

TRANSGENIC PLANTS COMPRISING CONSTRUCTS ENCODING PHOSPHOENOLPYRUVATE
CARBOXYKINASE AND/OR PYRUVATE ORTHOPHOSPHATE DIKINASE

The present invention relates to genetic constructs used in the preparation of transgenic plants. The constructs can have the ability to cause remobilisation of nitrogen during leaf senescence, such that nitrogen can be transported from the leaves to other regions of the plant. The invention extends to plant cells
5 transformed with such constructs, and to the transgenic plants themselves. The invention also relates to methods of producing transgenic plants, and to methods of increasing the rate of nitrogen remobilisation in senescent plants. The invention also relates to harvested plant leaves, for example tobacco leaves, that
10 have been transformed with the genetic constructs, and to smoking articles comprising such harvested plant leaves.

Leaf senescence is a phase of plant development during which the cells undergo distinct metabolic and structural changes prior to cell death. Physiological and
15 genetic studies indicate that senescence is a highly regulated process. The progression of a leaf through senescence is visibly marked by the loss of chlorophyll and consequent yellowing, which results from the disassembly of the chloroplasts. The decreasing levels of leaf chlorophyll, characteristic of this developmental stage, can be measured, e.g. by solvent extraction and
20 spectrophotometric measurement, or by a chlorophyll content meter. A decreased leaf chlorophyll level in comparison with an earlier leaf chlorophyll level recorded for the same plant, preferably grown under constant conditions, indicates senescence.

25 Molecular studies indicate that senescence is associated with changes in gene expression. The levels of mRNAs encoding proteins involved in photosynthesis decrease during senescence, whilst mRNA levels of genes encoding proteins thought to be involved in the senescence increase. Senescence is a highly organised process regulated by genes known as Senescence Associated Genes
30 (SAGs). Leaf senescence involves the degradation of proteins, nucleic acids and membranes, and the subsequent transport of the nutrients resulting from this degradation to other regions of the plant, such as the developing seeds, leaves or

- 2 -

storage organs. One problem of plant senescence is that many useful minerals and nutrients that are present in senescent leaves will remain in the leaves, and will be effectively lost as the leaves die. For example, nitrogen, which can be in the form of amine groups on amino acids, present in the senescent leaves, will go to waste, if it is not removed from dying leaves.

Therefore, increasing nitrogen remobilisation in plants, especially when they become senescent, could have important applications in crop production.

Firstly, nitrogen remobilised from leaves can be transported to the younger leaves as well as the developing seed. Increasing the efficiency of nitrogen exit from senescent leaves could therefore potentially increase nitrogen supply to seeds and younger parts of the plant, and thereby increase crop yield and nitrogen use efficiency. This is clearly a valuable goal when the world population is increasing but crop yields are not increasing sufficiently to meet demand. One potential target crop is *Brassica napus* (oilseed rape), which has poor nitrogen efficiency due to poor nitrogen remobilisation from vegetative tissue. Another target crop is wheat, as the potential benefits of increasing grain protein content are great. Grain protein content not only affects nutritive value of wheat, but also determines grain usage and therefore market value. For example, increased grain protein content results in increased bread volume. Also, an ability to increase nitrogen remobilisation could be very useful in the tobacco industry because it is known that residual nitrogen in tobacco leaves contributes to the formation of nitrosamines.

The enzymes phosphoenolpyruvate carboxykinase (PEPCK, or PCK) [EC 4.1.1.49] and pyruvate orthophosphate dikinase (PPDK) [EC 2.7.9.1] are known. PPDK is present in both prokaryotes and eukaryotes, and is conserved in terms of sequence and tertiary structure between bacteria and higher plants (Pocalyko et al., 1990, Biochemistry, 29, 10757–10765). The enzyme catalyses the reversible phosphorylation of pyruvate to form phosphoenolpyruvate (PEP) (Carroll et al., 1990, Federation of European Biochemical Societies, 274, 178–180; Hatch & Slack, 1968, Biochemical Journal, 106, 141–146), in the following reaction: Pyruvate + Pi

- 3 -

+ ATP = PEP + PPi + AMP. In both C3 and C4 plants, the PPDK gene has an unusual structure, with two transcripts resulting from the same gene. The longer transcript encodes a chloroplastic protein, with the first exon encoding a chloroplast transit peptide, while the shorter transcript is transcribed from a separate promoter within the first intron of the longer transcript, and therefore lacks the first exon encoding the chloroplast transit peptide. This shorter transcript generates a cytosolic isoform of PPDK. This gene structure has been reported in maize, rice, C3 and C4 *Flaveria* species, and *Arabidopsis thaliana*.

10 Phosphoenolpyruvate carboxykinase (PCK) catalyses a reversible reaction between ADP, carbon dioxide and phosphoenolpyruvate to form ATP and oxaloacetate, in the following reaction: $\text{oxaloacetate} + \text{ATP} = \text{PEP} + \text{ADP} + \text{CO}_2$. PCK has been shown to be present in the cytosol of cells of a wide range of plant tissues. These include developing seeds, trichomes and roots. In plants, PCK appears in tissues in which there is increased metabolism of nitrogenous compounds.

The inventors constructed a number of genetic constructs, in which genes encoding the enzymes PCK and/or PPDK were placed either alone or together under the control of a promoter, to determine what effect, if any, over-expression of these genes had on nitrogen remobilisation in senescent leaves.

According to a first aspect of the invention, there is provided a genetic construct comprising a senescence-specific promoter operably linked to at least one coding sequence, which encodes at least one polypeptide having phosphoenolpyruvate carboxykinase (PCK) activity and/or pyruvate orthophosphate dikinase (PPDK) activity.

According to a second aspect of the invention, there is provided a genetic construct comprising a promoter operably linked to at least one coding sequence, which encodes at least one polypeptide having phosphoenolpyruvate

- 4 -

carboxykinase (PCK) activity and pyruvate orthophosphate dikinase (PPDK) activity.

The inventors believed that the two enzymes PCK and PPDK may play a role in the interconversion of various amino acids during remobilisation of nitrogen from leaves during senescence. Although the inventors do not wish to be bound by hypothesis, a speculative biochemical pathway illustrating how PCK and PPDK may affect Nitrogen remobilisation is illustrated in Figure 18. They therefore considered that stimulating over-expression of these two enzymes in a plant, either independently or simultaneously, during senescence could play a role in the remobilisation of nitrogen.

As a result of their studies, the inventors surprisingly found that constructs according to the invention, encoding PCK and/or PPDK, resulted in increased rates of nitrogen remobilisation from senescent leaves. The inventors hypothesise that nitrogen may be being moved from senescent leaves in the form of amino acids to the younger parts of the plant, such as the plant seeds. Furthermore, the inventors also observed an increase in the amount of vegetative plant growth (which corresponds to an increase in crop yield), when these enzymes were over-expressed in senescent leaves. The inventors believe therefore that constructs according to the invention will be useful in the preparation of a transgenic plant, which may be able to exhibit increased rates of nitrogen remobilisation from senescent leaves and/or an increased growth rate.

The promoter in the genetic constructs of the first or second aspect may be capable of inducing RNA polymerase to bind to, and start transcribing, the at least one coding region encoding the at least one polypeptide having PCK and/or PPDK activity.

The promoter present in the construct of the second aspect may be constitutive, non-constitutive, or tissue-specific. Examples of suitable promoters include the cauliflower mosaic virus 35S promoter (full or truncated), the rubisco promoter,

- 5 -

the pea plastocyanin promoter, the nopaline synthase promoter, the chlorophyll r/b binding promoter, the high molecular weight glutenin promoter, the α , β -gliadin promoter, the hordein promoter or the patatin promoter.

- 5 The promoter present in the construct of the second aspect may be a senescence-specific promoter.

A “senescence-specific promoter” (SAG) can be a promoter, which is associated with controlling the expression of a senescence-associated gene. Hence, the
10 promoter can restrict expression of a coding sequence (i.e. a gene) to which it is operably linked substantially exclusively in senescing tissue. Therefore, a senescence-specific promoter can be a promoter capable of preferentially promoting gene expression in a plant tissue in a developmentally-regulated manner such that expression of a 3' protein-coding region occurs substantially
15 only when the plant tissue is undergoing senescence. It will be appreciated that senescence tends to occur in the older parts of the plant, such as the older leaves, and not in the younger parts of the plants, such as the seeds.

One example of a plant which is known to express numerous senescence-associated genes is *Arabidopsis*. Hence, the promoter in a construct according to
20 the first or second aspect may be isolated from a senescence-associated gene in *Arabidopsis*. Gepstein *et al.* (The Plant Journal, 2003, 36, 629-642) conducted a detailed study of SAGs and their promoters using *Arabidopsis* as a model. The genetic construct may comprise a promoter from any of the SAGs disclosed in
25 this paper. For example, a suitable promoter may be selected from a group consisting of SAG12, SAG13, SAG101, SAG21 and SAG18, or a functional variant or a functional fragment thereof.

