1* to 1 in each tissue and normalizing the expression of other members of the *PtdAKINγ* gene family to *PtdAKINγ1.1*. (A) Shoot tips (ST). (B) Mature leaves. (MF) (C). Secondary phloem (2P). (D) Secondary xylem (2X). (E) Roots (R). Error bars show standard deviation. N=6

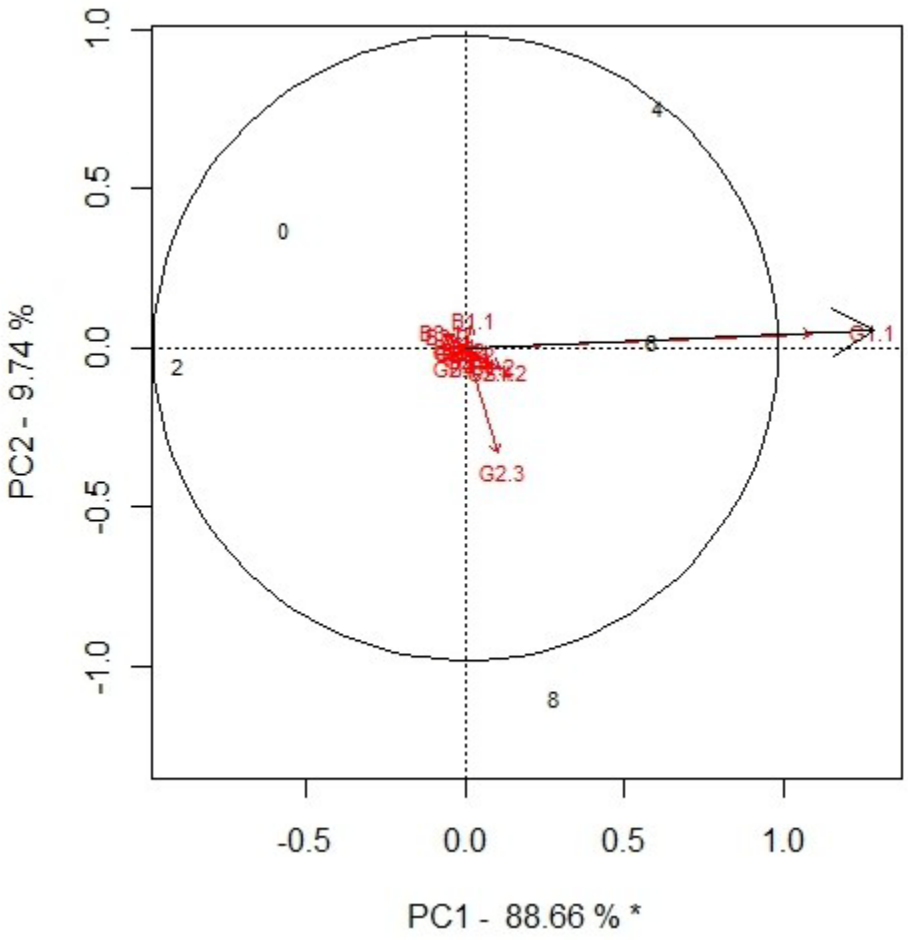

Figure 3.54. Principal component analysis of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in shoot tips of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Principal components which are significant based on the broken stick distribution are denoted with *. Magnitude of the contribution of a particular gene to the variation of the principle components is denoted by the length and direction of the arrow. Arrows which exceed the equilibrium the circle significantly contribute to the variation of the principal components. *PtdAKINγ1.4* was not included as PCA does not accommodate null data entries. Members of the *PtdSnRK1* gene family are denoted with "S" followed by the gene member number. Members of the *PtdAKINβ* gene family are denoted with "B" followed by the gene member number. Members of the *PtdAKINγ* gene family are denoted with "G" followed by the gene member number. Number of weeks in short day are denoted by 0, 2, 4, 6 and 8.

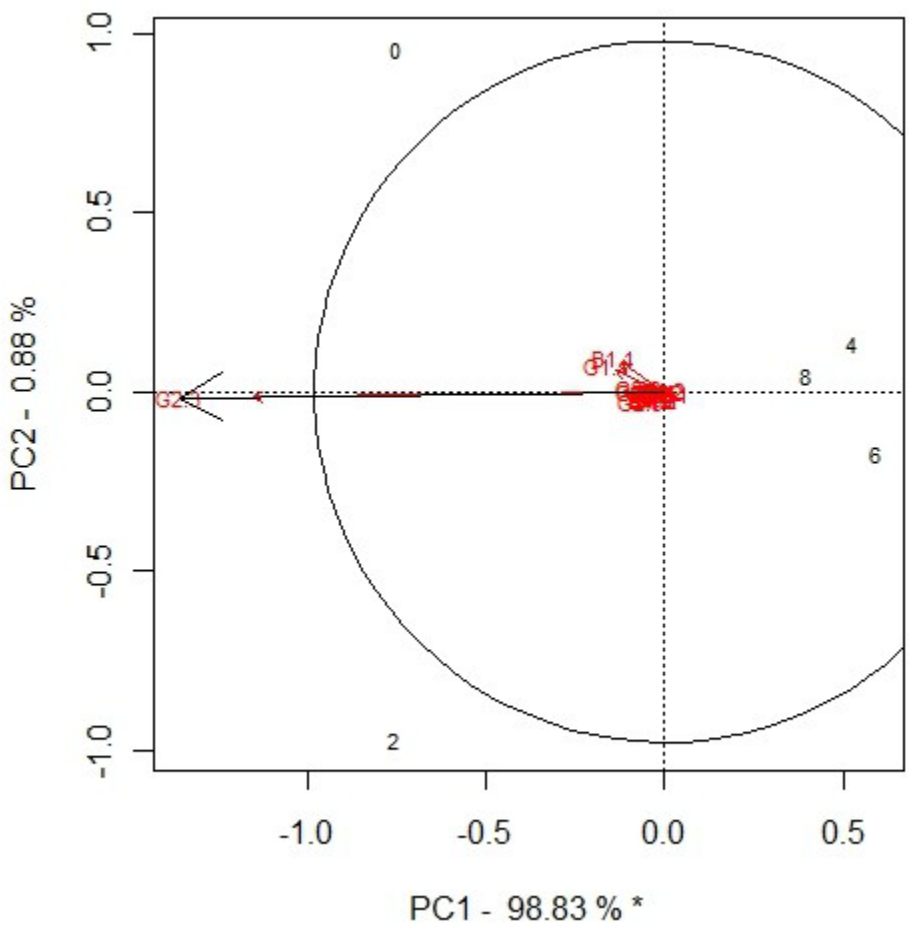

Figure 3.55. Principal component analysis of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in mature leaves of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Principal components which are significant based on the broken stick distribution are denoted with *. Magnitude of the contribution of a particular gene to the variation of the principle components is denoted by the length and direction of the arrow. Arrows which exceed the equilibrium the circle significantly contribute to the variation of the principal components. Members of the *PtdSnRK1* gene family are denoted with "S" followed by the gene member number. Members of the *PtdAKINβ* gene family are denoted with "B" followed by the gene member number. Members of the *PtdAKINγ* gene family are denoted with "G" followed by the gene member number. Number of weeks in short day are denoted by 0, 2, 4, 6 and 8.

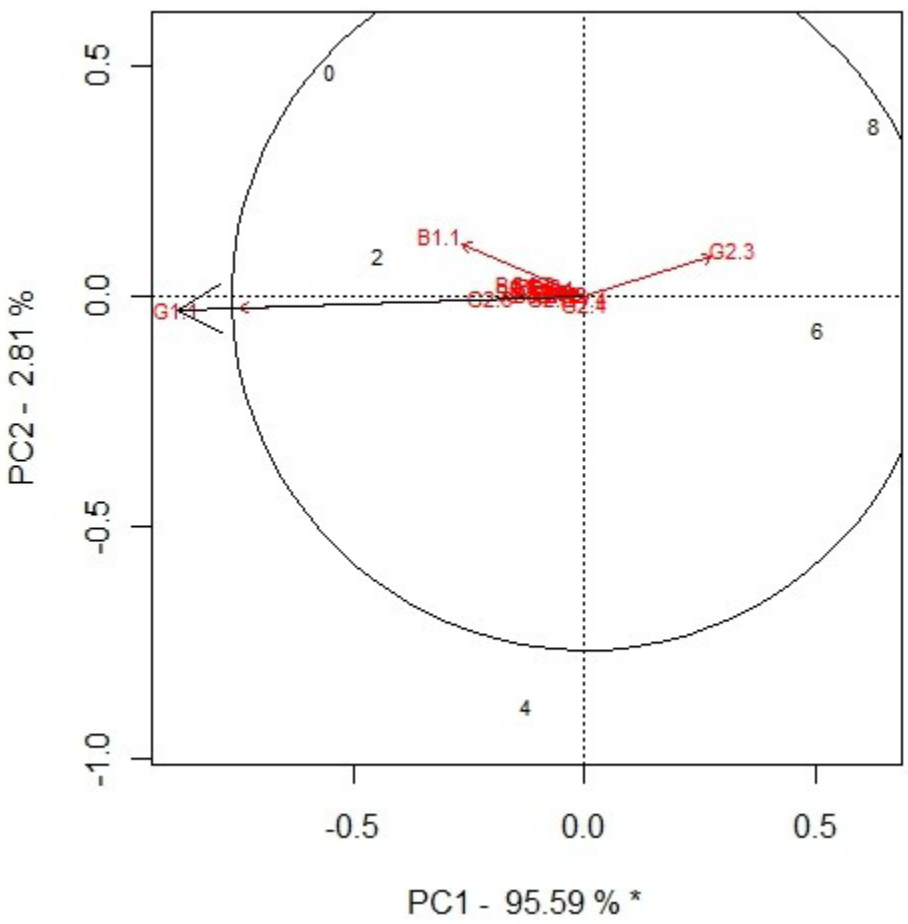

Figure 3.56. Principal component analysis of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in secondary phloem of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Principal components which are significant based on the broken stick distribution are denoted with *. Magnitude of the contribution of a particular gene to the variation of the principle components is denoted by the length and direction of the arrow. Arrows which exceed the equilibrium the circle significantly contribute to the variation of the principal components. *PtdAKINγ2.1* and *PtdAKINγ2.2* were not included as PCA does not accommodate null data entries. Members of the *PtdSnRK1* gene family are denoted with "S" followed by the gene member number. Members of the *PtdAKINβ* gene family are denoted with "B" followed by the gene member number. Members of the *PtdAKINγ* gene family are denoted with "G" followed by the gene member number. Number of weeks in short day are denoted by 0, 2, 4, 6 and 8.

