APPLYING HIGH THROUGHPUT TECHNIQUES IN THE STUDY OF ADENOSINE KINASE IN PLANT METABOLISM AND DEVELOPMENT

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TABLE OF CONTENTS

1. ABSTRACT

Adenosine kinase (ADK, EC 2.7.1.20) is a purine salvage enzyme, which phosphorylates adenosine (Ado) to AMP. It may also contribute to the interconversion of cytokinin ribosides and nucleotides. Recent microarray analyses have provided new insights into the impact of ADK activity towards plant metabolism and development. The majority of these findings reflect ADK's role in the metabolism of Ado produced from transmethylation reactions in addition to providing necessary nucleotides for the synthesis of nucleic acids and nucleotide cofactors. As such, ADK was found to increase during events associated with high transmethylation activity, such as cell wall synthesis and seed filling. Differences between plant organs were also detected, with *ADK* transcript levels found highest in siliques and roots and lowest in callus, leaves and buds. Transcript profiling of Arabidopsis expression using microarrays, reveals a predominance of *ADK1* expression relative to that of *ADK2*. In the majority of the studies, the isoforms appeared to behave in a similar pattern of expression, with the exception being microgametogenesis where *ADK1* was up-regulated when *ADK2* was not. What specialized function the *ADK1* could be providing to these cells during development and whether or not this is occurring in other biochemical processes has yet to be determined.

2. INTRODUCTION

Like all living things, plants rely on adenylate nucleotides for the majority of their cellular energy requirements as well as for the synthesis of nucleic acids and nucleotide cofactors. The enzymes involved in the synthesis and recycling of adenylates are thus essential to all cells and constitutively expressed, leading them to be called housekeeping enzymes. Adenosine kinase (ADK, ATP:adenosine 5'-phosphotransferase; EC 2.7.1.20) is such a housekeeping enzyme. By catalyzing the phosphorylation of adenosine (Ado) to adenosine monophosphate (AMP), ADK salvages Ado released as a by-product of secondary metabolism into the adenylate nucleotide pool. The energetic logic of the ADK-catalyzed reaction may be puzzling at first glance because ATP (a "high-energy" compound) is used as the phosphoryl donor for each Ado molecule recycled, producing AMP along with ADP (a "lower-energy" compound relative to ATP due to its lower phosphoryl group-transfer potential). However, when this energy cost is considered relative to that of *de novo* synthesis of Ado, the salvage route is very efficient. Moreover, recent studies of plant metabolism and gene expression strongly suggest that ADK's principal role lies in reducing the concentration of Ado produced due to *S*-adenosylmethionine (SAM)-dependent methyltransferase (MT) activities, rather than providing an energetically favourable route for adenylate salvage. The fact that the Ado is recycled directly into AMP has an additional benefit: it links ADK activity to the cellular energy charge and signaling events sensitive to AMP homeostasis. This has apparently been exploited as part of a defense response against viral infection in *Nicotiana tabacum* (1).

Several lines of evidence suggest that ADK may also interconvert cytokinins (CKs) from the more active
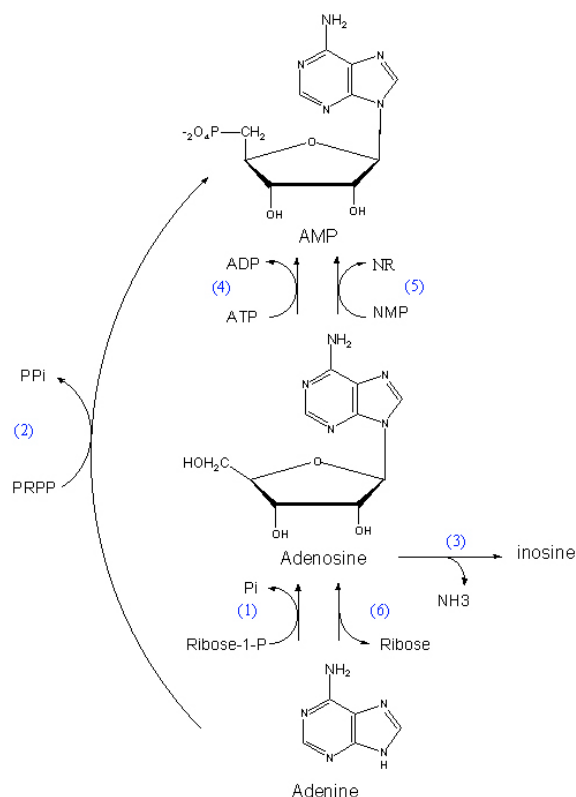
Figure 1. Enzymes used in adenosine and adenine salvage. The desired end product of purine salvage is the creation of more energetically and biologically favourable nucleotides. Adenine (Ade) bases can be converted into Adenonsine (Ado) nucleosides through use of the enzyme (A) Ado phosphorylase or directly into AMP by the way of (2) Ade phosphoribosyltransferase. There are several possible riutes for the salvage of Ado, one of which is making Ado into inosine using (3) Ado deaminase. Enzymatic reactions that result in the production of AMP are catalyzed by (4) Ado kinase (ADK) and (5) non-specific nucleoside phosphotransfereses. Finally, Ado can be made into Ade using (6) Ade nucleosidase, providing APT with usable substrates.

ribose forms to their corresponding nucleotides, which are thought to be less active as growth regulators (2, 3). Thus, the same housekeeping enzyme that is maintaining low cellular Ado levels may also contribute to determining the level of active cytokinin in plant cells.