Preferred promoters are SAG12 and SAG13 promoters. In one embodiment, the
30 promoter is a SAG12 promoter, which will be known to the skilled technician, or a functional variant or a fragment thereof (Gan & Amasino, 1997, Plant

Physiology, 113: 313-319). The DNA sequence encoding the SAG12 promoter is referred to herein as SEQ ID No.16, as follows:

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5  TCGAGACCCGATTGTTATTTTTAGACTGAGACAAAAAAGTAGAATCGTTGATTGTTAAATTTA
AAATTAGTTTCATTACGTTTCGATAAAAAAATGATTAGTTTATCATAGCTTAATTATAGCATTG
ATTTCTAAATTTGTTTTTTGACCACCCTTTTTTCTCTCTTTGGTGTTTTCTTAACATTAGAAGA
ACCCATAACAATGTACGTTCAAATTAATTA AAAACAATATTTCCAAGTTTTATATACGAAACTT
GTTTTTTTTTAATGAAAACAGTTGAATAGTTGATTATGAATTAGTTAGATCAATACTCAATATAT
10 GATCAATGATGTATATATATGAACCTAGTTGTTATACAAGAAATGAAAATGCTATTTAAATACA
GATCATGAAGTGTTAAAAAGTGTGAGAAATATGACATGAAGCGTTTTGTCTACCGGGTATTCTGA
GTTATAGGTTTGGATCTCTCAAGAATATTTTGGGCCATACTAGTTATATTTGGGCTTAAGCGTT
TTGCAAAGAGACGAGGAAGAAAGATTGGGTCAAGTTAACAAAACAGAGACACTCGTATTAGTTG
GTACTTTGGTAGCAAGTCGATTTATTTGCCAGTAAAAACTTGGTACACAACCTGACAACCTCGTAT
CGTTATTAGTTTGTACTTGGTACCTTTGGTTCAAGAAAAAGTTGATATAGTTAAATCAGTTGTG
15 TTCATGAGGTGATTGTGATTTAATTTGTTGACTAGGGCGATTCTTCACATCACAATAACAAAG
TTTTATAGATTTTTTTTTTTTATAACATTTTTGCCACGCTTCGTAAAGTTTGGTATTTACACCGCA
TTTTTCCCTGTACAAGAATTCATATATTTATTTATTTATATACTCCAGTTGACAATTATAAGTTT
ATAACGTTTTTACAATTATTTAAATACCATGTGAAGATCCAAGAATATGTCTTACTTCTTCTTT
GTGTAAGAAAACCTAACTATATCACTATAATAAAATAATTCTAATCATTATATTTGTAAATATGC
20 AGTTATTTGTCAATTTTGAATTTAGTATTTTAGACGTTATCACTTCAGCCAAATATGATTTGGA
TTTAAGTCCAAAATGCAATTTTCGTACGTATCCCTCTTGTCTGCTAATGATTATTTCAATATTTT
TTATATTATCCCTAACTACAGAGCTACATTTATATTGTATTCTAATGACAGGGAAACCTTCATA
GAGATTCAGATAGATGAAATTGGTGGGAAACATCATTGAACAGGAAACTTTTAGCAAATCATAT
CGATTTATCTACAAAAGAATACGTAGCGTAATGAAGTCCACTTGTGTGAATGACTATGATTTG
25 ATCAAATTAGTTAATTTTGTGCAATCATTTTTCTTTTTGATTTGATTAAGCTTTTAACTTGCAC
GAATGGTTCTCTTGTGAATAAACAGAATCTTTGAATCAAACCTATTTGATTAGTGAAAAGACAA
AAGAAGATTCCTTGTTTTTATGTGATTAGTGATTTTGATGCATGAAAGGTACCTACGTACTACA
AGAAAAATAACATGTACGTAACCTACGTATCAGCATGTAAAAGTATTTTTTTCCAAATAATTTA
TACTCATGATAGATTTTTTTTTTTTTGAAATGTCAATTAAAAATGCTTTCTTAAATATTAATTTT
30 AATTAATTAAATAAGGAAATATATTTATGCAAAACATCATCAACACATATCCAACCTTCGAAAAT
CTCTATAGTACACAAGTAGAGAAATTAAATTTTACTAGATACAACTTCCTAATCATCAAATAT
AAATGTTTACAAAACCTAATTAAACCCACCCTAAAAATTAATAAAAAATCCGAGCAAAGTGAGTG
AACAAGACTTGATTTTCAGGTTGATGTAGGACTAAAAATGACTACGTATCAAACATCAACGATCAT
TTAGTTATGTATGAATGAATGTAGTCATTACTTGTAAAACAAAAATGCTTTGATTTGGATCAAT
35 CACTTCATGTGAACATTAGCAATTACATCAACCTTATTTTCACTATAAAACCCCATCTCAGTAC
CCTTCTGAAGTAATCAAATTAAGAGCAAAAGTCATTTAACTTAGG

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SEQ ID NO:16

Therefore, the promoter in the construct of the invention may comprise a
40 nucleotide sequence substantially as set out in SEQ ID No.16, or a functional
variant or functional fragment thereof. The SAG12 promoter sequence may be
obtained from *Arabidopsis thaliana*, as described in US 5,689,042. This promoter
sequence can be seen in each of the genetic constructs in accordance with the
invention, as shown in Figure 3. In embodiments where the promoter is SAG12, it
45 will be appreciated that the promoter may comprise each of the bases 1-2093 of SEQ

- 7 -

ID No:16. However, functional variants or functional fragments of the promoter may also be used in the genetic constructs of the invention.

A “functional variant or functional fragment of a promoter” can be a derivative or a portion of the promoter that is functionally sufficient to initiate expression of any coding region that is operably linked thereto. For example, in embodiments where the promoter is based on SAG12, the skilled technician will appreciate that SEQ ID No:16 may be modified, or that only portions of the SAG12 promoter may be required, such that it would still initiate gene expression in the construct.

Functional variants and functional fragments of the promoter may be readily identified by assessing whether or not transcriptase will bind to a putative promoter region, and then lead to the transcription of the coding region into the polypeptide having PCK and/or PPDK activity. Alternatively, such functional variants and fragments may be examined by conducting mutagenesis on the promoter, when associated with a coding region, and assessing whether or not gene expression may occur.

The genetic construct of the first aspect may be capable of causing, during senescence, expression of at least one polypeptide exhibiting PCK activity and/or PPDK activity. Therefore, the genetic construct may comprise at least one coding sequence, which encodes (i) a phosphoenolpyruvate carboxykinase (PCK), or a functional variant or fragment thereof, and/or (ii) a pyruvate orthophosphate dikinase (PPDK), or a functional variant or fragment thereof. As described in the Examples, the inventors have developed a range of genetic constructs based on polypeptides having either PCK and/or PPDK activities, and these are shown in Figures 3.

In a first embodiment of the genetic construct according to the first aspect, the promoter may induce expression of a coding sequence encoding a polypeptide exhibiting PCK activity. This is referred to herein as a “PCK construct”, and is shown in Figure 3. Hence, in the first embodiment, the genetic construct may

comprise the senescence-specific promoter and a coding sequence encoding a phosphoenolpyruvate carboxykinase (PCK), or a functional variant or fragment thereof. The genetic construct may not encode a polypeptide having PPDK activity.

5

In a second embodiment of the construct according to the first aspect, the promoter may induce expression of a coding sequence encoding a polypeptide exhibiting PPDK activity. This is referred to herein as a “PPDK construct”, and is shown in Figure 3. In the second embodiment, the genetic construct may

10 comprise the senescence-specific promoter and a coding sequence encoding a pyruvate orthophosphate dikinase (PPDK), or a functional variant or fragment thereof. The genetic construct may not encode a polypeptide having PCK activity.

15 In a third embodiment of the construct according to the first aspect, the promoter may induce expression of a single coding sequence encoding a polypeptide exhibiting both PCK activity and PPDK activity. This is referred to as a “PCK/PPDK construct 1”. In the third embodiment, the genetic construct may comprise the senescence-specific promoter and a coding sequence encoding

20 (i) phosphoenolpyruvate carboxykinase (PCK), or a functional variant or fragment thereof, and (ii) pyruvate orthophosphate dikinase (PPDK), or a functional variant or fragment thereof. The construct of the third embodiment may encode a single transcript exhibiting dual activity, i.e. both PCK and PPDK enzymatic activity. The PCK-coding region may be positioned on the 3' side of

25 the PPDK-coding region. However, preferably the PCK-coding region is positioned on the 5' side of the PPDK-coding region.

In a fourth embodiment of the construct according to the first aspect, the promoter may induce expression of (i) a first coding sequence encoding a first

30 polypeptide, which exhibits PCK activity, and (ii) a second coding sequence encoding a second polypeptide, which exhibits PPDK activity. This is referred to as a “PCK/PPDK construct 2”. Hence, in the fourth embodiment, the

- 9 -

genetic construct may comprise at least one senescence-specific promoter and (i) a first coding sequence encoding PCK, or a functional variant of fragment thereof, and (ii) a second coding sequence encoding PPDK, or a functional variant or fragment thereof, i.e. two transcripts are encoded, one for each enzyme.

As described in Example 6, the inventors have found that over-expressing either PCK or PPDK in a host cell (e.g. by transforming with either the “PCK construct” or the “PPDK construct”) caused an increase in nitrogen remobilisation in senescent leaves. Furthermore, they found that the PCK and PPDK single constructs resulted in an increase in vegetative growth.

As described in Example 8, the inventors have found that simultaneously over-expressing both PCK and PPDK in a host cell (e.g. by transforming with both the “PCK construct” and the “PPDK construct”) is surprisingly effective at inducing nitrogen remobilisation in senescent leaves. Hence, nitrogen may be transported out of senescent leaves, for example as transport amino acids.

Suitable transport amino acids may be glutamine and/or asparagine.

Furthermore, simultaneously over-expressing PCK and PPDK during senescence may also increase growth rate, which may result in an increase in vegetative growth. Hence, the construct of the first aspect may comprise a coding sequence which encodes both PCK and PPDK, or a functional variant or fragment thereof.

It will be appreciated that the construct of the second aspect comprises a coding sequence which encodes both PCK and PPDK, or a functional variant or fragment thereof. The two enzymes may be encoded as a single polypeptide having dual activity, or as two polypeptides, one having PCK activity and the other having PPDK activity.

The phosphoenolpyruvate carboxykinase (PCK) or a functional variant or fragment thereof, and the pyruvate orthophosphate dikinase (PPDK) or a

functional variant or fragment thereof, may each be derived from any suitable source, such as a plant. The coding sequence of each enzyme may be derived from a suitable plant source, for example from *Arabidopsis*. Therefore, the coding sequence, which encodes the polypeptide having PCK activity, may be derived from *Arabidopsis*. Furthermore, the coding sequence, which encodes the polypeptide having PPDK activity, may be derived from *Arabidopsis* spp., *Zea* spp., *Flaveria* spp., or *Cleome* spp.. The coding sequence, which encodes the polypeptide having PPDK activity, may be derived from *Arabidopsis thaliana*, *Zea mays*, *Flaveria trinervia*, *Flaveria bidentis*, *Flaveria brownie* or *Cleome gynandra*.