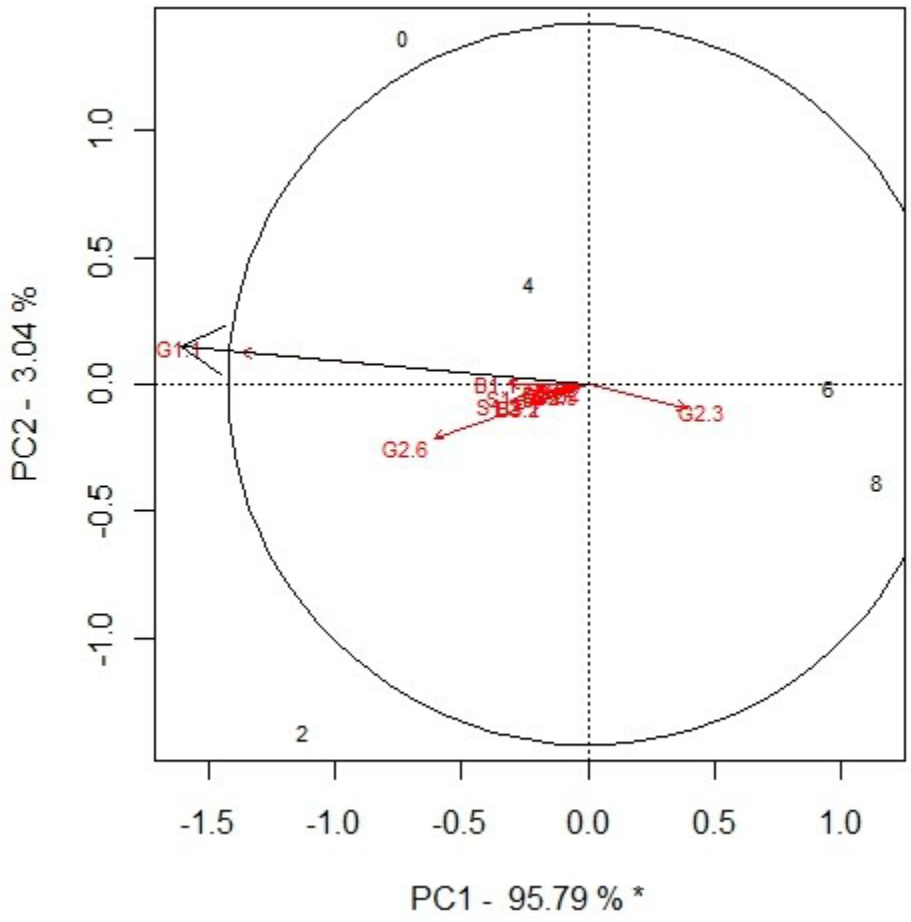

Figure 3.57. Principal component analysis of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in secondary xylem of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Principal components which are significant based on the broken stick distribution are denoted with *. Magnitude of the contribution of a particular gene to the variation of the principle components is denoted by the length and direction of the arrow. Arrows which exceed the equilibrium the circle significantly contribute to the variation of the principal components. *PtdAKINβ4.1*, *PtdAKINγ1.4*, *PtdAKINγ2.1* and *PtdAKINγ2.4* were not included as PCA does not accommodate null data entries. Members of the *PtdSnRK1* gene family are denoted with "S" followed by the gene member number. Members of the *PtdAKINβ* gene family are denoted with "B" followed by the gene member number. Members of the *PtdAKINγ* gene family are denoted with "G" followed by the gene member number. Number of weeks in short day are denoted by 0, 2, 4, 6 and 8.

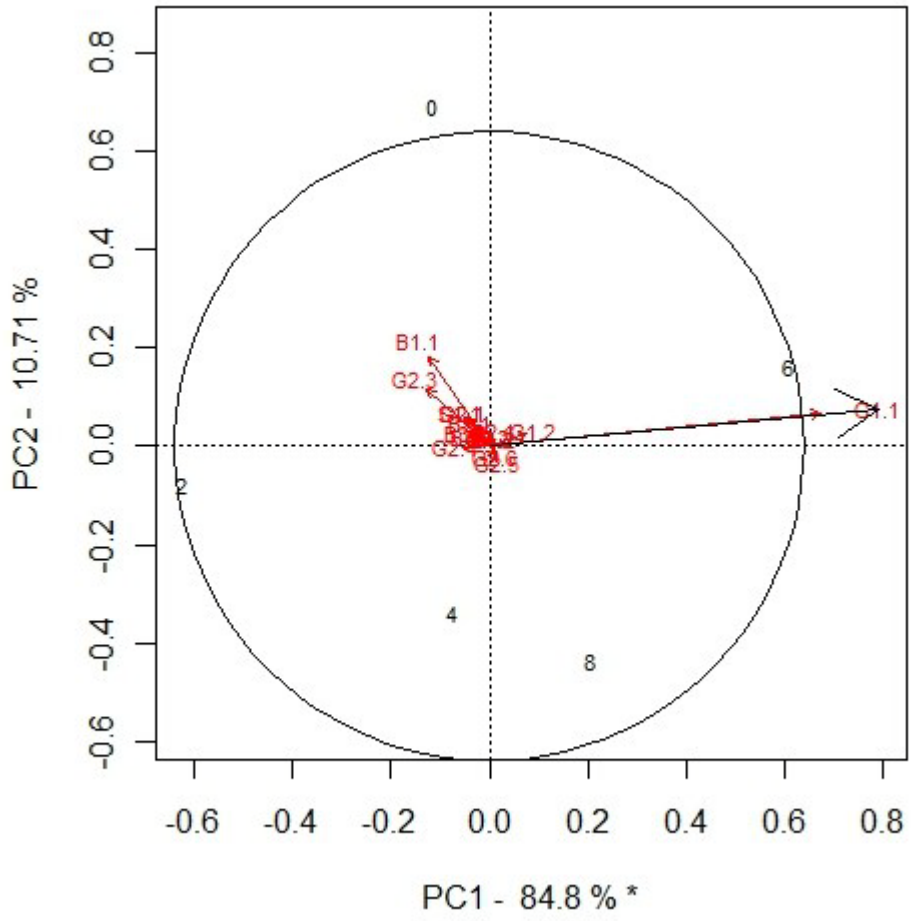

Figure 3.59. Principal component analysis of members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families in roots of poplars undergoing short day-induced dormancy. Poplars were grown in short day conditions (8h light; 16h dark) for up to 8 weeks. Principal components which are significant based on the broken stick distribution are denoted with *. Magnitude of the contribution of a particular gene to the variation of the principle components is denoted by the length and direction of the arrow. Arrows which exceed the equilibrium the circle significantly contribute to the variation of the principal components. *PtdAKINγ2.2* was not included as PCA does not accommodate null data entries. Members of the *PtdSnRK1* gene family are denoted with "S" followed by the gene member number. Members of the *PtdAKINβ* gene family are denoted with "B" followed by the gene member number. Members of the *PtdAKINγ* gene family are denoted with "G" followed by the gene member number. Number of weeks in short day are denoted by 0, 2, 4, 6 and 8.

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4.0. Conclusions

The major goals of this study were (1) to determine if the SnRK1 protein complex potentially plays a role in poplar during the nitrogen response and during dormancy acquisition, and if so (2) whether a subset of the genes encoding members of SnRK1 protein complexes in poplar could be identified for future in-depth study. I hypothesized that the genes for certain subunits would be differentially expressed under different levels of nitrogen availability and under dormancy-inducing short day conditions and that, furthermore, some genes would be expressed minimally while others would be expressed at high levels in different tissues. To test these hypotheses, I carried out gene expression profiling by qRT-PCR. I cloned three *PtdSnRK1* and nine *PtdAKINγ* cDNAs, and used these together with seven previously cloned *PtdAKINβ* cDNAs to design gene specific primers for qRT-PCR. Appropriate reference genes for qRT-PCR were also identified and robust qRT-PCR assays developed. I then used qRT-PCR to investigate the changes in gene expression profiles of the subunits of the PtdSnRK1 protein complex in several tissues of poplar in response to differential nitrogen availability and during dormancy acquisition in order to determine if the SnRK1 protein complex plays a role in processes involved in the response to nitrogen availability and during dormancy acquisition. Gene expression profiles were also directly compared between suites of different tissues from trees grown under standard conditions in order to provide further insight into possible roles for

subunits making up these SnRK1 protein kinase complexes. Data were analyzed by ANOVA and PCA as a means to determine whether the observed patterns were statistically significant.