This review will summarize the data on ADK expression acquired from numerous microarray and proteomic experiments as well as from specific reports of ADK activity to provide insight into the breadth of ADK's role in the cellular metabolism of plants.

3. ADENOSINE SALVAGE IN PLANTS

There are three enzymes that can potentially contribute to the metabolism of Ado in plants; Ado deaminase (ADA; EC 3.5.4.4), Ado nucleosidase (EC 3.2.29) and ADK. The pathways of such reactions are schematically shown in Figure 1. It is generally accepted that plants rely very little, if at all, on ADA activity for Ado recycling for it has yet to be unequivocally detected in plant extracts (4). However, this requires further study since sequences encoding putative ADA activities are transcribed in all major organs of *Arabidopsis thaliana* (Bianchi and Moffatt, unpublished results). Assuming active enzyme is produced in these cells, ADA may catalyze a minor route for Ado salvage, perhaps in specific subcellular compartments. A second route for Ado recycling is catalyzed by the sequential activities of two enzymes: Ado nucleosidase (EC 3.2.2.9; AN) and adenine phosphoribosyltransferase (EC 2.4.2.7; APT). Although this route is also considered of lesser importance in most plants, recent reports indicate that it does contribute to Ado metabolism in the leaves of tea (*Camellia sinensis* L.) (5, 6) and *Avicennia marina* (7). The latter case is particularly interesting. Control *A. marina* leaf discs contain 25-fold higher AN activity as compared to that of ADK, with APT activity being slightly above that of ADK. However, when the leaf discs were salt stressed, causing them to accumulate the methylated osmolyte glycine betaine and produce more Ado, ADK and AN activities increased 2- and 1.5-fold, respectively. Thus two Ado salvage routes are functional in this species, with ADK activity responding to a greater extent to increases in SAM-dependent methylation activity, although based on their relative specific activities AN contributes approximately 20-fold more Ado recycling capacity than does ADK.

Based on its high enzyme activity (8) and substantial level of expression throughout plant development ADK (2) is considered the predominant enzyme mediating Ado salvage in plants. The importance of ADK in Ado metabolism is evidenced by our failure to recover insertion mutants of *Arabidopsis thaliana* that completely lack ADK activity (Perry and Moffatt, unpublished results) whereas *A. thaliana* lines with less than 10% residual ADK activity are viable. These ADK-deficient lines grow to maturity but exhibit morphological abnormalities such as small, rounded and wavy leaves, compact structure and delayed senescence (9). Presumably, the other two routes for Ado salvage are not sufficiently active enough to compensate for ADK deficiency, in *A. thaliana*. The presence of ADK has been characterized from a wide range of eukaryotes, including yeast (10, 11), wheat (3), lupin (12), humans (13), peach (14) *Physcomitrella patens* (15) and *A. thaliana* (2). Kinetic studies conducted on these proteins were able to establish ADK's utilization of Ado versus other ribose sugars and the requirement for ATP and divalent cations such as Mg^{2+} to catalyze the phosphorylation of Ado to AMP. Further examination of the crystal structure of human ADK found it to closely resemble ribokinase, in addition to containing the substrate binding sites for ATP and divalent cations (16).

Up until recently, it was believed that ADK was only expressed in eukaryotes, however, the recent identification and characterization of an ADK in *Mycobacterium tuberculosis* has given thought to it being active in prokaryotes as well (18). This ADK had been categorized previously as a sugar kinase with unknown function due to its stronger sequence homology with
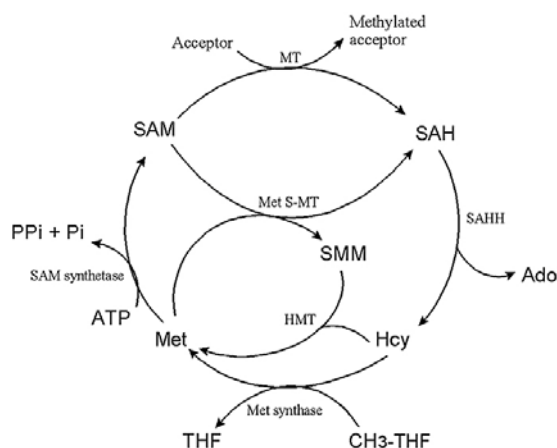
Figure 2. The activated methyl cycle in association with the SMM cycle. The activated methyl cycle (SAM cycle) provides the transmethylation of appropriate acceptor molecules by the (1) methyltransferase (1) medicated transfer of methyl group from *S*-adenosyl-methionine (SAM). The resulting by-product, *S*-adenosyl-L-homocysteine (SAH) is then broken down by (2) *S*-adenosyl-L-homocysteine hydrolase into adenosine (Ado) and homocysteine (Hcy). The Ado can be salavaged via several possible enzymes as indicated on Figure 1, while the Hcy is converted into methionine (Met) by (3) methionine syntex.The Met then has two potential fates: one it can be recycled back into Sam using (4) SAM synthetase or it can be react with SAM through the use of (5) Met *S*-methyltransferase to form S-methyl-Met (SMM). Upon donating the methyl group to Hcy via (6) Hcy *S*-methyltransferase (HMT), the SMM can once again form into Met. This additional cycle is possibly used for the regulation of SAM level.