There are believed to be three genes in *A.thaliana* coding for PCK. The genomic DNA sequence (including introns and exons) encoding one embodiment of *Arabidopsis* phosphoenolpyruvate carboxykinase (PCK) is provided herein as SEQ ID No:17, as follows:

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ATGTCGGCCGGTAACGGAAATGCTACTAACGGTGACGGAGGGTTTAGTTTCCCTAAAGGACCGG
TGATGCCGAAGATAACGACCGGAGCAGCAAAGAGAGGTAGCGGAGTCTGCCACGACGATAGTGG
TCCGACGGTGAATGCCACAACCATCGATGAGCTTCATTCGTTACAGAAGAAACGTTCTGCTCCT
ACCACACCGATCAACCAAAACGCCGCCGCTGCTTTTGCCGCCGTCTCCGAGGAGGAGCGTCAGA
AGATTCAGCTTCAATCTATCAGGTCCTTATAATAACTTCACATATACAGATTATTCATACGTTA
CTTTTGTTTATAACATACTTTATATCGAATTAAGGAAGATTATTGCGTTTTTCGTGTCGGATCAT
TTTCATGGAAAAAGTGTCTTTTAGCTAAATATATGGTGTAGTATTAAATATTTCTGACGTGATA
TACACTAAACTTGAAAAATTTCAATTACTATTTCTTCCTTTAATTCGGCAATATAATTTGTTTT
TGTTTTATTTTTGGATTAGACATTTATGGACAAGTTAATGCGCTATTGTGACTATTACCAGAAAA
TAATACTTTAATGTACATGACACGTGTTTAAACGACACGTGGAACTAATTTTGATTAATTGT
GAAACAGTGCATCGTTAGCATCGTTAACGAGAGAGTCAGGACCAAAGGTGGTGAGAGGAGATCC
GGCGGAGAAGAAGACCGATGGTTCAACTACTCCGGCGTACGCTCACGGCCAACATCATTCTATC
TTTTCTCCGGCTACTGGTGCTGTCAGTGATAGCTCCTTGAAGTTTACTCACGTCTCTACAATC
TTTCGCCTGCAGGTCAACAAATAAACCTAGAAATCCGAATCTGAATATTGATAAATGTTTCTGCA
ACGAGTTTGATAGATTTGGTTTTGTGATTTTGTGTTTGTAGAGCTTTATGAGCAAGCTATTAAG
TATGAGAAAGGTTTCGTTTATCACTTCTAATGGAGCTTTGGCGACGCTTTCTGGTGCTAAGACTG
GTCGTGCTCCCAGAGATAAGCGTGTTGTTAGAGATGCTACTACTGAGGATGAGCTTTGGTGGGG
AAAGTGAGTATTCCTAATCTCGATTTTGATTGATGGAGTTTTTGGGTTTATGCTCTGTTTTCGT
TTATTGATTTTGGAGTTTGATTTTGTATTTAGGGGTTTCGCCGAATATCGAAATGGATGAACATA
CTTTCATGGTGAACAGAGAAAGAGCTGTTGATTACTTGAATTCCTTGGAAAAGGTATTAAATTT
TGAAAACCTTTAATCAATGTTGTTGAGTGTAAGCTTTTGTGATCTAAGTTTATGAAATTTCTGTTG
TTGTTGGGGTTTTTGGTCTTTGTCAATGACCAATACTTAAACTGGGATCCAGAGAACAGAATC
AAAGTCAGGATTGTCTCAGCTAGAGCTTACCATTGTTTATGCACAACATGTAAGTAAAAT
CATTATTGACTCCTTGTATGTCAATCCATTATTGTGGGTGAAAGAAAACAACAAATTAGTAACT
GGGGAGGGTGTCAGGTGTATCCGACCAACTCAGGAGGAGCTTGAGAGCTTTGGTACTCCGGATT
TTACTATATACAATGCTGGGCAGTTTCCATGTAATCGTTACACTCATTACATGACTTCGTCCAC
TAGCGTAGACCTTAATCTGGCTAGGAGGGAAATGGTTATACTTGGTACTCAGTATGCTGGGGAA
ATGAAGAAGGCTCTTTTCAGTGTGATGCATTACCTTATGCCTAAGCGTCGTATTCTCTCCCTTC
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- 11 -

ATTCTGGATGCAATATGGGAAAAGATGGAGATGTTGCTCTCTTCTTTGGACTTTCAGGTATAGT
AGAGACAGTACCAACTATGGTGTGGGTGATGATGGAAGGAACGATAAATCAAATGATACAATA
CAATTACTGCTGAACTGACTTGAGAACTGCTTGCCTCTTTGTTGAGTTTAGCGGGTGAATTGAG
ATTGATGATTGTGTTTTTGTCTATGAATGATGATTTTAGGTACCGGGAAGACAACGCTGT
5 CTACTGATCACAACAGGTATCTTATTGGAGATGATGAGCATTGTTGGACTGAGACTGGTGTTC
GAACATTGAGGGTGGGTGCTATGCTAAGTGTGTTGATCTTTCGAGGGAGAAGGAGCCTGATATC
TGGAACGCTATCAAGTTTGGAAACAGGTAGAAAGACAGTACGTTGGAATTGTTTTTGAGAAAAA
ACATAAAGCAGTGATATAACAATAAGATTCTGATCTTGTTCAGTTTTGGAAAAATGTTGTGTTT
GATGAGCACACCAGAGAAGTGGATTACTCTGATAAATCTGTTACAGGTAAAACAATTGTTATTT
10 CTTTCATTCTCTTCGTCCTCACAATTAACAGAATGATCATTTCGATTCTCTTTGGTTGCAGAG
AACACACGTGCTGCCTACCCAATTGAGTTTCATTCCAAATGCGAAAATACCTTGTGTTGGTCCAC
ACCCGACAAATGTGATACTTCTGGCTTGTGATGCCTTTGGTGTCTCCCACCTGTGAGCAAGCT
GAATCTGGCACAACCATGTACCACCTTCATCAGTGGTTACACTGCTCTGGTAAGGCCAAAGTAA
AAGTCTTTATTTTGCACATCGTCTTCATAAATTTCAAAGCATAACCAAAGATGTGCAACATAT
15 ATAGGTTGCTGGCACAGAGGATGGTATCAAGGAGCCAACAGCAACATTCTCAGCTTGCTTTGGT
GCAGCTTTCATAATGTTGCATCCACAAAGTATGCAGCTATGTTAGCTGAGAAGATGAAGTCAC
AAGGTGCTACTGGTTGGCTCGTCAACACTGGTTGGTCTGGTGGCAGGTATATATGTCCTTCTAT
GGAAATCGATACAACAAAACGCTGCCTTGTAAACACATGTTTGTAGGCTATTAACATGATCTGTA
ATGTTTTATTTCTGCAGTTATGGTGTGGAAACAGAATCAAGCTGGCATACTAGAAAGATC
20 ATCGATGCAATCCATTTCGGGCAGTCTCTTGAAGGCAAACACTACAAGAAAACCGAAATCTTTGGAT
TTGAAATCCCACTGAGATCGAAGGGATACCTTCAGAGATCTTGGACCCCGTCAACTCCGTAAG
TTTCTGCAAATCTGTATAATGTAATTGCTTAAGTGATGATGAACAATTTTTTGTGATTTGGGT
TTAATGAAAATGCAGTGGTCTGATAAGAAGGCACACAAAGATACTCTGGTGAAACTGGGAGGTC
TGTTCAAGAAGAACTTCGAGGTTTTTGTCTAACCATAGATTGGTGTGATGGTAAGCTTACGGAG
25 GAGATTCTCGCTGCTGGTCCTATCTTTTAG

SEQ ID No:17

The cDNA sequence (exons only) encoding the *Arabidopsis* phosphoenolpyruvate carboxykinase (PCK) is provided herein as SEQ ID No:18, as follows:

30 ATGTCGGCCGGTAACGGAAATGCTACTAACGGTGACGGAGGGTTTAGTTTTCCCTAAAGGA
CCGGTGATGCCGAAGATAACGACCGGAGCAGCAAAGAGAGGTAGCGGAGTCTGCCACGAC
GATAGTGGTCCGACGGTGAATGCCACAACCATCGATGAGCTTCATTCTGTTACAGAAGAAA
CGTTCTGCTCCTACCACACCGATCAACCAAACGCCGCCGCTGCTTTTGCCGCCGCTCTCC
35 GAGGAGGAGCGTCAGAAGATTCAGCTTCAATCTATCAGTGCATCGTTAGCATCGTTAACG
AGAGAGTCAGGACCAAAGGTGGTGAGAGGAGATCCGGCGGAGAAGAAGACCGATGGTTCA
ACTACTCCGGCGTACGCTCACGGCCAACATCATTCTATCTTTTCTCCGGCTACTGGTGCT
GTCAGTGATAGCTCCTTGAAGTTTACTCACGTCTCTACAATCTTTCGCTGCAGAGCTT
TATGAGCAAGCTATTAAGTATGAGAAAGGTTTCGTTTATCACTTCTAATGGAGCTTTGGCG
40 ACGCTTTCTGGTGCTAAGACTGGTCTGTGCTCCCAGAGATAAGCGTGTTGTTAGAGATGCT
ACTACTGAGGATGAGCTTTGGTGGGGAAAGGGTTCGCCGAATATCGAAATGGATGAACAT
ACTTTCATGGTGAACAGAGAAAGAGCTGTTGATTACTTGAATTCCTTGGAAAAGGTCTTT
GTCAATGACCAATACTTAACTGGGATCCAGAGAACAGAATCAAAGTCAGGATTGTCTCA
GCTAGAGCTTACCATTCAATTGTTTATGCACAACATGTGTATCCGACCAACTCAGGAGGAG
45 CTTGAGAGCTTTGGTACTCCGGATTTTACTATATACAATGCTGGGCAGTTTCCATGTAAT
CGTTACACTCATTACATGACTTCGTCCACTAGCGTAGACCTTAATCTGGCTAGGAGGGAA
ATGGTTATACTTGGTACTCAGTATGCTGGGGAAATGAAGAAGGGTCTTTTCAGTGTGATG
CATTACCTTATGCCTAAGCGTCGTATTCTCTCCCTTCATTCTGGATGCAATATGGGAAAA
GATGGAGATGTTGCTCTCTTCTTTGGACTTTCAGGTACCGGGAAGACAACGCTGTCTACT
50 GATCACAACAGGTATCTTATTGGAGATGATGAGCATTGTTGGACTGAGACTGGTGTTCG
AACATTGAGGGTGGGTGCTATGCTAAGTGTGTTGATCTTTCGAGGGAGAAGGAGCCTGAT