As hypothesized, it was found that a subset of the investigated genes showed variation in transcript abundance across different tissues, under conditions of differential nitrogen availability and during dormancy acquisition. As also hypothesized, different members of the *PtdSnRK1*, *PtdAKINβ*, and *PtdAKINγ* gene families showed patterns of transcript abundance that were distinct from other members. For instance, in the nitrogen availability experiment, the putative paralogues *PtdAKINβ1.1* and *PtdAKINβ1.2* showed contrasting expression profiles, suggesting that neofunctionalization or subfunctionalization had occurred. There are also indications that functional redundancy may have been retained between paralogues, as some pairs of paralogous genes do show very similar patterns of gene expression to each other across the three different experiments. These include the catalytic subunits *PtdSnRK1.1* and *PtdSnRK1.2*. This does not mean, however, that neofunctionalization or subfunctionalization have not occurred, as post-translational regulation or response to other stimuli were not investigated. Furthermore, similarity of expression pattern does not take into account magnitude of transcript abundance. For instance, though the expression patterns of *PtdAKINγ1.1* and *PtdAKINγ1.2* are often similar, the abundance of transcripts of *PtdAKINγ1.1* is much higher than that of *PtdAKINγ1.2*. The differences in magnitude of abundance between putatively

paralogous genes could indicate that the gene which is expressed at lower levels has lost functions over time, perhaps becoming very specialized or perhaps simply losing function until eventually it will become a pseudogene. Alternatively, the gene which is expressed in greater quantity may have acquired new functions which require that it be expressed at higher levels.

The differential expression patterns exhibited by these genes suggest that SnRK1 complexes are modified during the nitrogen response and dormancy acquisition. These results suggest that SnRK1 complexes with specific subunits could be involved in regulating molecular and biochemical processes that occur during the nitrogen response and dormancy acquisition. During differential nitrogen availability, most genes in the tissues studied were expressed at lower abundance under conditions of high nitrogen availability, with the exceptions of *PtdAKINγ1.1*, *PtdAKINγ1.2* and *PtdAKINβ1.1*. The increased expression of these three genes in various tissues as well as the lower abundance of transcripts corresponding to other genes suggests that under conditions of high nitrogen availability there is a shift in plant processes, with processes regulated by *PtdAKINγ1.1*, *PtdAKINγ1.2* and *PtdAKINβ1.1* taking precedence over other processes which might be regulated by the other members of the *PtdSnRK1*, *PtdAKINβ* and *PtdAKINγ* gene families. This could also indicate that other genes are not entirely redundant and are involved in other processes which are not as urgent under conditions of high nitrogen availability, and are therefore downregulated. In either case, this suggests that the PtdSnRK1 protein complex is

composed of specific subunits, and that this composition plays a role in determining the function of the complex.

During dormancy acquisition, gene expression profiles showed more variability between tissues. For instance, in secondary phloem and secondary xylem, the abundance of nearly all genes decreased during short day conditions, while this was not the case with other tissues. In shoot tips, expression profiles of various genes changed at about the four week time point, coinciding with dynamic changes which have been identified in other studies. The variability of gene expression profiles between tissues suggests that there are tissue specific processes which occur during dormancy acquisition and that the PtdSnRK1 complex may be involved in the regulation of these different processes. As under conditions of differential nitrogen availability the PtdSnRK1 complex is likely composed of different α, β and γ subunits in its role, as different genes responded in different tissues. For instance, *PtdAKINγ1.4* was found to be expressed at relatively high levels only in shoot tips during the later weeks of dormancy acquisition, suggesting that there is an increased abundance of PtdSnRK1 protein complexes which include PtdAKINγ1.4. It would be interesting to investigate if the abundance of *PtdAKINγ1.4* persists during dormancy and if the abundance decreases during dormancy release.

In order to facilitate the identification of a subset of genes to be targeted for future study, principal component analyses were conducted to determine which gene expression profiles were most able to distinguish treatments from each other.

It was determined that *PtdAKINβ1.1*, *PtdAKINγ1.1* and *PtdAKINγ2.3* were often significantly responsible for distinguishing different treatments from each other. This allows the inference that *PtdAKINβ1.1*, *PtdAKINγ1.1* and *PtdAKINγ2.3* may participate in forming PtdSnRK1 protein complexes which merit further inquiry. It is interesting to note that, in many cases, *PtdAKINβ1.1*, *PtdAKINγ1.1* and *PtdAKINγ2.3* are often more abundant than other members of their respective gene families. It is possible that this is because other genes respond to more specific stimuli which were not explored in this study. It is also possible that *PtdAKINβ1.1*, *PtdAKINγ1.1* and *PtdAKINγ2.3* are involved in processes which were fundamental in the treatments of the three experiments of this study. It is certainly tempting to think that, because of the increased abundance, these three genes have even undergone neofunctionalization while others have undergone a gradual loss of function.

In summary, differential expression profiles indicate that the SnRK1 protein complex in poplar responds to differential nitrogen availability and dormancy acquisition in poplar. Furthermore, the subunits PtdAKINβ1.1, PtdAKINγ1.1 and PtdAKINγ2.3 may be involved in the formation of PtdSnRK1 protein complexes which are involved in roles associated with nitrogen response and dormancy acquisition. To further characterize PtdSnRK1, several experiments can be conducted:

1. Yeast two-hybrid assays can be used to confirm interaction of PtdAKINβ1.1, PtdAKINγ1.1 and PtdAKINγ2.3 with a PtdSnRK1

catalytic subunit and with each other.

2. Confirmation and specificity of kinase activity of the PtdSnRK1 protein complex can be confirmed using kinase assays with known SnRK1 targets.
3. Immunohistochemical staining can be used to determine the protein localization of PtdAKINβ1.1, PtdAKINγ1.1 and PtdAKINγ2.3 in tissues.
4. Transgenic poplars over- and underexpressing *PtdAKINβ1.1*, *PtdAKINγ1.1* and *PtdAKINγ2.3* can be characterized under conditions of differential nitrogen availability and during dormancy acquisition. Changes to the transcriptome could be investigated using microarrays.

5.0. Appendix

5.1. R Script provided by Patrick James

```
## Install Libraries

library(vegan) ## Library that performs PCA analysis
library(BiodiversityR) ## Additional library that computes PCA significance

##
rm(list=ls(all=TRUE)) ## deletes all files in work space - so that you start clean
gc(T) ## garbage collection (memory mgmt.)

## Set Working directory

baseDir <- "C:/INSERT_DESTINATION"  ## Assign variable baseDir to desires address
setwd(baseDir) ## Use function "setwd" to set the working directory

## Read in Data
X <- read.table("geneData1.txt", header=T, row.names=1)  # "geneData1.txt" is what I named the file.

head(X) ## to see the first 10 rows of the data set.
summary(X) ## summarizes the data set by column

## # Simple pca of gene responses in response to different treatments
# The "scale" argument is important - F (false) means that the responses are not standardized and assumes
# that the different responses are in the same units.
# If set to "T" (true) it scales the responses to mean=0, and var=1.  Try both and note the differernes.
```

```
RDA1 <- vegan::rda(X, scale=F)

# Here is the opposite plot - that is, a PCA of treatments, organized according to different genes.
# Here the "t" = matrix transpose.  Same issues related to scaling apply.  Type in "?rda" to get the full story.

# RDA1 <- vegan::rda(t(X), scale=F)

## Assess significance of axes
bj <- PCAsignificance(RDA1)   ## from BiodiversityR

## Biplot - plot with both sites and sspecies

## 1 - Set plotting parameters
par(mfrow=c(1,1), pty='s')

## 2 - Set axes labels using values from 'bj'
lab1 <- paste("PC1 - ", round(bj[2,1], 2), "% *")
lab2 <- paste("PC2 - ", round(bj[2,2], 2), "%")

  ## 3 - Set main title
  mainTitle <- "PCA - Gene Expression"

## 4 - Call actual biplot function
plot1 <- biplot(RDA1, type='text', main=mainTitle, xlab=lab1, ylab=lab2, cex=0.4)

## 5 - Include actual points - can be commented out.
points(RDA1, display='sites', pch=19, col='blue')
```

6 - Draw significance circle - loadings that exceed the circle are 'significant' relative to the broken stick criterion.

```
ordiequilibriumcircle(RDA1, plot1)
```