ribokinase and fructokinase than other ADKs. Whether or not other prokaryotes are also expressing ADK activities requires further study.

4. CONTRIBUTIONS OF ADK ACTIVITY TO PLANT METABOLISM

4.1. Maintenance of nucleotide pools

Plants as well as animals are capable of regenerating their nucleotide pools through salvage pathways. Nucleotide bases and nucleosides resulting from the breakdown of nucleic acids (18), the hydrolysis of *S*-adenosyl-L-homocysteine (SAH) within the SAM cycle and from by-products formed by the conversion of methionine (Met) to ethylene, are all potential sources for the salvage of Ado and adenine (19). How these are recycled is dependent upon which enzyme is used by the plant. It has been suggested that overall, Ado nucleosides are used more in the maintenance of adenylate pools than are the Ade bases (20). The advantages associated with the salvaging of nucleotides as opposed to the *de novo* pathway are the recycling of limited nitrogen sources and energy conservation (21) (Five of the 12 steps of *de novo* purine nucleotide synthesis require the hydrolysis of ATP or GTP).

4.2. Reducing the intracellular levels of Ado

As well as contributing to the adenylate pools, ADK activity prevents the accumulation of Ado that would

otherwise lead to the inhibition of methyltransferase (MT) activities. Within the activated methyl cycle, methyl groups are donated from SAM to acceptors by way of MTs (EC 2.1.1), each of which is thought to be specific for one substrate. Upon methylation of the acceptor, a molecule of *S*-adenosyl-L-homocysteine (SAH) is produced, which in plants is cleaved into L-homocysteine (Hcy) and Ado by SAH hydrolase (SAHH; EC 3.3.1.1). The SAHH-catalyzed reaction serves two important functions: it provides the precursor for the regeneration of Met and SAM, thereby maintaining the availability of these compounds for cellular metabolism, and it also reduces the accumulation of SAH which would otherwise competitively inhibit MT activities (22). Since MT activities are essential for the synthesis/ functionality of hundreds of compounds in plant cells including DNA and mRNAs, pectin, lignin and phosphatidylcholine, cells must remove SAH continuously to maintain SAM-dependent methylation. However, since the reaction catalyzed by SAHH is reversible and its equilibrium lies in the direction of SAH formation, it must be drawn in the direction of SAH hydrolysis through the constant removal of both products, Ado and Hcy, which would otherwise inhibit SAHH and subsequently MT activities as well. As shown in Figure 2, Hcy is metabolized to Met, either by Met synthase (EC 2.1.1.13) or Hcy *S*-methyltransferase (EC 2.1.1.10), and the resulting Met is subsequently converted to SAM, using SAM synthetase (EC 2.5.1.6) or back to *S*-methylmethionine (SMM) by Met S-methyltransferase (EC 2.1.1.12). The purpose of re-diverting Met through the SMM cycle is possibly used as a means of SAM regulation (23).

In plant cells, the majority of the Ado produced resulting from SAM-dependent methyltransferase activities is salvaged by ADK (8). Moreover, during periods of increased methylation activity in spinach, such as during the synthesis of glycine betaine in response to salinity stress, ADK and SAHH expression and activities increase 2-3 fold (24). These increases match well with the change in the methyltransferase activity required to synthesize glycine betaine indicating that ADK expression is responsive to the increased flux through the activated methyl cycle (24).

A. thaliana lines that are deficient in ADK activity due to transgene silencing have provided direct evidence that this enzyme is essential for maintaining SAM-dependent transmethylation activities in this plant. The most affected lines have up to 40-fold higher levels of SAH than that of the wild-type parent, presumably due to the reversal of SAHH activity resulting from excess Ado. The increased SAH levels in these lines are correlated with the severity of their phenotype and with the inhibition of the MTs acting on pectin and DNA (8, Perry and Moffatt, unpublished results). The observation that tobacco plants deficient in SAHH activity have morphological changes similar to those observed in the ADK-deficient lines and their genomic DNA is also hypomethylated (25) supports the conclusion that the changes in the ADK-deficient lines are associated with ADK's role in the activated methyl cycle.