- 12 -

ATCTGGAACGCTATCAAGTTTGGAAACAGTTTTGGAAAATGTTGTGTTTGATGAGCACACC
 AGAGAAGTGGATTACTCTGATAAATCTGTTACAGAGAACACACGTGCTGCCTACCCAATT
 GAGTTCATTCCAAATGCGAAAATACCTTGTGTTGGTCCACACCCGACAAATGTGATACTT
 CTGGCTTGTGATGCCTTTGGTGTCTCCACCTGTGAGCAAGCTGAATCTGGCACAAACC
 5 ATGTACCACTTCATCAGTGGTTACACTGCTCTGGTTGCTGGCACAGAGGATGGTATCAAG
 GAGCCAACAGCAACATTCTCAGCTTGCTTTGGTGCAGCTTTCATAATGTTGCATCCCACA
 AAGTATGCAGCTATGTTAGCTGAGAAGATGAAGTCACAAGGTGCTACTGGTTGGCTCGTC
 AACACTGGTTGGTCTGGTGGCAGTTATGGTGTGTTGGAAACAGAATCAAGCTGGCATACT
 AGAAAGATCATCGATGCAATCCATTTCGGGCAGTCTCTTGAAGGCAAACTACAAGAAAACC
 10 GAAATCTTTGGATTTGAAATCCCAACTGAGATCGAAGGGATACCTTCAGAGATCTTGGAC
 CCCGTCAACTCCTGGTCTGATAAGAAGGCACACAAAGATACTCTGGTGAACTGGGAGGT
 CTGTTCAAGAAGAACTTCGAGGTTTTTGGCTAACCATAAGATTGGTGTGATGGTAAGCTTA
 CGGAGGAGATTCTCGCTGCTGGTCCTATCTTTTAG

SEQ ID No:18

15

Accordingly, the coding sequence, which encodes the polypeptide having PCK
 activity, may comprise a nucleic acid sequence substantially as set out in SEQ ID
 No:17 or SEQ ID No.18, or a functional variant or fragment thereof.

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The polypeptide sequence of *Arabidopsis* PCK is provided herein as SEQ ID
 No:19, follows:

MSAGNGNATNGDGGFSFPKGPVMPKITTGAAKRSGVCHDDSGPTVNATTIDELHSLQKK
 RSAPTTPINQNAFAAFAVSEERQKIQLQSIASLASLTRESGPKVVVRGDPAEKKTDGS
 25 TTPAYAHGQHHSIFSPATGAVSDSSLKFTHVLYNLSPAELYEQAIKYEKGSFITSNGALA
 TLGAKTGRAPRDKRVVRDATTEDELWWGKGSPNIEMDEHTFMVNRERAVDYLNLSLEKVF
 VNDQYLNWDPENRIKVRIVSARAYHSLFMHNMCIPTQEELESFGTPDFTIYNAGQFPCN
 RYTHYMTSSTSVDLNLARREMVILGTQYAGEMKKGLFSVMHYLMPKRRILSLHSGCNMGK
 DGDVALFFGLSGTGKTTLSTDHNRYLIGDDEHCWTETGVSNIIEGGCYAKCVDLSREKEPD
 30 IWNAIKFGTVLENVVFDEHTREVDYSDKSVTENTRAAYPIEFIPNAKIPCVGPHPTNVIL
 LACDAFGVLPVSKNLNAQTMYHFISGYTALVAGTEDGIKEPTATFSACFGAAAFIMLHPT
 KYAAMLAEKMKSQGATGWLVTGWSSGSYGVGNRIKLAYTRKIIDAIHSGSLLKANYKKT
 EIFGFEIPTIEGIPSEILDVNSWSDKKAHKDTLVKLGLFKKNFEVFANHKIGVMVSL
 RRRFSLVLVSF

35

SEQ ID No:19

Accordingly, the polypeptide having PCK activity may comprise an amino acid
 sequence substantially as set out in SEQ ID No:19, or a functional variant or
 fragment thereof.

40

Arabidopsis is believed to have at least two forms of PPK, a chloroplastic form
 and a cytosolic form, both of which are encoded by the same gene with minor splicing