end./

5.2. Melt curves for *PtdSnRK1* and *PtdAKINγ* gene family members

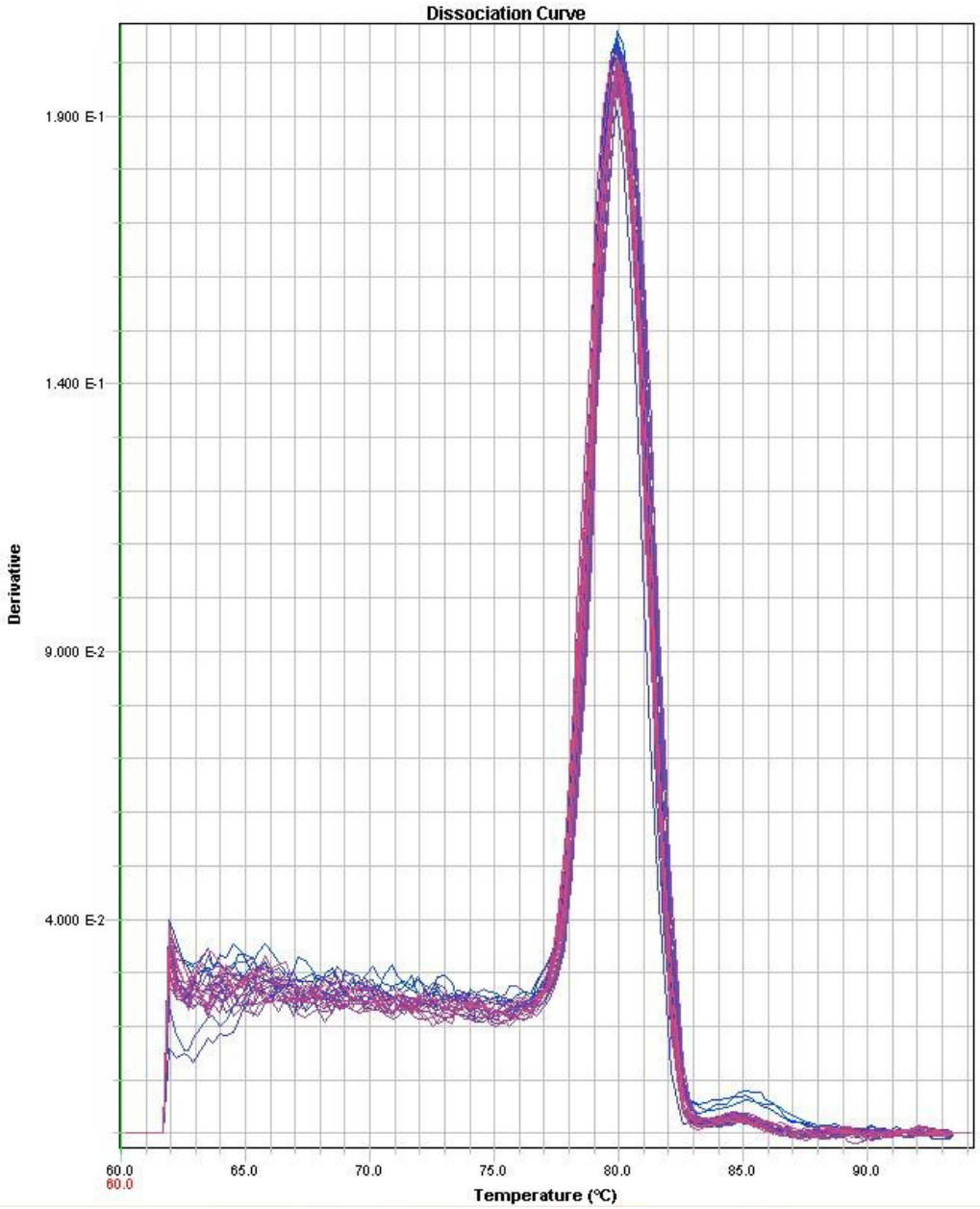

Figure A.1. Melt curve of amplification product using *PtdSnRK1.1* specific qRT-PCR primers. A qRT-PCR assay using *PtdSnRK1.1* qRT-PCR primers and a dilution series composed of members of the *PtdSnRK1* gene family was conducted to determine if multiple products were amplified. A single peak indicates the amplification of a single product.

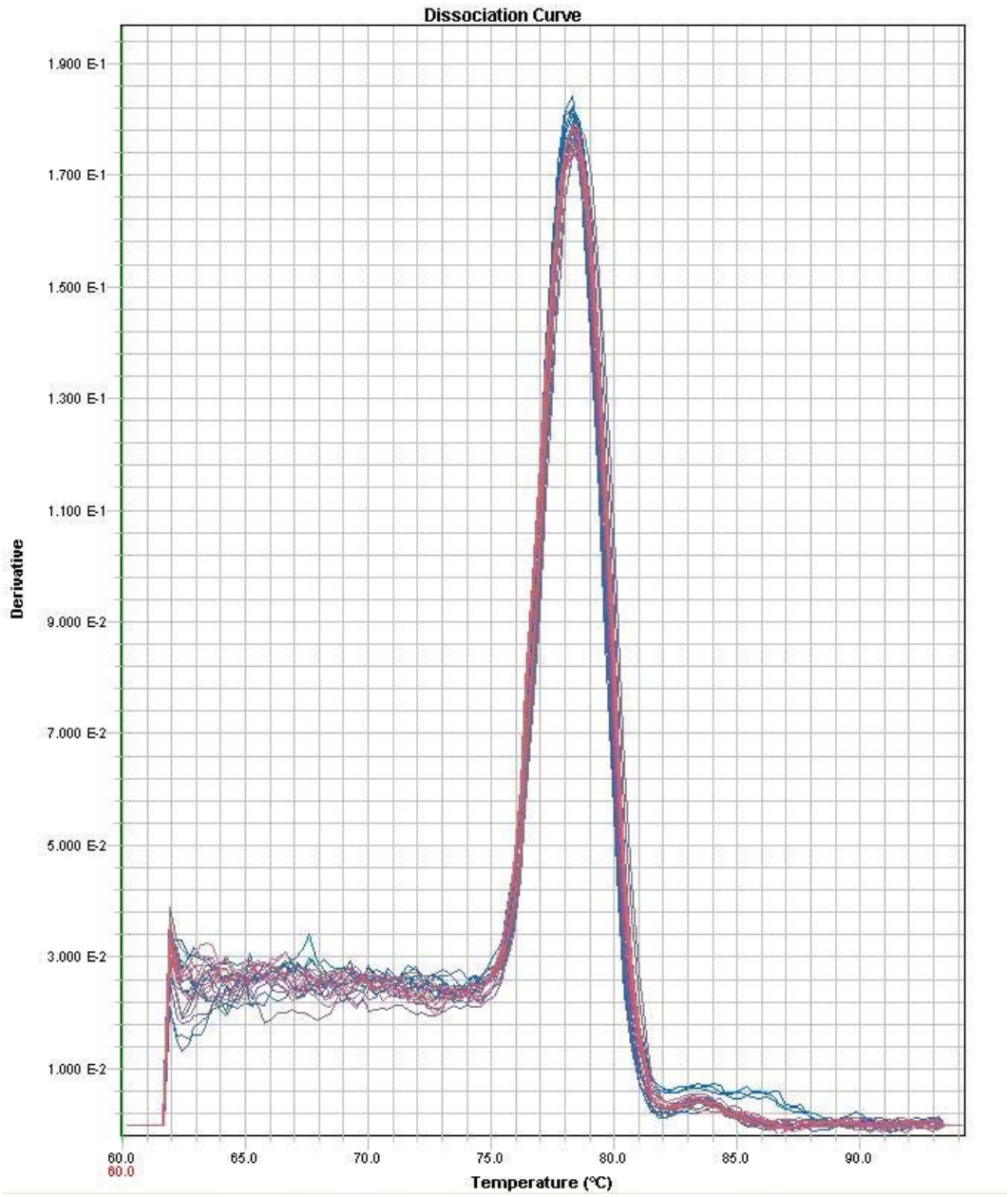

Figure A.2. Melt curve of amplification product using *PtdSnRK1.2* specific qRT-PCR primers. A qRT-PCR assay using *PtdSnRK1.2* qRT-PCR primers and a dilution series composed of members of the *PtdSnRK1* gene family was conducted to determine if multiple products were amplified. A single peak indicates the amplification of a single product.

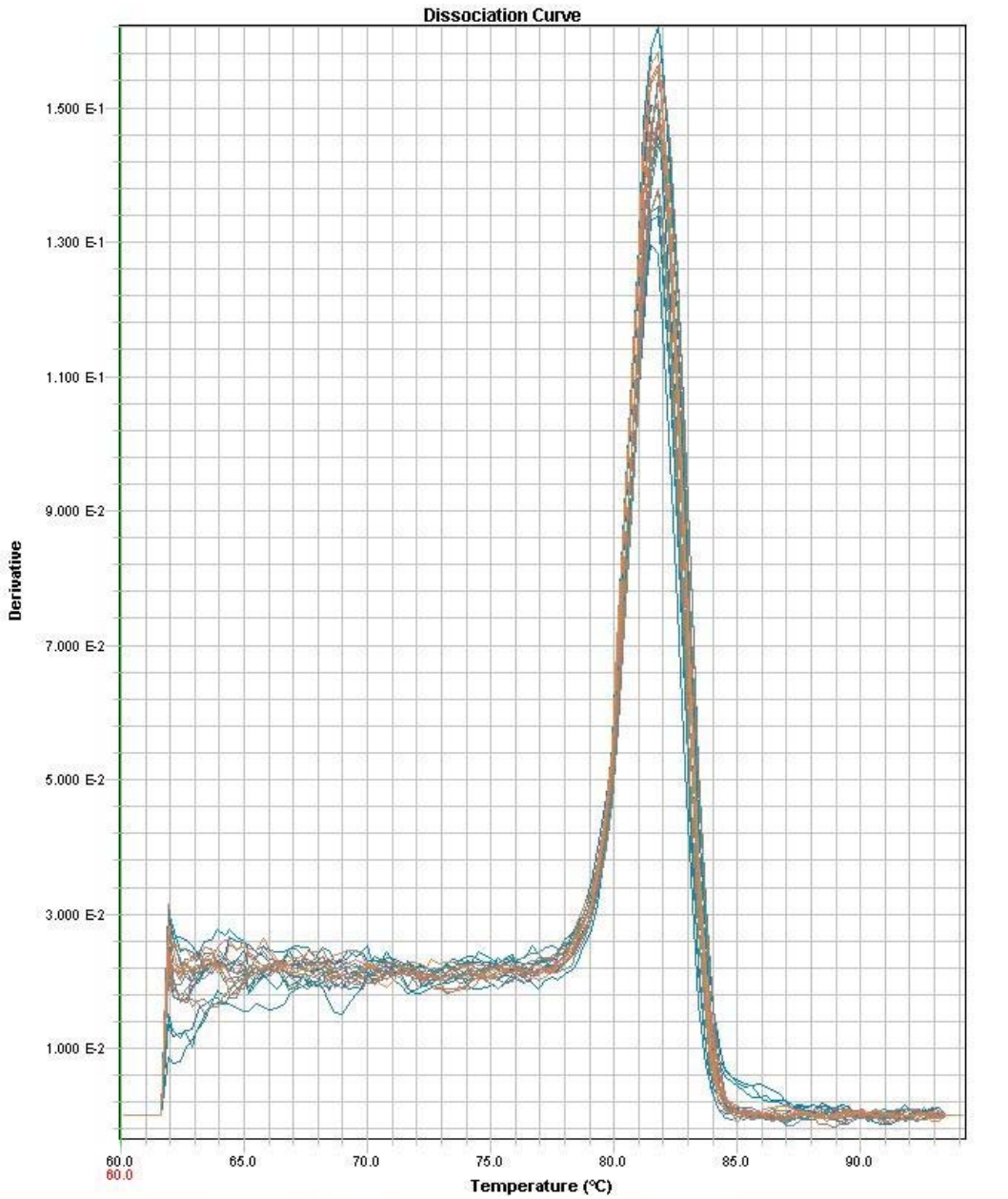

Figure A.3. Melt curve of amplification product using *PtdSnRK1.3* specific qRT-PCR primers. A qRT-PCR assay using *PtdSnRK1.3* qRT-PCR primers and a dilution series composed of members of the *PtdSnRK1* gene family was conducted to determine if multiple products were amplified. A single peak indicates the amplification of a single product.