4.3. Cytokinin interconversion

CKs are a class of plant-specific growth regulators that play a central role in controlling the cell cycle and influencing numerous developmental programs including cell signaling for apoptosis (26), male development in response to gibberellin (27), meristem activity and morphology (28), chloroplast biogenesis (29) and cell division (29). The most predominant class of CKs are N^6-substituted adenines, which are generally thought to be synthesized via the addition of isopentenyl to AMP creating isopentenyladenosine monophosphate (iPMP, 30). Modifications of the side chain lead to the formation of zeatin monophospate (ZMP) and other derivatives. An alternate iPMP-independent pathway has been shown to exist in *A. thaliana* in which ZMP is formed directly by the addition of an alternate side chain to AMP (31).

It is thought that the free bases and riboside forms of CKs are the biologically active compounds and their interconversion to the nucleotide and conjugation with sugars, amino acids or phosphorylation creates storage, transport or inactivated forms of the molecule (32). Based on the results of *in vitro* assays, ADK may contribute to the interconversion of CK ribosides and nucleotides (2, 3), although it is not clear how much of a contribution this makes *in vivo* since the affinity of *A. thaliana* ADK for a CK riboside, as estimated by its K_m, is 10-fold lower than for Ado and its catalytic efficiency (V_{max}/K_m) is reduced 250-fold. ADK's *in vivo* role in CK metabolism has only been documented in a few cases to date. Chloronemal tissues of the moss *Physcomitrella patens* incorporate exogenously fed isopentenyladenosine into its nucleotide, via a route that must depend on ADK activity (15). Analysis of CK metabolism and CK composition in the ADK-deficient lines of *A. thaliana* may provide further insight into the contribution of ADK to cytokinin interconversion. The importance of ADK in such a role was exhibited in a recent study of isopentenyladenosine-induced apoptosis in tobacco BY-2 cultured cells. This effect was dependent on the intracellular phosphorylation of iPA to iPMP by ADK (32).

4.4. Plant pathogen responses & gene silencing

As well as participating in a plant's response to abiotic stressors, ADK is also involved in pathogen resistance. A study conducted by Wang *et al*. (1), indicates that in *Nicotiana tabacum,* ADK activity increases in response to stresses that deplete cellular ATP, most likely to provide alternate routes for the synthesis of adenylate nucleotides. The rise in ADK activity is mediated by the action of the metabolic regulator SNF kinase which is induced by increased AMP:ATP ratios. Interestingly, infection of *N. tabacum* by *Begomovirus* and *Curtovirus* geminiviruses leads to the expression of viral proteins (AL2 and L2) that inactivate both SNF kinase and ADK in order to disable this plant defense system.

It has become evident in the last few years that RNA silencing is a key mechanism plants utilize to limit viral infections. In RNA silencing, small RNAs resulting from the cleavage of double-stranded RNA, induce epigenetic gene silencing in both the cytoplasm and the nucleus (33). The double-stranded RNA can be provided by either foreign viral RNA undergoing replication or transposable elements (TE) naturally found within plants. The gene silencing itself can occur by the post-transcriptional degradation of complementary mRNAs, and in the case of plants, transcriptional gene silencing of homologous DNA sequences. Plant viruses can then serve as both a trigger and a target of RNA silencing as their double-stranded RNA is detected by the plant and cleaved into single-stranded RNA (34). To adapt to this defense mechanism, many plant viruses develop proteins, such as AL2 and L2, that interfere with silencing pathway components and as a result counter the silencing activity of the host (35, 36). Plants may also suppress viral replication through the methylation of viral DNA (37) in which case the virus may avoid the defense mechanism by inhibiting ADK and thereby SAM-dependent DNA methylation.

5. EXPRESSION OF ADK GENES IN PLANTS

To understand the mechanism regulating ADK expression, it is important to examine the expression of ADK genes in the major organs of different plants. This is because the demands for ADK will vary in specific tissues, to sustain their nucleotide pools, activated methyl cycle flux and possibly, CK interconversion. The possible presence of more than one ADK coding sequence in the genome can further complicates these analyses. For example, the *A. thaliana* genome contains two genes encoding ADK, designated as *ADK1* (At3g09820) and *ADK2* (At5g03300), which have 92% amino acid and 89% nucleotide identities (2).

To date there have been limited reports of the transcript abundance of *ADK* in plants, with most being related to its expression in specific stress conditions rather than specific tissues or organs. However, the results of these studies are consistent with the hypothesis that that transcription of *ADK* increases in association with SAM-dependent methylation activities (5, 23). For example, a study on *ADK* transcript abundance in the major organs of *A. thaliana* by northern analysis showed that they were expressed constitutively, with higher steady-state mRNA levels in stem and root; *ADK1* transcript levels were generally higher than those of *ADK2* (2). In all cases, *ADK* transcript abundance increased in association with changes in *SAHH* transcript levels, presumably due to changes in the flux of the activated methyl cycle (M. Todorova, unpublished results).