variations at the 5' end of the gene giving rise to the two forms. The genomic DNA sequence (including introns and exons) encoding both forms of *Arabidopsis* pyruvate orthophosphate dikinase (PPDK) is provided herein as SEQ ID No:20, as follows:

```
5  ATGACAAGTATGATCGTGAAGACAACGCCGGAGCTCTTCAAAGGAAATGGAGTGTTCCGTACGG
   ATCATCTCGGAGAAAACCGAATGGTTAGTCGATCAAACCGGCTAGGTGATGGATCAAACCGTTT
   CCCTAGAACCGGTACAATCCATTGCCAACGGTTAAGCATAGCAAAGACCGGTTTGCATCGTGAG
10  ACGAAGGCTCGAGCCATACTTAGCCCTGTGTCCGATCCGGCCGCTTCCATAGCCCCAAAAGGTAA
   GCCTTTCCATTTCAATCATTCTGGTGTATTTTACCATAAAATTTTATACACTTTTTTATTACG
   TTTTGTTTTATGATTCTGACGTGAGATTCTTGAGAGAACTATCACCGATCATTGGGTCTGAACC
   ATCTAGCAGCTCAATTATTATCGGTTATAACCCTACCGGTTATAGAATACAAAACAGGTTACGC
   CATTGTGACATTTGCTTTGTGATCTTGTGAGACGATTAATTATTTGATGTTGATTGGTTTCGTT
   ACTCTTGTTTAAACAATCGAACGGTTCAAATAATACACACATGTGATGTGAGATCATTTCGGT
15  AGTAATACCAAATAGCGTCTGGCCTAAATTATGAAAGTACTATTTTGAATTAAATTATTGTGGA
   AACATGAACTTATTTAAATTCAAGTATTTTCGAAATTTGTAATAAAAAAAAACTTTTCTCTAG
   ATTCATTAGCCCTACTTTTCGTAGAAACAACCTTAATGTATTCAAAGACCACTTGCTGCTTAA
   GTCAGACTCTTGTGCCACTTGGTAGATCCACCAATGCCACGTTTTGTTATTGTGCCAAAGAATA
   CGTGAATATGTCCAAACGGCAATCAAATTCCTTGGCGTAAAACACAAAAATTATGATACTAGTTT
20  AAATCCACAATTCACCTCACCATAAAGAATTCATGTATTAGAGATGGTATGACAAGAACTGGT
   TGAATTTGATGACATTTGTTTGCTATTGTTTTGGTTAAGTAAAAGTTTTGTTAAAAAGGAAAAT
   AGCATCGGTAGTGGCAGATAGCAAGTGTGTGAGTGAGATCAGATATGGTTGACACATCTATGAC
   GAGTCATCGCAACGAACTTCTTTAATTTTGGTCAATTATATTACAATTTAGCATTTTCGAGGTT
   GGAATTTTGGAAATGATCTCTTGATAAGATAATAATGTATTTTTGATGACGTATCCATCAAACT
25  ATAAATGATTTATATTAAATATGAAATTTTCGACTGTATACAAGTTTTTATATTATAAAATTATT
   CGATGTACATATGATCATAATAACTTTACTATATATATAGATACGTATATGTGTTCTTAACTT
   GCACAAACATTTCTGCAATCTAAACCTCAATCAAAACAAACAAACAAAAAACCATGATGCAGCG
   AGTATTCACCTTTGGAAAAGGAAGAAGCGAAGGCAACAAGGGCATGAAGTCCTTGGTATGTTAC
   CAATACCATCATCATGATCATATCAATTCATTAATAATTTAGTGTTTGCTATTTTTCAAGAACCA
30  TTTATCAAAAATGTTAATTGTTGTTGTGTATGAAGTTGGGAGGGAAAGGAGCCAACCTGGCGGA
   GATGGCTAGCATAGGCTTGTCGGTGCCGCCGGGGCTAACCATATCGACGGAGGCTTGTCAGCAG
   TATCAGATCGCCGGCAAAAAGCTTCCAGAAGGTTTATGGGAAGAGATCTTAGAAGGTCTTAGCT
   TCATCGAACGTGACATTGGAGCTTCCCTCGCTGATCCCTCCAAGCCACTCCTCCTCTCTGTTCTG
   CTCGGCGCGCCGCGTAAGTTAATTATAACTTTTTTTCTTGACTATTTTTATTTTAAGGATTTTT
35  TCTAATGTAAATTTCTGTTTTTTTTCTTTCTATGTTTTCTTTAATCTTTTGAAGATTTTTTG
   ACGCAGATTTTGACTTGTTAGATTTCTTTTATTGAAGTTGAGATCCAAATATTTTTTTGGTTAT
   TTTGCCATTTGGCCGTTTTTGGAAAGAGTTTAAAATGTACTAGATAGAAAATGAATAAGTTTTGT
   GGCTATTGAAAGACCTAATGATTTTGGTATTCAACTATAACGTAGAAAATGAAGATCTTTCGT
   TTATCTATTTTTTAAAACAGAACTACATTGACTTGTCTTTGATCGATATTTTGCATTGTAGATCT
40  CAATGCCTGGTATGATGGACACTGTACTTAACCTTGGCTTGAACGACCAAGTCGTCGTTGGTCT
   GGCCGCAAAAAGCGGAGAGCGTTTTTGCTTACGATTCGTTCCGGCGTTTTTCTTGATATGTTTGGT
   GATGTTGTAAGTCCTCTGTTTTTTCAATACTATTTTCAGGTAACCTTGCATGACAAGAAAATCTTTT
   GACCTACCTTATAATTGTTTTCTTGATCAATAAAAGGTGATGGGAATTCCACACGCCAAGTTTG
   AAGAGAAGTTAGAGAGAATGAAGGAGAGGAAAGGAGTTAAAAATGACACTGACTTAAGCGCGGC
45  TGATCTCAAGGAATTGGTTGAGCAGTACAAGAGTGTTTACTTAGAGGCCAAGGGTCAAGAGTTT
   CCTTCAGGTTTGTTTTGATTCCTACTTGAGGTCAAGTGATAAAAATTAGTTATTAGTTACAAAT
   GTTTAAACGGGGTTAATTGCAGATCCAAAGAAGCAATTGGAGCTAGCGATTGAAGCGGTATTTCG
   ATTCTTGGGATAGCCCGAGAGCGAACAAGTACAGAAGTATTAACCAGATAACTGGATTGAAAGG
   AACCGCGGTTAACATTCAGTGTATGGTGTGTTGGAAACATGGGGGACACTTCAGGGACTGGTGT
50  CTCTTCACTAGGAACCTAGCACAGGAGAGAAGAAGCTTTATGGCGAGTTTCTAGTTAATGCTC
   AGGTTTGGCATCTATCACAAATGTGTGAATCTCATATCAACAAGTAAGCCCATACTCATTAAATA
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- 14 -

TTGGTTTTGGGACAGGGAGAGGATGTGGTTGCAGGGATAAGAACCAGAAAGATTTGGATACAA
TGAAGAGATTTATGCCTGAGGCTTACGCTGAACTTGTTGAGAACTGCAACATCTTAGAAAGACA
TTACAAAGACATGATGGTTGATACACATAAACAATACTTCAATTAGTCCTCATCAACAATTCTT
TAGTAATTTAAACAAAATCTCAAATGTGTATTGCAGGATATTGAATTCACAGTACAAGAAGAGA
5 GATTGTGGATGCTGCAATGCAGAGCGGGTAAGCGAACGGGTAAAGGCGCCGTGAAGATAGCAGT
TGATATGGTAGGTGAAGGGCTTGTTGAGAAATCTTCTGCTATCAAAATGGTGGAGCCTCAACAT
CTTGATCAACTACTTCACCCACAGGTACAACTCAAATATTCATCTTCTTCTTTTTTCATAGTC
ATAAACTTGATGTTGAAACCAAAATTCGAAACTTACTGGTAATGATTGGTTCACTTGAACAAGA
ACTAATGGGTTTTAAGACGTTTAGGGTTTAGGAGTAAAAGCAGAGATGATTGTCTGACACGTAAC
10 CGATGAATAGGGTTTTGGAAATTTTGATTGAGAGGTCAATGAAGGTTTTTTTTTTTTTTTTTAT
TGATGGATTAGTTTCATGATCCATCGGGGTATCGTGAAAAAGTGGTGGCCAAAGGCTTACCTGC
GTCACCAGGAGCGGCGGTGGACAGGTTGTGTTACGGCGGAGGAAGCCGAAGCTTGGCATTCT
CAGGGTAAAACGTGATTCTGGTTCGAACTGAGACAAGCCCTGACGATGTGGGAGGTATGCACG
CAGCGGAAGGTATATTGACGGCTAGAGGAGGAATGACGTCACACGCGGCTGTTGTTGCTCGCGG
15 TTGGGGAAAATGTTGCATTGCTGGTTGTTCCGAGATTCTGTGTCGACGAGAACCACAAGGTTTTT
GGATTGATTTTTAGAACTTGTCTATATAAGTTAGGGGAAGATTGTTTCTAAAGTTAGGGTTTAA
AAATTTTCAGGTTCTATTGATTGGAGATTTGACGATTAATGAAGGCGAATGGATCTCAATGAAC
GGATCAACCGGTGAGGTTATATTAGGGAAACAAGCATTTGGCTCCTCCGGCTTTAAGTCCAGATT
TGGAGACTTTCATGTCTGGGCTGATGCAATCAGACGTCTCAAGGTGTTTATGAGTTTCTGTTC
20 CTTTAACTTGTTTGATATTTTTAACTTTCTAACTCAAATGTTTCGATGACCGATAAGGTTATGG
CGAATGCGGATACACCTGAAGACGCCATTGCAGCTAGGAAAAACGGAGCTCAAGGAATCGGGCT
TTGTAGGACAGAGCATATGGTAACTCCTCCTCTGTACTTGATTTTCATGTTTTTGATGATTTAGA
TTGTTTGTATCCAAATGTTTAATGTTGTCTTTGGTTTGGTTTTTAAGTTCTTTGGAGCAGATAG
GATTAAAGCAGTGAGAAAGATGATAATGGCGGTAACAACAGAGCAAAGGAAAGCTTCTCTCGAC
25 ATCTTGCTTCCTTACCAACGTTTCGGATTTCGAAGGGATCTTCCGTGCTATGGATGGTAAATGTT
TTGAGTCGTCTCTCTAAATGTATCACAACTTAAAACATGCCTAAACCTTTTTATTTTTCTAGG
TTTACCGGTAACAATCCGTTTGTAGACCCTCCGCTTCACGAGTTTCTCCCGGAAGGCGACTTG
GACAACATTGTACATGAGCTAGCTGAAGAACTGGTGTGAAAGAAGATGAAGTCTTGTACGGA
TAGAGAACTCTCTGAAGTGAATCCAATGCTTGGTTTCCGCGGTTGCAGGTTTCTTACTCTCTT
30 TGTTTCTCTGTCTCTTGCACCTGAAGAACAATCTGATGATCGGTAAACTTGTACGTTATAG
GCTCGGAATATCGTATCCAGAGCTAACGGAGATGCAAGCGCGTGCAATTTTTGAAGCTGCAGCG
TCAATGCAGGACCAAGGTGTTACTGTCAATTCCTGAGATTATGGTTCCACTTGTAGGAACTCCTC
AGGAATTGGGTCACCAAGTTGATGTAATTCGTAAAGTTGCAAAGAAAGTATTTGCTGAGAAGGG
TCATACCGTGAGCTACAAGTTGGGACAATGATTGAGATCCCTCGAGCCGCGCTCATTGCAGAT
35 GAGGTAAATGTAACAAGACACAAAATGTGTTTTAGGCACCTGAAACCATGTTGCTATTTGCTAA
GTAGGAACCTTTTTCTTTTGACAGATTGCGAAAGAGGCGGAGTTTTTCTCGTTCTGGGACAAACG
ACTTGACGCAGATGACGTTTGGATACAGTAGAGACGATGTCGGCAAGTTTCTACCGATTACCT
CGCCAAAGGAATCTTACAGCACGACCCTTTTGAGGTATAATGACTACCATTTCTGTTTGTCTCT
ATCCATAGGATAAAATCTTGATAGCCATTTTTTTGTGTTTGGACCAGGTTCTTGATCAGCAAGG
40 TGTAGGGCAATTGATCAAGATGGCGACAGAAAAAGGACGAGCAGCTAGGCCTAGCCTCAAGGTT
GGGATATGTGGAGAACATGGAGGAGATCCATCTTCTGTGGGATTCTTTGCTGAAGCAGGACTTG
ACTATGTCTCTTGTCTCTTTTCAGGTAATTGATTAATTTCAAACCAATAAACACTTTTTTTA
CAACACTATTGTATAACTCAGATTGATGTAATTTTGGGATTTCTGTTGTTGTTGTTGTTGTTG
TGTTGTTGCAGGGTTCCAATTGCAAGGCTTGACGCTGCTCAAGTAGTTGTTGCATGA
45

SEQ ID No:20

The cDNA sequence encoding the cytosolic form of the *Arabidopsis* pyruvate
orthophosphate dikinase (PPDK) is provided herein as SEQ ID No:21, as
50 follows:

- 15 -

ATGATGCAGCGAGTATTCACCTTTGGAAAAGGAAGAAGCGAAGGCAACAAGGGCATGAAG
TCCTTGTGGGAGGGAAAGGAGCCAACTGGCGGAGATGGCTAGCATAGGCTTGTCTGGTG
CCGCCGGGGCTAACCATATCGACGGAGGCTTGTCTAGCAGTATCAGATCGCCGGCAAAAAG
5 CTTCCAGAAGGTTTATGGGAAGAGATCTTAGAAGGTCTTAGCTTCATCGAACGTGACATT
GGAGCTTCCCTCGCTGATCCCTCCAAGCCACTCCTCCTCTCTGTTCTCGCTCCGGCGCCGCC
ATCTCAATGCCTGGTATGATGGACACTGTACTTAACCTTGGCTTGAACGACCAAGTCGTC
GTTGGTCTGGCCGCAAAAAGCGGAGAGCGTTTTTGCTTACGATTCTGTTCCGGCGTTTTCTT
GATATGTTTGGTGTGTTGTGATGGGAATTCACACGCCAAGTTTGAAGAGAAGTTAGAG
10 AGAATGAAGGAGAGGAAAGGAGTTAAAAATGACACTGACTTAAGCGCGGCTGATCTCAAG
GAATTGGTTGAGCAGTACAAGAGTGTTTACTTAGAGGGCCAAGGGTCAAGAGTTTCCTTCA
GATCCAAAGAAGCAATTGGAGCTAGCGATTGAAGCGGTATTCGATTCTTGGGATAGCCCG
AGAGCGAACAAGTACAGAAGTATTAACCAGATAACTGGATTGAAAGGAACCGCGGTAAAC
ATTCAAGTGTATGGTGTGTTGGAAACATGGGGGACACTTCAGGGACTGGTGTCTCTTCACT
15 AGGAACCCTAGCACAGGAGAGAAGAAGCTTTATGGCGAGTTTCTAGTTAATGCTCAGGTT
TGGCATCTATCACAATGTGTGAATCTCATATCAACAAGGATAAGAACACCAGAAGATTTG
GATACAATGAAGAGATTTATGCCTGAGGCTTACGCTGAACTTGTTGAGAACTGCAACATC
TTAGAAAAGACATTACAAAGACATGATGGATATTGAATTCACAGTACAAGAAGAGAGATTG
TGGATGCTGCAATGCAGAGCGGGTAAAGCGAACGGGTAAAGGCGCCGTGAAGATAGCAGTT
20 GATATGGTAGGTGAAGGGCTTGTGTTAGAAATCTTCTGCTATCAAAATGGTGGAGCCTCAA
CATCTTGATCAACTACTTCACCCACAGTTTCATGATCCATCGGGGTATCGTGAAAAAGTG
GTGGCCAAAGGCTTACCTGCGTCACCAGGAGCGGCGGTTGGACAGGTTGTGTTACAGGCG
GAGGAAGCCGAAGCTTGGCATTCTCAGGGTAAAACGTGTGATTCTGGTTCGAACTGAGACA