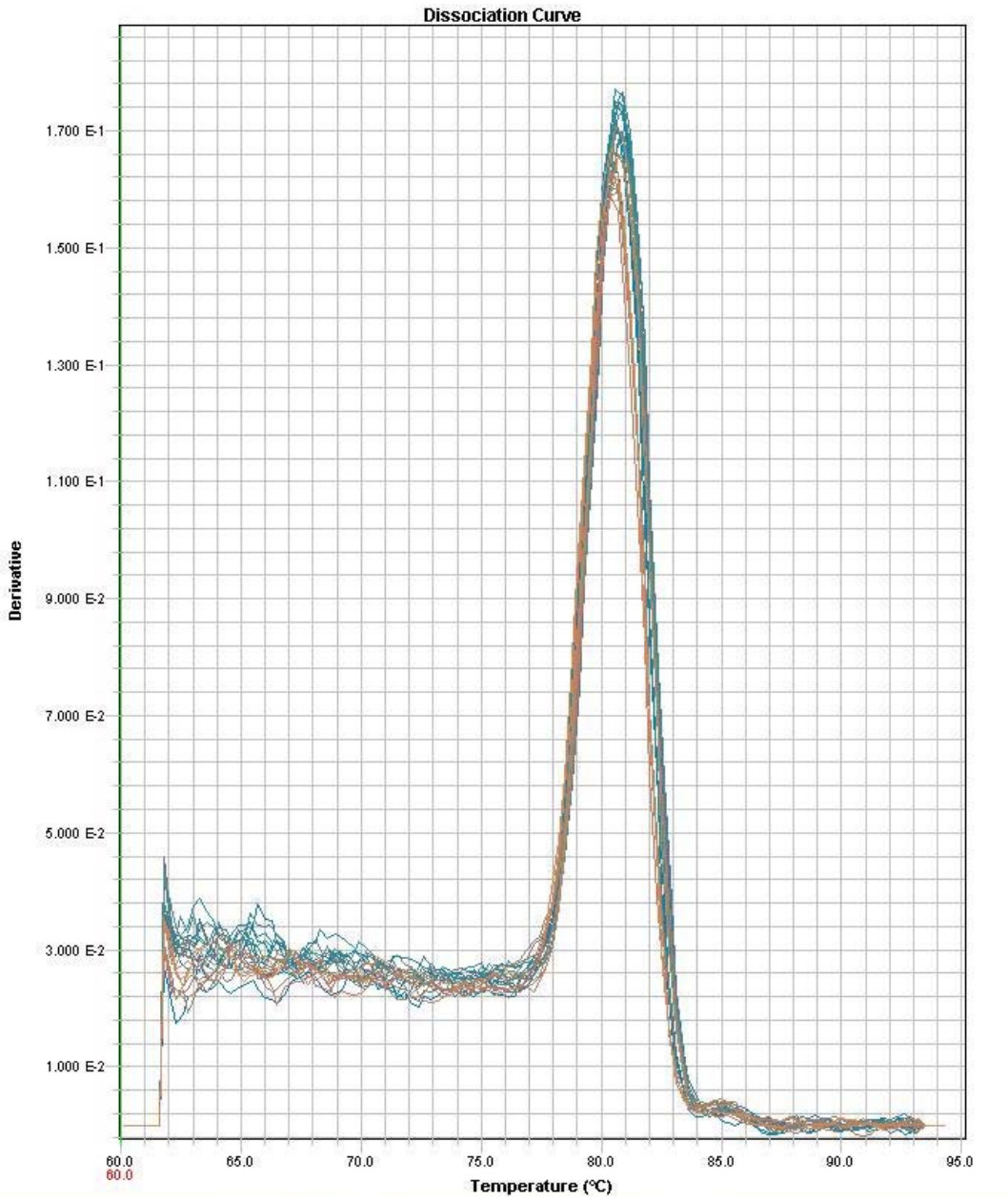

Figure A.4. Melt curve of amplification product using *PtdAKINγ1.1* specific qRT-PCR primers. A qRT-PCR assay using *PtdAKINγ1.1* qRT-PCR primers and a dilution series composed of members of the *PtdAKINγ* gene family was conducted to determine if multiple products were amplified. A single peak indicates the amplification of a single product.

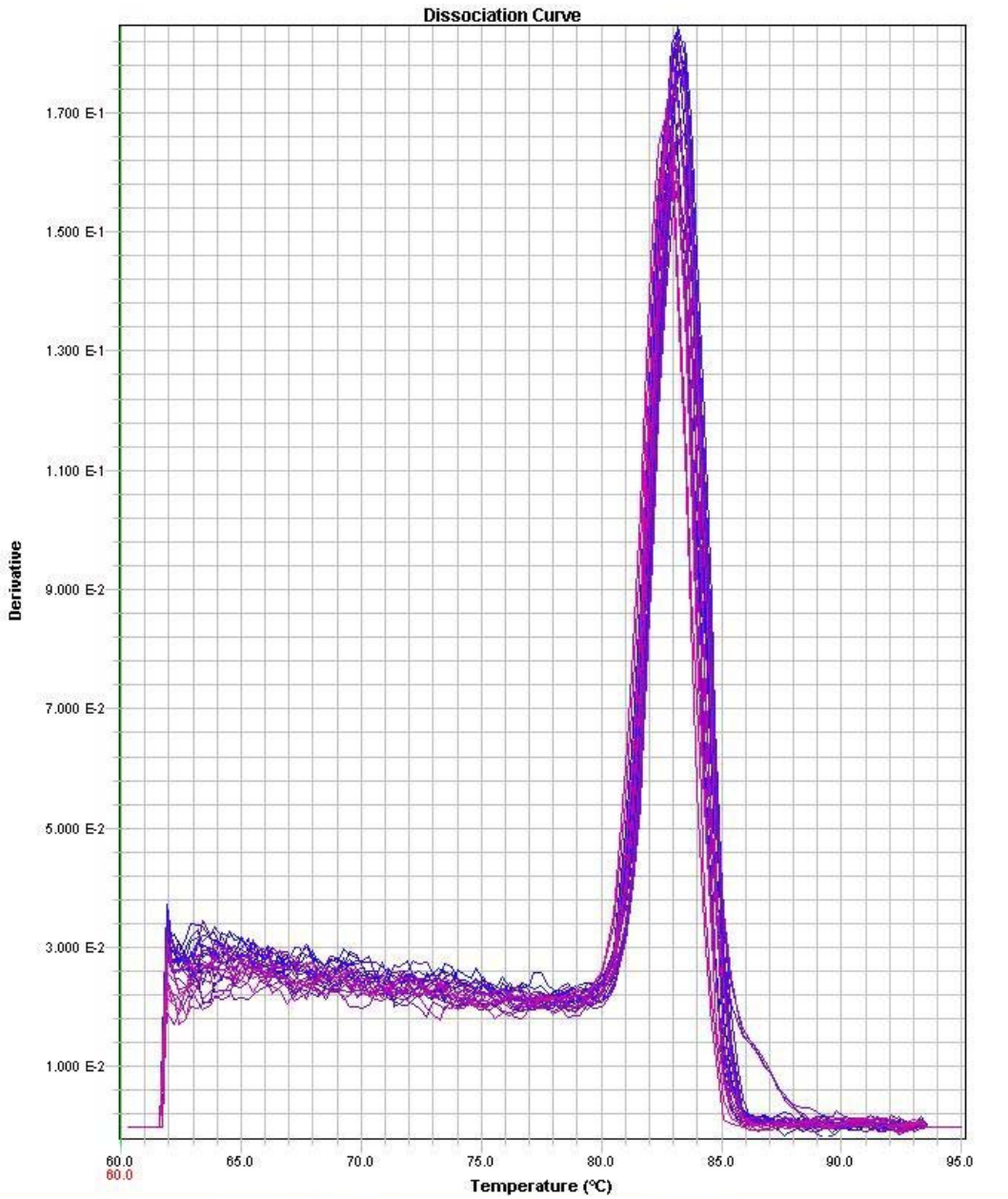

Figure A.5. Melt curve of amplification product using *PtdAKINγ1.2* specific qRT-PCR primers. A qRT-PCR assay using *PtdAKINγ1.2* qRT-PCR primers and a dilution series composed of members of the *PtdAKINγ* gene family was conducted to determine if multiple products were amplified. A single peak indicates the amplification of a single product.