The analysis of expressed sequence tags (EST) libraries is another method for assessing transcript abundance arising from a gene of interest. ESTs are created by randomly sequencing cDNAs from a variety of tissues (38). The sequences are deposited in public databases allowing researchers to quantify expression from of a given sequence in that library. This *in silico* analysis can provide valuable insight into the conditions affecting the gene's transcription. However, ESTs of a particular organ or tissue may not be fairly represented, leading to false conclusions about a gene's transcriptional activity. Even with normalization, these discrepancies can occur so that only

estimates based on a reasonable number of ESTs should be used. The Institute for Genomic Research (TIGR) gene index provides a straightforward interface to analyze the expression profile of any *A. thaliana* gene, in addition to other plant species, based on the available EST collections (http://www.tigr.org/tbd/tgi/plant.shtml.).

6. ANALYZING ADK EXPRESSION USING HIGH THROUGHPUT TECHNOLOGIES

Using high throughput methods, gene expression in a particular cell type or organ can be monitored at either the transcriptional or the proteomic level. The subsequent biochemical responses can be detected by a metabolomic analysis of the same cells. Each of these methods has their own technical challenges with transcript profiling by microarrays being the most accessible and metabolomics being the most sophisticated and less established of the three types of studies.

Microarrays consist of DNA targets (cDNA inserts or oligonucleotides) hybridized to a complex probe prepared from RNA extracted from a specific tissue or cell line (39). The probes are created by reverse transcription of mRNA or total RNA, which can then be labelled with radioactive or fluorescent tags. By comparing the binding of probes prepared from two populations of cells, to the array, it is possible to estimate the relative transcript abundance of the labelled mRNA sequences in the two populations. The benefits of large-scale genome transcription profiling through use of microarrays include parallel monitoring of gene expression, high sensitivity and the use of random probes (40, 41). Because so many data points can be assayed at the same time, transcripts associated with a specific gene or pathway can also be detected and analysed. In addition, random probes do not require the sequence of the gene to be known, allowing novel genes to be evaluated.

Along with the noted benefits, there are also difficulties associated with using microarrays. The results of individual microarray experiments are highly variable due to cross-contamination as well as printing and hybridisation errors. These errors result in misclassifications, inaccurate estimates of expression and false positives, ultimately resulting in replicate readings with inconsistent expressed gene lists (42). It has been estimated that there is a 5% chance of false negatives and 10% of false positives arising due to a single experiment (43). These discrepancies can be resolved, however, by performing at least three biological replicates and pooling their data (42). Incorporating controls into the array and replicating the samples decreases the chance of errors by improving the power of statistical analyses and thereby removing bad samples from the data pool.

Since the first published study using microarrays to measure tissue expression of *Arabidopsis* in 1998 by Ruan *et al*. (44), the use of transcript profiling to study the plant genome has been making rapid advances. Plant microarrays have been successfully used to quantify gene expression in various tissues (45), subcellular compartments (40, 46), developmental stages (41, 45, 47, 48, 49, 50) and stress reactions (41, 51-55). There are numerous other microarray data sets that are publicly available, which can be easily screened for the expression of specific *A. thaliana* sequences using the Microarray Expression Search algorithm provided by the Arabidopsis Information Resource centre (TAIR; www.arabidopsis.org) and Nottingham Arabidopsis Stock Centre (NASCArrays; http://ssbdjc2.nottingham.ac.uk/narrays/experimentbrowse. pl). The experiments addressing *ADK* transcriptional changes as well as recent reports of the role of ADK activity in plant development are discussed below.

6.1. Seed development

Seeds of all plants share the universal function of providing a means of propagation and distribution for the species. This commonality of purpose accounts for their characteristic accumulation of large quantities of storage materials, ability to enter quiescent states and upon entry into this rest period, displaying a resistance to adverse environment conditions. Once favourable conditions are reached, the seed leaves it's state of dormancy and germinates.

A comprehensive study performed by Girke *et al*. in 2002 (45) compared the transcription levels of 2600 seed expressed cDNAs in the seeds, leaves and roots of *A. thaliana* through use of microarray analysis. Of the 2600 hybridized genes, 25% had slightly higher seed expression ($\geq$ 2-fold increase) and 10% were found to be seed specific (displayed an expression ratio of $\geq$ 10). The majority of these seed specific genes were identified as storage proteins with a small minority being oil synthesis genes. Because lipids are used for housekeeping purposes in addition to energy storage, 91% of the available 113 lipid biosynthetic genes were also found in high levels within leaves and roots. Genes that were not exclusively found in seeds but measured at higher levels ($\geq$ 4 relative expression) included more storage proteins and other genes associated with seeds such as oleosins, fatty acid elongase and lipoxygenase as well as transcription factors, kinases, phosphatases and developmental proteins. Most of the genes associated with carbohydrate and secondary metabolism, which ADK is involved with, displayed relative expression levels between 2- and 4-fold higher in seeds. *ADK* itself had transcript levels $\geq$-fold higher in seeds than over that of roots or leaves, indicating that as for the gene products involved in carbohydrate and secondary metabolite synthesis, *ADK* is abundantly, but not exclusively, expressed in seeds. However, the metabolic changes associated with embryo maturation result in increased *ADK* expression.