AGCCCTGACGATGTGGGAGGTATGCACGCAGCGGAAGGTATATTGACGGCTAGAGGAGGA
25 ATGACGTCACACGCGGCTGTTGTTGCTCGCGGTTGGGGAAAATGTTGCATTGCTGGTGTG
TCCGAGATTCTGTGTCGACGAGAACCACAAGGTTCTATTGATTGGAGATTTGACGATTAAT
GAAGGCGAATGGATCTCAATGAACGGATCAACCGGTGAGGTTATATTAGGGAAACAAGCA
TTGGCTCCTCCGGCTTTAAGTCCAGATTTGGAGACTTTCATGTCTGGGCTGATGCAATC
AGACGTCTCAAGGTTATGGCGAATGCGGATACACCTGAAGACGCCATTGCAGCTAGGAAA
30 AACGGAGCTCAAGGAATCGGGCTTTGTAGGACAGAGCATATGATTGTTTGTATCCAAATG
TTTAATGTTGTCTTTGGTTTTGGTTTTTAAAGTTCTTTGGAGCAGATAGGATTAAAGCAGTG
AGAAAGATGATAATGGCGGTAACAACAGAGCAAAGGAAAGCTTCTCTCGACATCTTGCTT
CCTTACCAACGTTCCGATTTTGAAGGGATCTTCCGTGCTATGGATGGTTTTACCGGTAACA
ATCCGTTTGTAGACCCCTCCGCTTCACGAGTTTCTCCCGGAAGGCGACTTGGACAACATT
35 GTACATGAGCTAGCTGAAGAACTGGTGTGAAAGAAGATGAAGTCTTGTACGGATAGAG
AACTCTCTGAAGTGAATCCAATGCTTGGTTTCCGCGGTTGCAGGCTCGGAATATCGTAT
CCAGAGCTAACGGAGATGCAAGCGCGTGCAATTTTTGAAGCTGCAGCGTCAATGCAGGAC
CAAGGTGTTACTGTCATTCCTGAGATTATGGTTCCACTTGTAGGAACCTCCTCAGGAATTG
GGTCACCAAGTTGATGTAATTCGTAAAGTTGCAAAGAAAGTATTTGCTGAGAAGGGTCAT
40 ACCGTGAGCTACAAGGTGGGACAATGATTGAGATCCCTCGAGCCGCGCTCATTGCAGAT
GAGATTGCGAAAGAGGCGGAGTTTTTCTCGTTCCGGGACAAACGACTTGACGCAGATGACG
TTTGGATACAGTAGAGACGATGTGCGCAAGTTTTCTACCGATTTACCTCGCCAAAGGAATC
TTACAGCACGACCCTTTTGGAGTTCTTGATCAGCAAGGTGTAGGGCAATTGATCAAGATG
GCGACAGAAAAAGGACGAGCAGCTAGGCCTAGCCTCAAGGTGGGATATGTGGAGAACAT
45 GGAGGAGATCCATCTTCTGTGGGATTCTTTGCTGAAGCAGGACTTGACTATGTCTCTTGT
TCTCCTTTCAGGGTTCCAATTGCAAGGCTTGCAGCTGCTCAAGTAGTTGTTGCATGA

SEQ ID No:21

The cDNA sequence encoding the chloroplastic form of the *Arabidopsis* pyruvate orthophosphate dikinase (PPDK) is provided herein as SEQ ID No:22, as follows:

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5  ATGACAAGTATGATCGTGAAGACAACGCCGGAGCTCTTCAAAGGAAATGGAGTGTTCCGT
   ACGGATCATCTCGGAGAAAACCGAATGGTTAGTCGATCAAACCGGCTAGGTGATGGATCA
   AACCGTTTCCCTAGAACCGGTACAATCCATTGCCAACGGTTAAGCATAGCAAAGACCGGT
   TTGCATCGTGAGACGAAGGCTCGAGCCATACTTAGCCCTGTGTCCGATCCGGCCGCTTCC
10  ATAGCCCAAAAGCGAGTATTACCTTTGGAAAAGGAAGAAGCGAAGGCAACAAGGGCATG
   AAGTCCTTGTTGGGAGGGAAAGGAGCCAACCTGGCGGAGATGGCTAGCATAGGCTTGTCG
   GTGCCGCCGGGGCTAACCATATCGACGGAGGCTTGTCAGCAGTATCAGATCGCCGGCAAA
   AAGCTTCCAGAAGGTTTATGGGAAGAGATCTTAGAAGGTCTTAGCTTCATCGAACGTGAC
   ATTGGAGCTTCCCTCGCTGATCCCTCCAAGCCACTCCTCCTCTCTGTTTCGCTCCGGCGCC
15  GCCATCTCAATGCCTGGTATGATGGACACTGTACTTAACCTTGGCTTGAACGACCAAGTC
   GTCGTTGGTCTGGCCGCAAAAAGCGGAGAGCGTTTTTGCTTACGATTCTGTTCCGGCGTTTT
   CTTGATATGTTTGGTGATGTTGTGATGGGAATTCCACACGCCAAGTTTGAAGAGAAGTTA
   GAGAGAATGAAGGAGAGGAAAGGAGTTAAAAATGACACTGACTTAAGCGCGGCTGATCTC
   AAGGAATTGGTTGAGCAGTACAAGAGTGTTTACTTAGAGGCCAAGGGTCAAGAGTTTCCT
20  TCAGATCCAAAGAAGCAATTGGAGCTAGCGATTGAAGCGGTATTTCGATTCTTGGGATAGC
   CCGAGAGCGAACAAGTACAGAAGTATTAACCAGATAACTGGATTGAAAGGAACCGCGGTT
   AACATTCAGTGTATGGTGTTTGGAAACATGGGGGACACTTCAGGGACTGGTGTTCTCTTC
   ACTAGGAACCCTAGCACAGGAGAGAAGAAGCTTTATGGCGAGTTTCTAGTTAATGCTCAG
   GTTTGGCATCTATCACAATGTGTGAATCTCATATCAACAAGGATAAGAACACCAGAAGAT
25  TTGGATACAATGAAGAGATTTATGCCTGAGGCTTACGCTGAACTTGTTGAGAAGTGAAC
   ATCTTAGAAAGACATTACAAAGACATGATGGATATTGAATTCACAGTACAAGAAGAGAGA
   TTGTGGATGCTGCAATGCAGAGCGGGTAAGCGAACGGGTAAAGGCGCCGTGAAGATAGCA
   GTTGATATGGTAGGTGAAGGGCTTGTTGAGAAATCTTCTGCTATCAAAATGGTGAGCCT
   CAACATCTTGATCAACTACTTCACCCACAGTTTTCATGATCCATCGGGGTATCGTGAAAAA
30  GTGGTGGCCAAAGGCTTACCTGCGTCACCAGGAGCGGCGGTTGGACAGGTTGTGTTACAG
   GCGGAGGAAGCCGAAGCTTGGCATTCTCAGGGTAAAACCTGTGATTCTGGTTTCGAAGTGA
   ACAAGCCCTGACGATGTGGGAGGTATGCACGCAGCGGAAGGTATATTGACGGCTAGAGGA
   GGAATGACGTACACGCGGCTGTTGTTGCTCGCGGTTGGGGAAAATGTTGCATTGCTGGT
   TGTTCCGAGATTCTGTGTCGACGAGAACCACAAGGTTCTATTGATTGGAGATTTGACGATT
35  AATGAAGGCGAATGGATCTCAATGAACGGATCAACCGGTGAGGTTATATTAGGGAAACAA
   GCATTGGCTCCTCCGGCTTTAAGTCCAGATTTGGAGACTTTCATGTCCTGGGCTGATGCA
   ATCAGACGTCTCAAGGTTATGGCGAATGCGGATACACCTGAAGACGCCATTGCAGCTAGG
   AAAACGGAGCTCAAGGAATCGGGCTTTGTAGGACAGAGCATATGATTGTTTGTATCCAA
   ATGTTTAATGTTGTCTTTGGTTTGGTTTTTAAGTTCTTTGGAGCAGATAGGATTAAAGCA
40  GTGAGAAAGATGATAATGGCGGTAACAACAGAGCAAAGGAAAGCTTCTCTCGACATCTTG
   CTTCTTACCAACGTTTCGGATTTTCAAGGGATCTTCCGTGCTATGGATGGTTTACCGGTA
   ACAATCCGTTTGTAGACCCTCCGCTTCACGAGTTTCTCCCGGAAGGCGACTTGGACAAC
   ATTGTACATGAGCTAGCTGAAGAACTGGTGTGAAAGAAGATGAAGTCTTGTCACGGATA
   GAGAACTCTCTGAAGTGAATCCAATGCTTGGTTTCCGCGGTTGCAGGCTCGGAATATCG
45  TATCCAGAGCTAACGGAGATGCAAGCGCGTGCAATTTTTGAAGCTGCAGCGTCAATGCAG
   GACCAAGGTGTTACTGTCATTCTGAGATTATGGTTTCCACTTGTAGGAACTCCTCAGGAA
   TTGGGTCACCAAGTTGATGTAATTCGTAAAGTTGCAAAGAAAGTATTTGCTGAGAAGGGT
   CATAACGTGAGCTACAAGGTTGGGACAATGATTGAGATCCCTCGAGCCGCGCTCATTGCA
   GATGAGATTGCGAAAGAGGCGGAGTTTTTCTCGTTCCGGACAAACGACTTGACGCAGATG
50  ACGTTTGGATACAGTAGAGACGATGTCGGCAAGTTTCTACCGATTTACCTCGCCAAAGGA
   ATCTTACAGCACGACCCTTTTGAGGTTCTTGATCAGCAAGGTGTAGGGCAATTGATCAAG
   ATGGCGACAGAAAAAGGACGAGCAGCTAGGCCTAGCCTCAAGGTTGGGATATGTGGAGAA
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- 17 -

CATGGAGGAGATCCATCTTCTGTGGGATTCTTTGCTGAAGCAGGACTTGACTATGTCTCT
TGTTCCTTTTCAGGGTTCCAATTGCAAGGCTTGCAAGTCAAGTAGTTGTTGCATGA

SEQ ID No:22

5

Accordingly, the coding sequence, which encodes a polypeptide having PPK activity, may comprise a nucleic acid sequence substantially as set out in SEQ ID No:20, SEQ ID No:21 or SEQ ID No:22, or a functional variant or fragment thereof.

10

The polypeptide sequence of the cytosolic form of *Arabidopsis* PPK is provided herein as SEQ ID No:23, as follows:

MMQRVFTFGKGRSEGNKGMKSLGKGANLAEMASIGLSVPPGLTISTEACQQYQIAGKK
15 LPEGLWEEIILEGLSFIERDIGASLADPSKPLLLSVRSGAAISMPGMMDTVNLGLNDQVV
VGLAAKSGERFAYDSFRRFLDMFGDVVMGIPHAKFEEKLERMKERKGVKNDTDLAADLK
ELVEQYKSVYLEAKGQEFPSDPKKQLELAIEAVFDSWDSPRANKYRSINQITGLKGTAVN
IQCMVFGNMGDTSGTGVLFTRNPTSTGEKKLYGEFLVNAQVWHLSCVNLISTRIPTEDL
DTMKRFMPEAYAELENVCNILERHYKDMMDIEFTVQEERLWMLQCRAGKRTGKGAVKIAV
20 DMVGEGLVEKSSAIKMVEPQHLDQLLHPQFHDPSGYREKVVAKGLPASPGAAVGQVVFTA
EEAEAWHSQGKTVILVRTETSPDDVGGMHAAEGILTARGGMTSHAAVARGWGKCCIAGC
SEIRVDENHKVLLIGDLTINEGEWISMNGSTGEVILGKQALAPPALSPDLETFMWADAI
RRLKVMANADTPEDAIAARKNGAQGIGLCRTEHMIVCIQMFNVVFGLVFKFFGADRIKAV
RKMIMAVTTEQRKASLDILLPYQRSDFEGIFRAMDGLPVTIRLLDPPLHEFLPEGDLNI
25 VHELAEETGVKEDEVLSRIEKLSEVNPMLGFRGCRGLGISYPELTEMQARAIFEAAASMQD
QGVTVIPEIMVPLVGTPELGHQVDVIRKVAKKVFAEKGHTVSYKVGTMIEIPRAALIAD
EIAKEAEFFSFGTNDLTQMTFGYSRDDVGKFLPIYLAKGILQHDPFEVLDQQGVGQLIKM
ATEKGRAARPSLKVGICGEHGGDPSSVGFFAEAGLDYVSCSPFRVPIARLAAAQVVVA

30

SEQ ID No:23

The polypeptide sequence of the chloroplastic form of *Arabidopsis* PPK is provided herein as SEQ ID No:24, as follows:

MTSMIVKTTPELFKNGVFRTHLGENRMVSRSNRLGDGSNRFPRGTIHCQRLSIAKTG
35 LHRETKARAILSPVSDPAASIAQKRVFTFGKGRSEGNKGMKSLGKGANLAEMASIGLS
VPPGLTISTEACQQYQIAGKKLPEGLWEEIILEGLSFIERDIGASLADPSKPLLLSVRSGA
AISMPGMMDTVNLGLNDQVVVGLAAKSGERFAYDSFRRFLDMFGDVVMGIPHAKFEEKL
ERMKERKGVKNDTDLAADLKELVEQYKSVYLEAKGQEFPSDPKKQLELAIEAVFDSWDS
40 PRANKYRSINQITGLKGTAVNIQCMVFGNMGDTSGTGVLFTRNPTSTGEKKLYGEFLVNAQ
VWHLSCVNLISTRIPTEDLDTMKRFMPEAYAELENVCNILERHYKDMMDIEFTVQEER
LWMLQCRAGKRTGKGAVKIAVDMVGEGLVEKSSAIKMVEPQHLDQLLHPQFHDPSGYREK

VVAKGLPASPGAAVGQVVFTAEAEAWHSQGKTVILVRTETSPDDVGGMHAAEGILTARG
 GMTSHAAVVARGWGKCCIAGCSEIRVDENHKVLLIGDLTINEGEWISMNGSTGEVILGKQ
 ALAPPALSPDLETFMSWADAIRRLKVMANADTPEDAIAARKNGAQGIGLCRTEHMIVCIQ
 MFNVVFGVLVFKFFGADRIKAVRKMIMAVTTEQRKASLDILLPYQRSDFEGIFRAMDGLPV
 5 TIRLLDPPLHEFLPEGDLNIVHELAEETGVKEDEVLSRIEKLSEVNPMLGFRGCRLGIS
 YPELTEMQARAIFEEAASMQDQGVTVIPEIMVPLVGTPOELGHQVDVIRKVAKKVFAEKG
 HTVSYKVGTMIEIPRAALIADDEIAKEAEFFSFSGTNDLTQMTFGYSRDDVGKFLPIYLAGK
 ILQHDPFEVLDQQGVGQLIKMATEKGRAARPSLKVIGICGEHGGDPSSVGFFAEAGLDYVS
 CSPFRVPIARLAAAQVVVA

10

SEQ ID No:24

Accordingly, the polypeptide having PPDK activity may comprise an amino acid
 sequence substantially as set out in SEQ ID No:23 or SEQ ID No:24, or a
 functional variant or fragment thereof. Since highest PPDK abundance was
 15 observed almost exclusively in cell lines harbouring genomic DNA encoding PPDK, it
 appears that introns may have a positive effect on PPDK expression. As such, in one
 embodiment, the construct may comprise the cDNA of the genes encoding either
 PPDK and/or PCK.