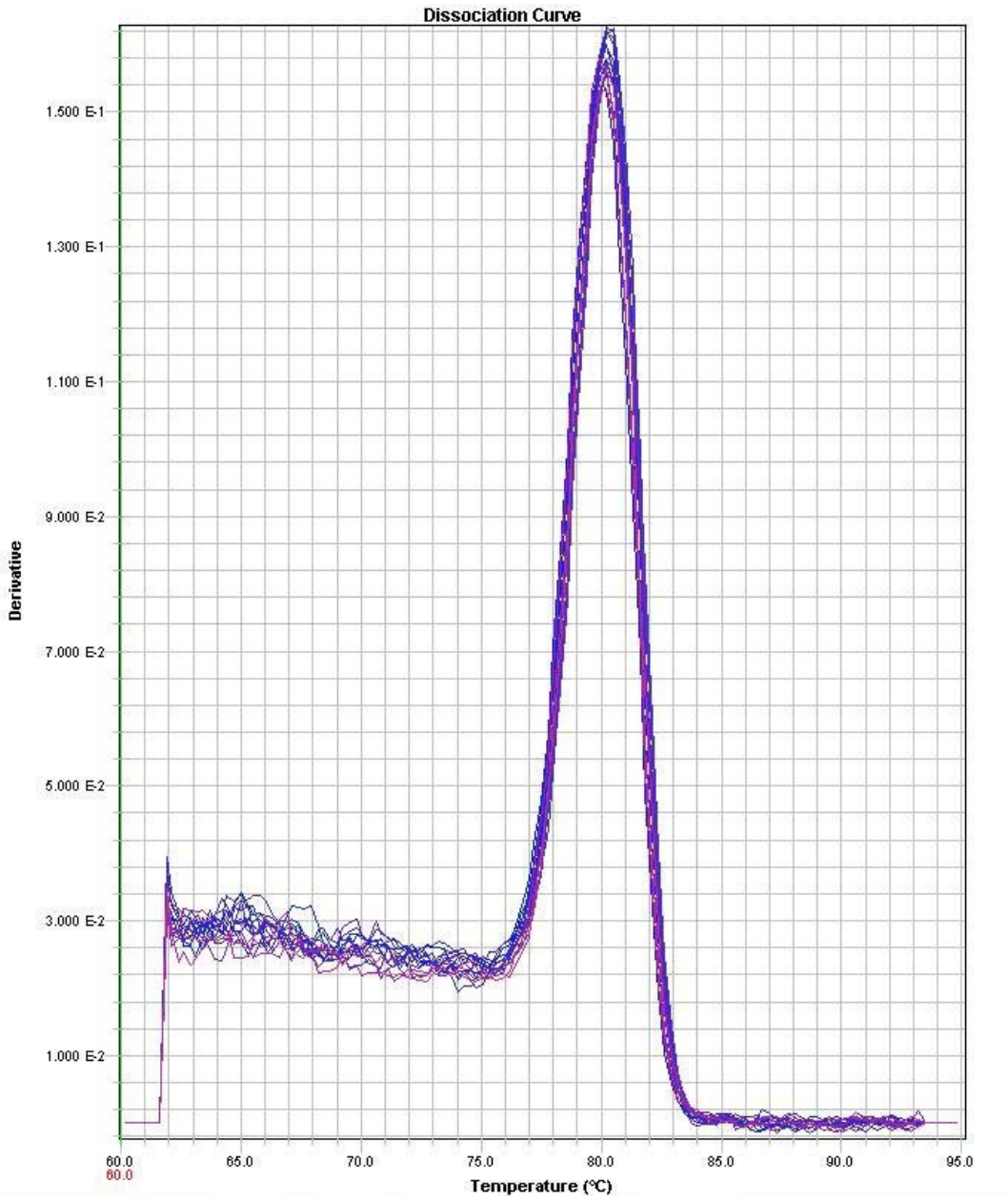

Figure A.6. Melt curve of amplification product using *PtdAKINγ1.4* specific qRT-PCR primers. A qRT-PCR assay using *PtdAKINγ1.4* qRT-PCR primers and a dilution series composed of members of the *PtdAKINγ* gene family was conducted to determine if multiple products were amplified. A single peak indicates the amplification of a single product.

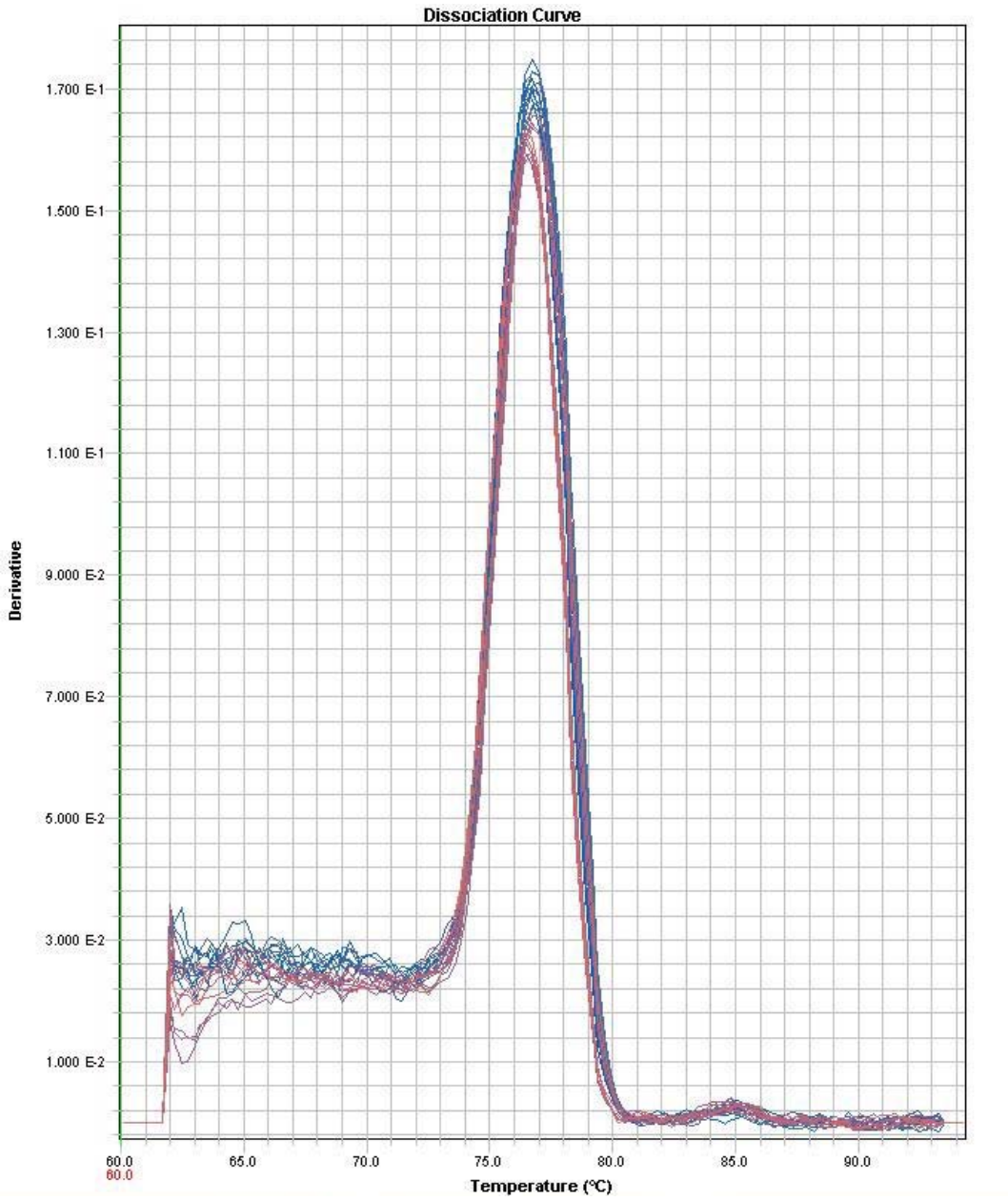

Figure A.7. Melt curve of amplification product using *PtdAKINγ2.1* specific qRT-PCR primers. A qRT-PCR assay using *PtdAKINγ2.1* qRT-PCR primers and a dilution series composed of members of the *PtdAKINγ* gene family was conducted to determine if multiple products were amplified. A single peak indicates the amplification of a single product.