6.1.1. Desiccation

During the desiccation stage of embryo development, the seed becomes 90-95% dehydrated, allowing it to cease growing and withstand unfavourable growth conditions. To survive this period, the embryo acquires desiccation tolerance and accumulates important quantities of storage compounds in order to subsequently resume growth (50). In most seeds, the storage compounds consist of carbohydrates such as starch and oils (triacylglycerols), and specialized storage proteins, which

may ultimately account for 90% of the seed's dry weight (49).

Proteomic, in addition to EST analysis, was performed on 120 proteins isolated from *Medicago truncatula* seeds undergoing desiccation (12-18 days after pollination [DAP]) (56). In order to assess the changes in protein levels, normalized volume (NV) or normalized spot abundance values, were compared. With the exception of one protein, the majority of the highly abundant storage protein transcripts (normalized volumes > 10 000) increased up to 20 DAP before decreasing, indicating storage proteins accumulate early on in seed development. The less abundant proteins (21 to 10,000 normalized volumes) exhibited varying expression patterns. Several proteins associated with methyl recycling were in this category. ADK abundance was relatively steady (NV maximum= 180) until it dropped between 18 and 20 DAP. SAM synthetase also displayed a decreasing trend, but unlike ADK, it was only found 12-16 DAP after which very low levels of SAM synthetase were present. Two other components of the SAM cycle, SAHH and Met synthase, remained relatively constant through 12-18 DAP but increased at 18-DAP to reach 35 and 28 NV, respectively. Because SAM synthetase levels were very low by this developmental period, the ongoing activity of SAHH, Met synthase and ADK may quickly use up the available SAM. This is significant to the developing plant because SAM, along with serving as a cofactor for methylation, is a precursor for ethylene, biotin, and polyamines along with being an effector in the regulation of Thr, Lys and Met synthesis. Therefore, a decrease in SAM levels may possibly serve as a repressor of metabolic activity, facilitating the onset of quiescent state in the seed (56).

A similar pattern emerges from the analysis of transcript levels in developing tomato seeds using arrays of 11,100 unique ESTs. The data for this experiment is available at the Tomato Expression Database (http://ted.bti.cornell.edu/). These arrays show *SAHH* transcripts remaining stable up until 27 DAP at which point their levels steadily increase until peaking at 43 DAP (a 3.9-fold increase) and subsequently decreasing, until by 57 DAP, the levels are similar to those at 7 DAP. The SAM synthetase transcript levels displayed a similar trend, only rising in abundance until 39 DAP (3.1-fold increase) then dropping below the amount originally present. *ADK* expression was not measured in these arrays, although the *APT* and adenosine deaminase transcript levels were measured and remained constant.

A transcript profiling study by Ruuska *et al*. (49) focused on the temporal expression that occurs during seed filling, between 5 and 13 days post flowering. The microarray contained > 3500 unique gene sequences compiled of ESTs derived from developing seeds as well as cDNA clones of genes found in carbohydrate and lipid pathways. The developmental events occurring during this period encompass the accumulation of storage oils and proteins. The microarray data revealed that approximately 35% of the genes represented on the array changed in expression at least two-fold, but a larger fraction (65%)

showed little or no change in expression. Of the expressed genes, *ADK2* and *SAHH1* increased less than two-fold; *ADK1* was not included in the analysis. A subsequent microarray found a decrease in *ADK* transcript levels in white pine seeds prior to desiccation in correlation with increased polyamine synthesis (58). Taken along with the results of proteomic experiments conducted on *M. truncatula* showing a decline in *ADK* following desiccation at 18 DAP, it could be proposed that ADK activity and adenosine salvage are not as important in the production of storage compounds as they are the events leading up to embryo quiescence.

6.2. ADK expression in mature and developing organs

More than one high throughput method has been used to assess changes in transcript abundance in different organs; several of which detected changes in *ADK* expression. Massively Parallel Signature Sequencing (MPSS) is a relatively new technique developed by Lynx Therapeutics (58, 59) and applied to evaluate Arabidopsis expression (http://mpss.udel.edu/at/java.html). MPSS produces short sequence signatures originating from a defined position within an mRNA, and the relative abundance of these signatures in a given library represents a quantitative estimate of expression of that gene. In all the tissues tested, with the exception of siliques, *ADK1* transcripts were twice as high as those of *ADK2*. The range of expression from highest to lowest in both isoforms is as follows: root>flower buds (stage 11-12)>callus>leaf. In expanding green siliques (stage 16-17) *ADK1* transcript levels were four-fold higher than those of *ADK2*.