20 Genetic constructs of the invention may be in the form of an expression
 cassette, which may be suitable for expression of the at least one coding
 sequence in a host cell. The genetic construct of the invention may be introduced in
 to a host cell without it being incorporated in a vector. For instance, the genetic
 construct, which may be a nucleic acid molecule, may be incorporated within a
 25 liposome or a virus particle. Alternatively, a purified nucleic acid molecule (e.g. histone-
 free DNA, or naked DNA) may be inserted directly into a host cell by suitable means,
 e.g. direct endocytotic uptake. The genetic construct may be introduced directly in to
 cells of a host subject (e.g. a plant) by transfection, infection, microinjection, cell fusion,
 protoplast fusion or ballistic bombardment. Alternatively, genetic constructs of the
 30 invention may be introduced directly into a host cell using a particle gun.
 Alternatively, the genetic construct may be harboured within a recombinant
 vector, for expression in a suitable host cell.

Hence, in a third aspect, there is provided a recombinant vector comprising the
 35 genetic construct according to the first or second aspect.

The recombinant vector may be a plasmid, cosmid or phage. Such recombinant vectors are highly useful for transforming host cells with the genetic construct of the invention, and for replicating the expression cassette therein. The skilled technician will appreciate that genetic constructs of the invention may be combined with many types of backbone vector for expression purposes. The backbone vector may be a binary vector, for example one which can replicate in both *E. coli* and *Agrobacterium tumefaciens*. For example, a suitable vector may be a pBIN plasmid, such as pBIN19. However, a preferred backbone vector is BNP1380000001, which is based on pBINPLUS (F. A. van Engelen et al. Transgenic Research (1995) 4, 288-290), and which harbours the SAG12 promoter.

Recombinant vectors may include a variety of other functional elements in addition to the promoter (e.g. a senescence-associated promoter), and the at least one coding sequence (encoding PCK and/or PPK). For instance, the recombinant vector may be designed such that it autonomously replicates in the cytosol of the host cell. In this case, elements which induce or regulate DNA replication may be required in the recombinant vector. Alternatively, the recombinant vector may be designed such that it integrates into the genome of a host cell. In this case, DNA sequences which favour targeted integration (e.g. by homologous recombination) are envisaged.

The recombinant vector may also comprise DNA coding for a gene that may be used as a selectable marker in the cloning process, i.e. to enable selection of cells that have been transfected or transformed, and to enable the selection of cells harbouring vectors incorporating heterologous DNA. The vector may also comprise DNA involved with regulating expression of the coding sequence, or for targeting the expressed polypeptide to a certain part of the host cell, e.g. the chloroplast. Hence, the vector of the third aspect may comprise at least one additional element selected from a group consisting of: a selectable marker gene (e.g. an antibiotic resistance gene); a polypeptide termination signal; and a protein targeting sequence (e.g. a chloroplast transit peptide).

- 20 -

Examples of suitable marker genes include antibiotic resistance genes such as those conferring resistance to Kanamycin, Geneticin (G418) and Hygromycin (*npt-II*, *hyg-B*); herbicide resistance genes, such as those conferring resistance to phosphinothricin and sulphonamide based herbicides (*bar* and *smI* respectively; EP-A-242246, EP-A- 0249637); and screenable markers such as beta-glucuronidase (GB2197653), luciferase and green fluorescent protein (GFP).

The marker gene may be controlled by a second promoter (which may not be a senescence-associated promoter), which allows expression in cells, which may or may not be in the seed, thereby allowing the selection of cells or tissue containing the marker at any stage of development of the plant. Suitable second promoters are the promoter of nopaline synthase gene of *Agrobacterium* and the promoter derived from the gene which encodes the 35S cauliflower mosaic virus (CaMV) transcript. However, any other suitable second promoter may be used.

The various embodiments of genetic constructs of the invention may be prepared using the cloning procedure illustrated in Figure 1, which may be summarised as follows. The genomic and cDNA versions of the genes encoding PCK and PPDK may be amplified from the genomic or cDNA templates by PCR using suitable primers. PCR products may be examined using agarose gel electrophoresis. The PCR products may then be ligated into a suitable vector for cloning purposes, for example the pCR4 Blunt-TOPO vector (Invitrogen). Vectors harbouring the PCR products may be grown up in a suitable host, such as *E. coli*. *E. coli* colonies may then be screened by PCR using suitable primers, and inserts in plasmids showing the correct restriction enzyme digest pattern may be sequenced using suitable primers.

E. coli colonies carrying TOPO-cDNA (PCK or PPDK) or TOPO-gDNA (PCK or PPDK) may be cultured to produce a suitable amount of each plasmid, which may then be purified. The plasmids may then be digested to release a DNA fragment encoding PPDK or PCK, which may then be cloned into a vector harbouring a suitable promoter, for example a SAG promoter, such as a pBNP plasmid. The resultant PPDK constructs were named BNP-PPDKcDNA and BNP-PPDKgDNA, and the resultant PCK constructs were named pALBNP1 (cDNA) and pALBNP2 (gDNA).

Embodiments of the vector according to the third aspect may be substantially as set out in Figures 3.

5 In a fourth aspect, there is provided a method of increasing the concentration of PCK and/or PPDK in a leaf of a test plant to above that of the corresponding concentration of PCK and/or PPDK in a wild-type plant cultured under the same conditions, the method comprising altering plant metabolism in the test plant to achieve increased levels of PCK and/or PPDK in plant leaves after the
10 initiation of leaf senescence.

In a fifth aspect, there is provided a method of decreasing the concentration of nitrogen in the leaves of a test plant to below that of the corresponding concentration of nitrogen in a wild-type plant cultured under the same
15 conditions, the method comprising altering plant metabolism in the test plant to achieve increased levels of PCK and/or PPDK in plant leaves after the initiation of leaf senescence.

In a sixth aspect, there is provided a method of increasing the growth rate of a
20 test plant compared to the corresponding growth rate of a wild-type plant cultured under the same conditions, the method comprising altering plant metabolism in the test plant to achieve increased levels of PCK and/or PPDK in plant leaves after the initiation of leaf senescence.

25 Methods for determining the level of nitrogen in plant leaves, and plant growth rates, are set out in the Examples. The methods of the fourth, fifth or sixth aspects may comprise transforming a test plant cell with a genetic construct according to the first or second aspect, or a vector according to the third aspect. The genetic construct or the vector may be introduced into a host cell by any suitable means.

In a seventh aspect, there is provided a cell comprising the genetic construct according to the first or second aspect, or the recombinant vector according to the third aspect.

- 5 The cell may be a plant cell. As the inventors have observed that over-expressing both PCK and/or PPDK in a host cell is surprisingly effective at inducing nitrogen remobilisation in senescent leaves, the cell of the seventh aspect may comprise one or more constructs of the first or second aspect, or one or more vectors of the third aspect, such that both PCK and/or PPDK are over-