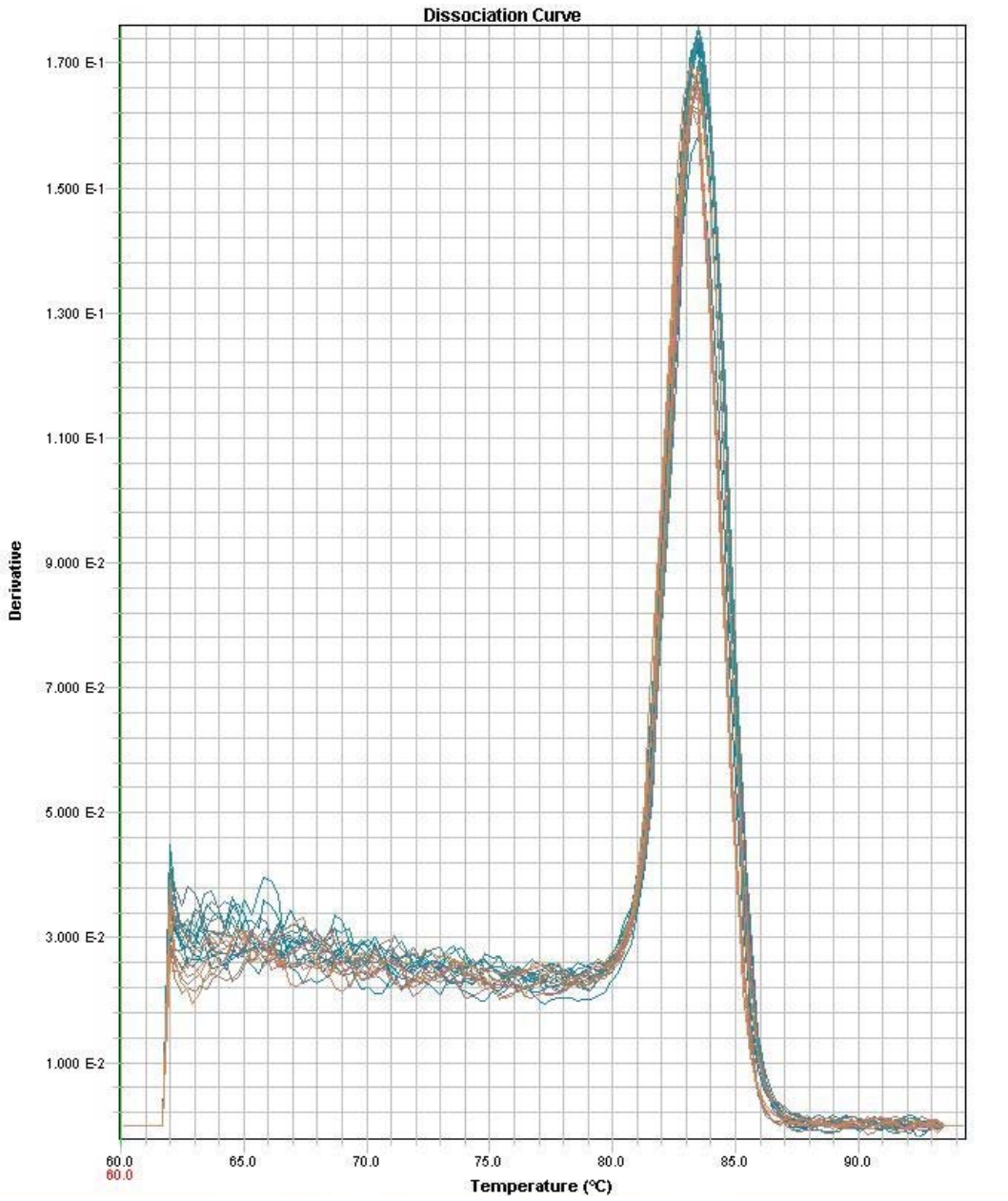

Figure A.8. Melt curve of amplification product using *PtdAKINγ2.2* specific qRT-PCR primers. A qRT-PCR assay using *PtdAKINγ2.2* qRT-PCR primers and a dilution series composed of members of the *PtdAKINγ* gene family was conducted to determine if multiple products were amplified. A single peak indicates the amplification of a single product.

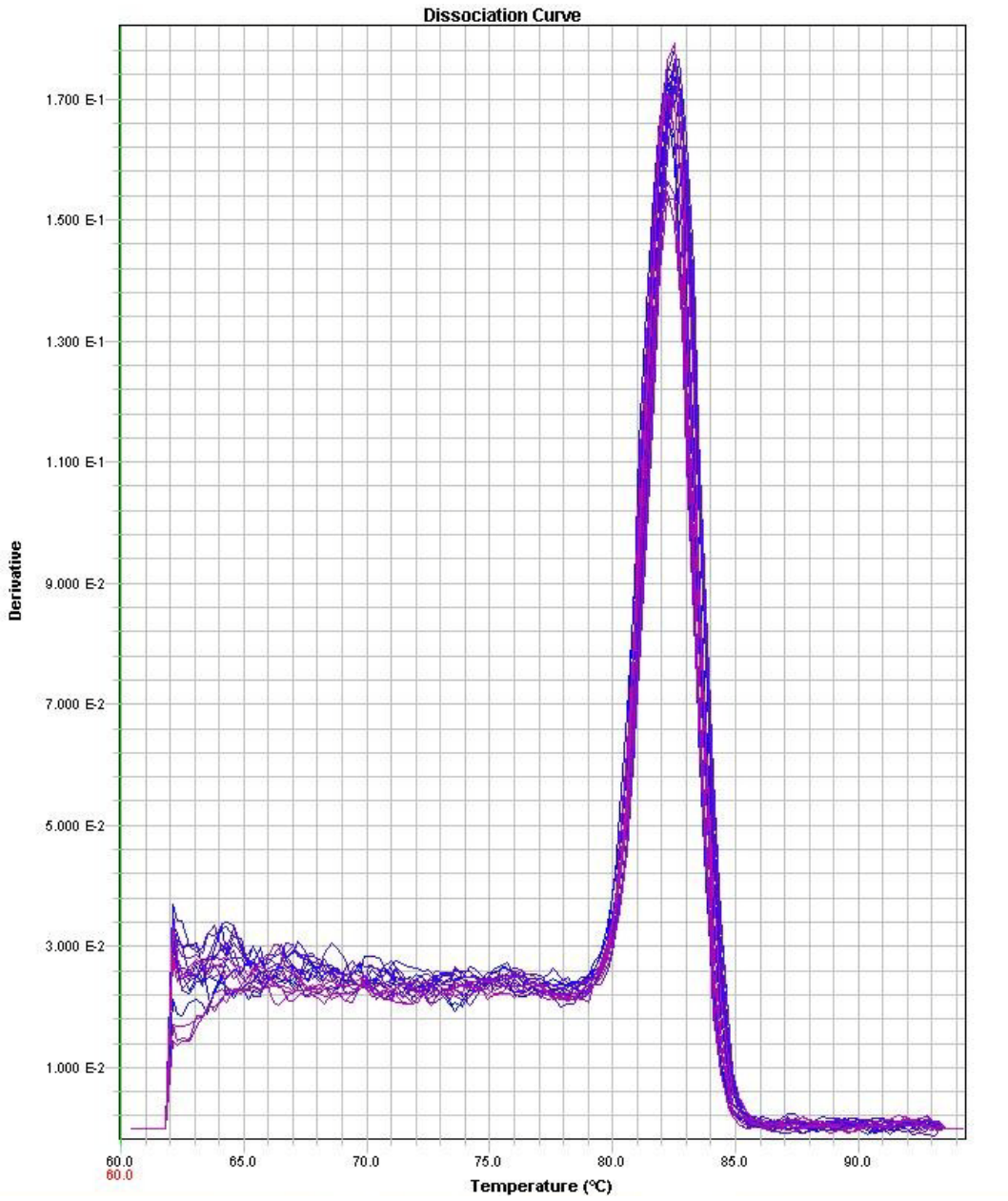

Figure A.9. Melt curve of amplification product using *PtdAKINγ2.3* specific qRT-PCR primers. A qRT-PCR assay using *PtdAKINγ2.3* qRT-PCR primers and a dilution series composed of members of the *PtdAKINγ* gene family was conducted to determine if multiple products were amplified. A single peak indicates the amplification of a single product.

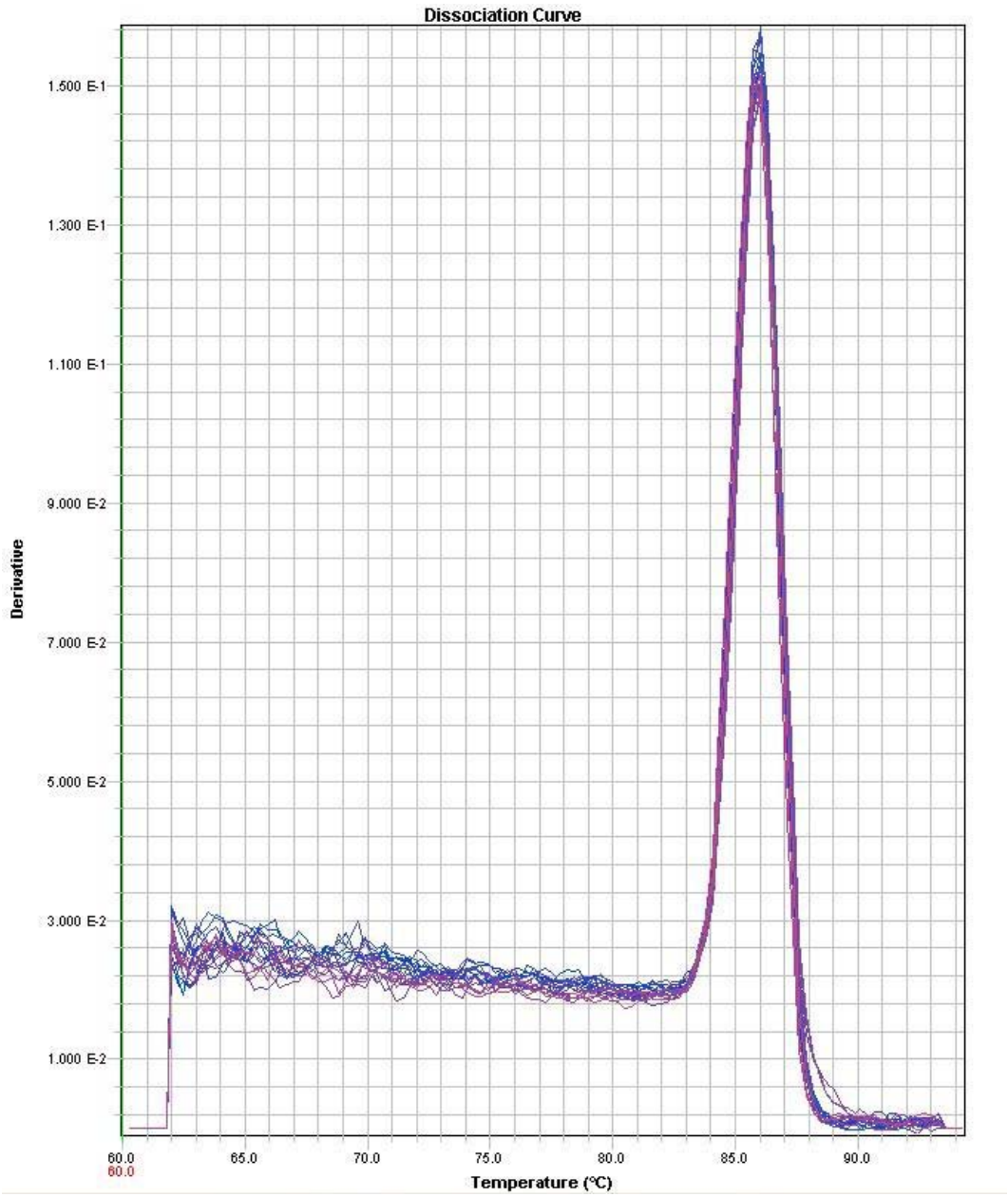

Figure A.10. Melt curve of amplification product using *PtdAKINγ2.4* specific qRT-PCR primers. A qRT-PCR assay using *PtdAKINγ2.4* qRT-PCR primers and a dilution series composed of members of the *PtdAKINγ* gene family was conducted to determine if multiple products were amplified. A single peak indicates the amplification of a single product.