The analysis of microarray data provided by the TAIR Microarray database (TAIR Microarray Expression Search; http://www.arabidopsis.org/servlet/MultiServlet) also indicates higher *ADK1* transcript abundance in meristems and roots as compared to flowers and leaves.

Affymetrix ATH1 genome arrays have been used to generate transcript profiles of stigmas and ovaries (60). This study revealed two-fold higher levels of *ADK* and *SAHH* transcripts in the stigma versus the ovaries. In both organs, *ADK1* transcript abundance was twice that of *ADK2*. (R. Swanson, personal communication). Interestingly, two of the highest expressed genes in the stigma are *SAHH1* and *2*, suggesting that there is a high production of SAH in these cells and the increase in ADK expression serves to maintain SAH hydrolysis.

6.3. Microgametogenesis

Recent microarrays performed by Honys and Twell (61, 62) have examined the identity of pollen-specific genes and their expression throughout development. The first microarray performed used an Arabidopsis 8K Affymetrix GeneChip hybridized to separate RNA samples extracted from mature pollen grains and sporophyte tissue (61). Pollen-specific genes were then determined by selecting those that did not exhibit significant transcript levels within in the sporophyte tissue hybridizations. In order to verify their results, the researchers checked the transcript abundance of specific genes using reverse transcription polymerase

chain reactions (RT-PCR) and compared them to previously characterized gene expression profiles of other organs. Of the 7,792 genes tested, 992 were established to be pollen specific. Functional analysis of the pollen transcriptome revealed a large majority of mRNAs encoding genes for cell wall metabolism, cytoskeleton, and signaling, while the lowest levels of mRNAs were found to encode for transcription and protein synthesis. Not surprisingly, *ADK* was not found to be pollen specific. The second microarray performed by Honys and Twell (62) used an Affymetrix ATH1 genome array to follow the profile the transcripts present in uninucleate microspores, bicellular pollen, tricellular pollen and mature pollen. This data shows a modest increase (2.2-fold) in *ADK1* expression from the uninucleate to mature pollen stages, while *ADK2* transcripts dropped to negligible levels following the formation of bicellular pollen. In the assumption that the transcript abundance is a reflection of enzyme activity, these findings suggest that *ADK1* sustains the Ado/CK salvage requirements of the later stages of microsporogenesis while *ADK2* becomes an insignificant contributor. *ADK1* gene expression during this period is likely associated with Ado salvage since its expression pattern mimics that of *SAHH*2 and *SAM* synthetase and five of the six most abundantly expressed genes in pollen are involved in cell wall synthesis, which is a methyl-requiring process.

6.4. Cell wall synthesis

By constraining the rate and direction of cell growth, the cell wall helps control the development and morphology of plants. As the needs of the plant changes so does the composition of the cell wall. The primary cell wall is created in the cell plate during division and is composed of an independent, yet interacting, cellulose framework, embedding a pectic matrix and in some cases structural proteins and phenylpropanoids (63). The pectin serves as both a structural support for the cellulose and a regulator of plant processes such as cell expansion, defense and cell signaling. Pectin is made up of some of the most complex polysaccharides found in plants, making the synthesis of the compound difficult to study. The pectin carbohydrate subunits of pectin are initially esterfied with methyl and other moieties and are later de-esterified, after being incorporated into the cell wall, to facilitate the formation of calcium cross-bridges, which affect the wall's porosity and tensile strength.

To identify genes associated with pectin synthesis, transcript profiles of wild type and 26-dichlorobenzonitrile-treated *A. thaliana* cell cultures were compared (64). The herbicide treatment inhibits cellulose synthesis, resulting in cell walls predominantly composed of pectin. Under these conditions, the transcripts of both isoforms of *ADK* and *SAHH* increased between 1.5-3.5 fold, most likely to accommodate the increased flux through the activated methyl cycle associated with the synthesis of methylated pectin precursors.

Once plant cells stop growing, the secondary wall is formed to reinforce cell structure. Secondary walls are highly variable amongst plant species, usually exhibiting specialized functions. One distinguishing feature is the deposition of lignins, complex networks of phenylpropanoids that, except for a few cases, are only formed during secondary wall synthesis (63) to provide structural support and a defense against pathogens. Although the exact pathway of lignin biosynthesis remains contentious (65), it is well known that it is composed of methylated monolignol precursors derived from the phenylpropanoid pathway.

Recent studies have been performed to try to further our understanding of cell wall synthesis through use of poplar trees as a model due to their clearly defined developmental stages of wood formation. Wood formation originates in the vascular cambium where it then forms into xylem cells through cell division, expansion, secondary wall formation, lignification and programmed cell death (PCD).

A functional analysis of 5,692 ESTs from the cambial meristem and developing derivatives of poplar was carried out to identify genes involved in wood formation (66). These sequences were recovered from distinct cDNA libraries prepared from cells of the cambial region and developing xylem. The comparison of the ESTs in each collection revealed that xylem contains a higher abundance of cell wall synthesis-related genes, while the cambial region expresses genes associated with protein synthesis to a higher level than xylem. Enzymes associated with pectin synthesis such as pectin acetylesterase and methylesterase, were more abundant in the cambial region while lignin biosynthetic genes were detected in both tissues, but predominantly expressed within the developing xylem. Additionally, different components of lignin biosynthesis showed differential expression, with peroxidase detected at high levels in the cambial region library and laccase including *SAM* synthetase in the developing xylem. Overall, *SAM* synthetase represents 2.5% of the ESTs found in the developing xylem library. Both SAM synthetase and Met synthase are indicators of an active SAM cycle and an Ado salvage requirement. Consistent with these activities ADK transcript expression was detected at higher levels within the xylem.

Temporal expression of poplar has also been examined, using DNA microarray analysis with 2,995 ESTs derived from various developmental stages undergoing xylogenesis (48). The different developmental phases were comprised of (A) meristemic cells, (B) early expansion, (C) late expansion, (D) secondary wall formation and (E) late cell maturation, with phloem samples taken as negative controls. Of the 2,995 ESTs examined, 539 genes displayed developmentally-regulated expression of at least 8-fold (max expression ratio/min expression ratio), 1,246 genes had a 4-fold change and 386 appeared to be expressed constitutively (max ratio/min ratio < 2). As found in Sterky *et al*. (66), enzymes associated with the production of pectin (pectin esterases, methylesterases and pectate lyases) were detected in the stages involved in primary wall synthesis, A-C. The initiation of secondary wall metabolite transcription began in stage C

and continued until E, where genes used during lignification were predominantly expressed. ADK appears to be involved in lignin formation, as it's transcript levels started increasing in early expansion until peaking at the end of secondary wall formation.

6.5. Programmed cell death

Plant cell death is a genetically controlled method of cellular suicide that is a necessary component of healthy development in both plants and animals. Previous studies have linked the down-regulation of adenine nucleotide translocase (ANT) to the initiation of PCD in both plants and animals (54, 69, 70). A reduction in ANT would result in the inhibition of ATP synthesis as well as the movement of newly synthesized ATP from the mitochondria to the cytosol. This reduction in cellular energy could then account for the side effects associated with apoptosis such as mitochondrial swelling, cytochrome *c* release and the opening of permeability transition pores (69).

Swidzinski *et al*. (70) used a microarray consisting of 100 cDNAs previously implicated in PCD-related responses to identify those required in the induction of PCD in *A. thaliana* cell suspension cultures. To differentiate between the genes initiating PCD as opposed to those being induced, transcripts from 14 day-old cell cultures undergoing senescence (already exhibiting PCD) and in 6-day cultures 1 hour post-treatment with heat, (which induced PCD) were each compared with transcripts in healthy 6-day old log phase cells. Although heat treatment resulted in the substantial decrease in expression of *ANT* genes, which is indicative of PCD induction, there was no significant difference in *ADK1* or *ADK2* transcript levels. However, in the senescing cultures that are undergoing PCD, the abundance of transcripts arising from both *ADK* genes decreased 5.5-fold, likely due to reduced metabolic demand for salvage activity. The activity of the SAM cycle also appears to decrease as indicated by the lower expression levels of *SAHH* and *SAM* synthetase.

7. PERSPECTIVE

The salvage of Ado is an indirect requirement for plant development and survival due to its role in maintaining the flux through the activated methyl cycle and contributing to adenylate nucleotide pools. The principal enzyme involved in Ado recycling in plants is Ado kinase. By altering the expression of ADK, the metabolic requirements of specific developmental stages and cell types can be accommodated. Although the need for SAM-dependent methylation may vary widely in some cell types, such as in cells accumulating methylated osmolytes, *ADK* transcript abundance does not change more than 3-fold (24). Because of this variable expression and involvement with numerous pathways, ADK expression at the genomic level can be difficult to follow using traditional assay techniques.

The application of high-throughput technologies for transcript profiling to the analysis of plant gene expression provides researchers with the means to identify co-ordinate changes in transcript levels of different genes throughout plant development. Several of these analyses have detected changes in *ADK* transcript abundance, which are consistent with its association with activated methyl cycle and nucleotide synthesis. None of the studies addressed ADK's potential involvement in cytokinin metabolism.

Despite the wealth of data generated by these studies, they are really the starting point for documenting changes in gene expression on a genomic scale. It is essential to validate these results, by RT-PCR or RNA blots, particularly for genes such as ADK where relatively modest differences in expression have been shown to be physiologically relevant. Validation is also required to determine whether increases in ADK transcript levels reflect changes in enzyme activity. Further studies should examine the balance of ADK activity with that of other Ado salvage enzymes and identify the specific contributions of different *ADK* isoforms. In addition to applying these measures to the pathways already examined, the role of ADK in the activities of other metabolic pathways such those involved in the synthesis of defense compounds and mineral acquisition as well as in gene silencing mechanisms are deserving of study.

8. ACKNOWLEDMENTS

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