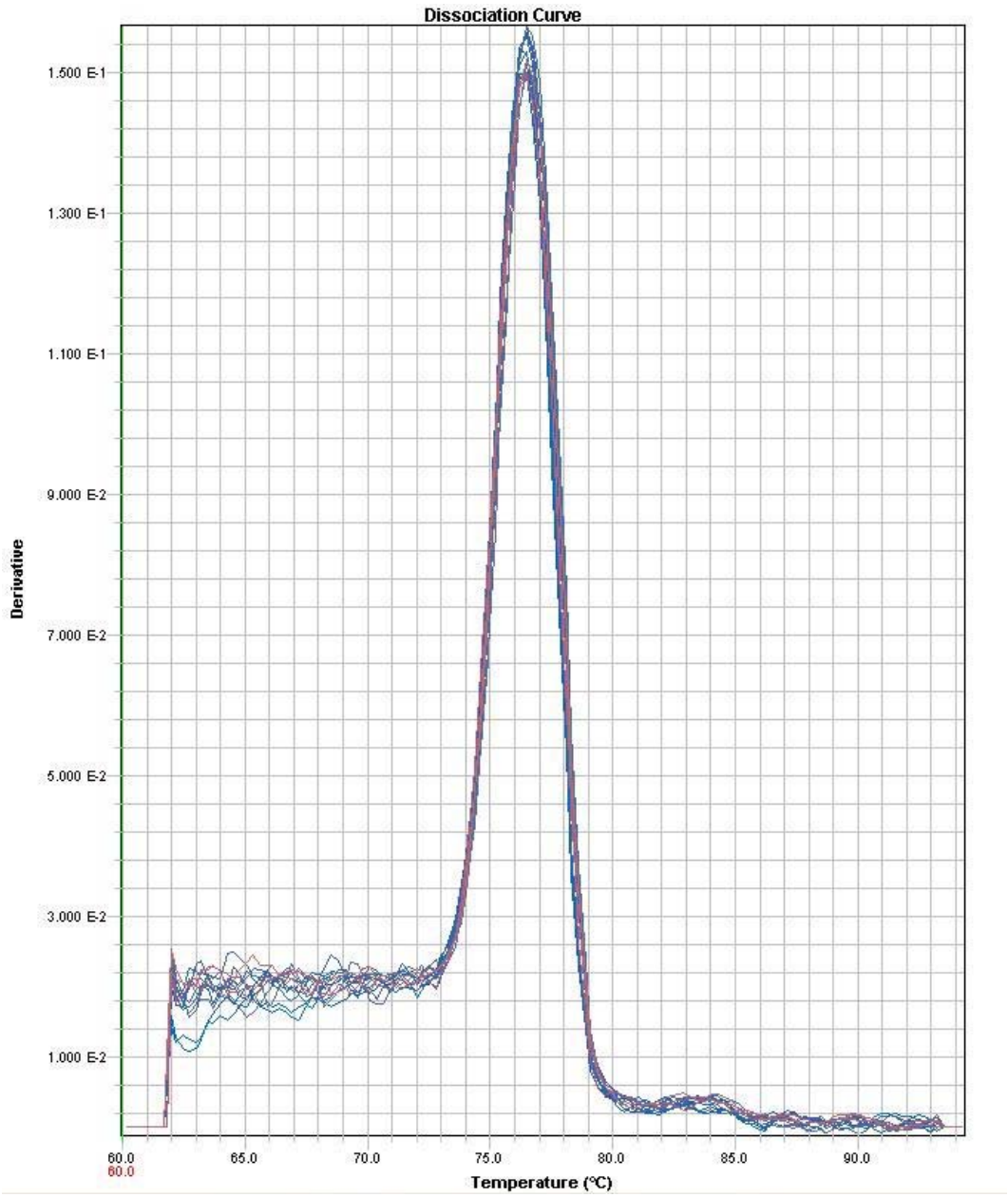

Figure A.11. Melt curve of amplification product using *PtdAKINγ2.5* specific qRT-PCR primers. A qRT-PCR assay using *PtdAKINγ2.5* qRT-PCR primers and a dilution series composed of members of the *PtdAKINγ* gene family was conducted to determine if multiple products were amplified. A single peak indicates the amplification of a single product.

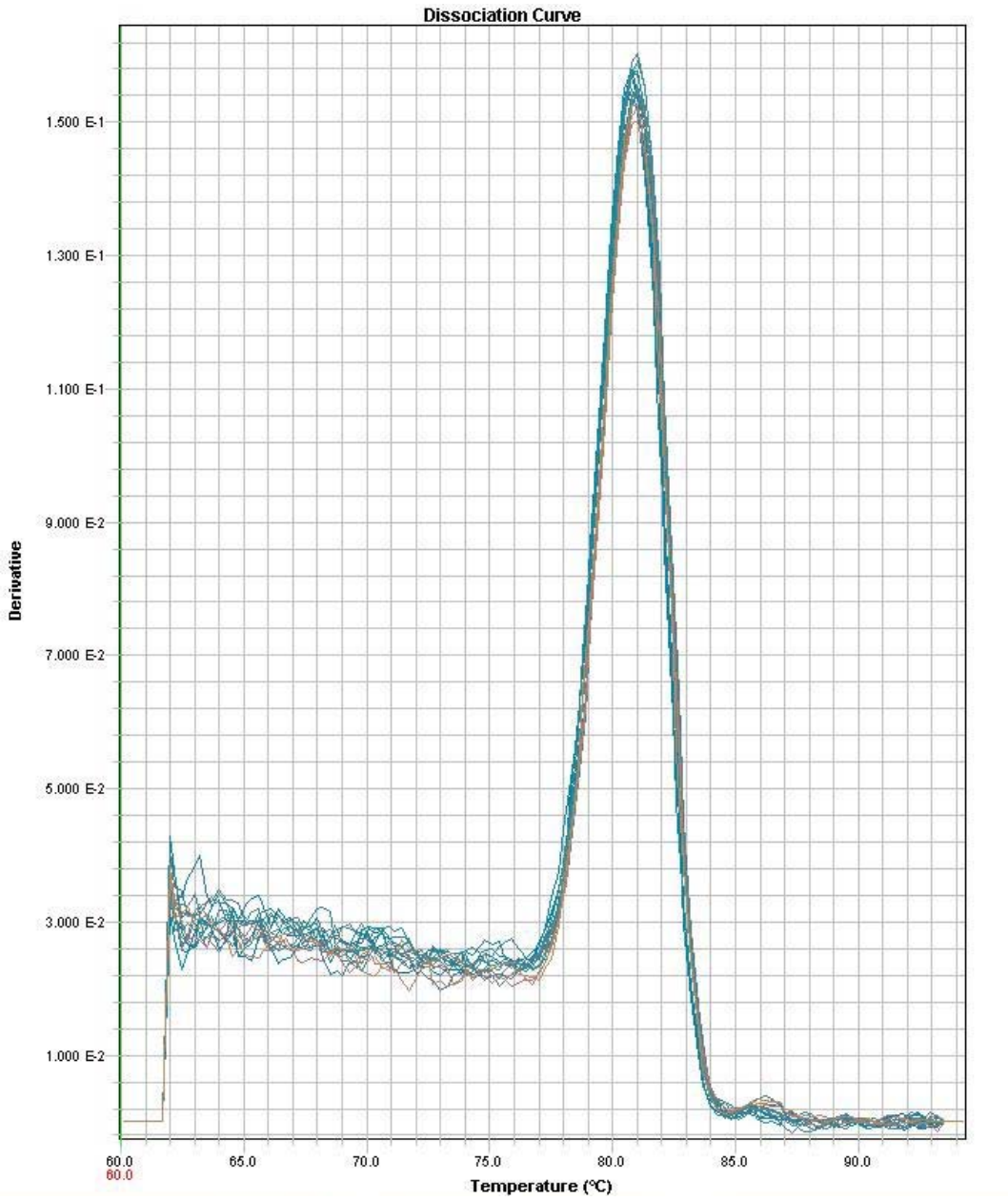

Figure A.12. Melt curve of amplification product using *PtdAKINγ2.6* specific qRT-PCR primers. A qRT-PCR assay using PtdAKINγ2.6 qRT-PCR primers and a dilution series composed of members of the PtdAKINγ gene family was conducted to determine if multiple products were amplified. A single peak indicates the amplification of a single product.