10 expressed.

For example, the host cell may be transformed with the first embodiment of the genetic construct of the first aspect (i.e. the PCK construct) only. Alternatively, the host cell may be transformed with the second embodiment of the genetic
15 construct of the second aspect (i.e. the PPDK construct) only. In another embodiment, the host cell may be transformed with the first and second embodiments of the genetic construct of the first aspect, such that both PCK and PPDK are expressed in the host cell. The host cell may alternatively be transformed with the third or fourth embodiments of the construct of the first
20 aspect (i.e. PCK/PPDK constructs 1 or 2), such that both PCK and PPDK are expressed in the host cell. It is also envisaged that the host cell may be transformed with the construct of the second aspect, which encodes both PCK and PPDK.

- 25 The cell may be transformed with the genetic construct or the vector according to the invention, using known techniques. Suitable means for introducing the genetic construct into the host cell may include use of a disarmed Ti-plasmid vector carried by *Agrobacterium* by procedures known in the art, for example as described in EP-A-0116718 and EP-A-0270822. A further method may be to
30 transform a plant protoplast, which involves first removing the cell wall and introducing the nucleic acid, and then reforming the cell wall. The transformed cell may then be grown into a plant.

In an eighth aspect, there is provided a transgenic plant comprising the genetic construct according to the first or second aspect, or the vector according to the third aspect.

5

The transgenic plant according to the eighth aspect may include the Brassicaceae family, such as *Brassica* spp.. The plant may be *Brassica napus* (oilseed rape).

Further examples of transgenic plants according to the eighth aspect include the
10 family Poales, such as *Triticeae* spp. The plant may be *Triticum* spp. (wheat).

Increasing the grain protein content in wheat may result in increased volume of food products comprising wheat, such as bread.

Further examples of suitable transgenic plants according to the eighth aspect
15 include the Solanaceae family of plants which include, for example jimson weed, eggplant, mandrake, deadly nightshade (belladonna), capsicum (paprika, chilli pepper), potato and tobacco. One example of a suitable genus of Solanaceae is *Nicotiana*. A suitable species of *Nicotiana* may be referred to as tobacco plant, or simply tobacco. Various methods for transforming plants with the genetic
20 construct of the first or second aspect, or vector of the third aspect, are known and can be used in the present invention.

For example, tobacco may be transformed as follows. *Nicotiana tabacum* is transformed using the method of leaf disk co-cultivation essentially as described
25 by Horsch *et al.* (Science 227: 1229-1231, 1985). The youngest two expanded leaves may be taken from 7 week old tobacco plants and may be surface sterilised in 8% Domestos™ for 10 minutes and washed 6 times with sterile distilled water. Leaf disks may be cut using a number 6 cork borer and placed in the *Agrobacterium* suspension, containing the appropriate binary vectors (according to
30 the invention), for approximately two minutes. The discs may be gently blotted between two sheets of sterile filter paper. Ten disks may be placed on LS 3% sucrose + 2µM BAP + 0.2µM NAA plates, which may then be incubated for 2

- 24 -

days in the growth room. Discs may be transferred to plates of LS + 3% sucrose + 2 μ M BAP + 0.2 μ M NAA supplemented with 500 g/l claforan and 100 g/l kanamycin. The discs may be transferred onto fresh plates of above medium after 2 weeks. After a further two weeks, the leaf disks may be transferred onto
5 plates containing LS + 3% sucrose + 0.5 μ M BAP supplemented with 500 mg/l claforan and 100 mg/l kanamycin. The leaf disks may be transferred onto fresh medium every two weeks. As shoots appear, they may be excised and transferred to jars of LS + 3% sucrose supplemented with 500 mg/l claforan. The shoots in jars may be transferred to LS + 3% sucrose + 250 mg/l claforan after
10 approximately 4 weeks. After a further 3-4 weeks the plants may be transferred to LS + 3% sucrose (no antibiotics) and rooted. Once the plants are rooted they may be transferred to soil in the greenhouse.

In a ninth aspect, there is provided a plant propagation product obtainable from
15 the transgenic plant according to the eighth aspect.

A "plant propagation product" may be any plant matter taken from a plant from which further plants may be produced. Suitably, the plant propagation product may be a seed.
20

In a tenth aspect of the invention, there is provided a method of producing a transgenic plant which remobilises nitrogen at a higher rate than a corresponding wild-type plant cultured under the same conditions, the method comprising the steps of:-

- 25 i) transforming a plant cell with the genetic construct according to the first or second aspect, or the vector according to the third aspect; and
ii) regenerating a plant from the transformed cell.

In an eleventh aspect, there is provided a method of producing a transgenic plant
30 having a higher growth rate than a corresponding wild-type plant cultured under the same conditions, the method comprising the steps of:-

- i) transforming a plant cell with the genetic construct according to the first

- 25 -

or second aspect, or the vector according to the third aspect; and

ii) regenerating a plant from the transformed cell.

Preferably, and advantageously, the methods according to the invention do not
5 compromise the health or fitness of the test plant that is generated. Preferably,
the methods comprise transforming the test plant, and preferably its leaves, with
the genetic construct of the first or second aspect, or the vector of the third
aspect. The inventors have observed that over-expressing both PCK and PPDK
in a host cell is effective at inducing nitrogen remobilisation in senescent leaves.

10 Hence, it is preferred that the methods of the tenth and eleventh aspect
comprise transforming the test plant with one or more constructs of the
invention such that both PCK and PPDK are over-expressed. For example, the
test plant may be transformed with the first embodiment of the genetic construct
of the first aspect of the invention (i.e. the PCK construct) and in addition the
15 second embodiment of the construct of the first aspect (i.e. the PPDK
construct). Hence, transformation of these two constructs result in over-
expression of both enzymes. Alternatively, the test plant may be transformed
with the third or fourth embodiments of the construct of the first aspect of the
invention, each of which encode PCK and PPDK. Alternatively, the test plant
20 may be transformed with the construct of the second aspect of the invention,
which encodes both PCK and PPDK.

The inventors have observed that a plant leaf of a test plant which has been
transformed with construct(s) which encode both PCK and PPDK exhibits
25 increases in nitrogen remobilisation upon the onset of senescence such that the
concentration of nitrogen decreases in that leaf. Furthermore, they observed an
increase in vegetative growth rate. Although they are not bound by hypothesis,
inventors believe that the decrease in nitrogen concentration in the leaves may
induce the increase in growth rate, and hence crop yield.

30 In a twelfth aspect of the invention, there is provided a harvested leaf containing
a lower level of nitrogen than the corresponding level of nitrogen in a harvested

- 26 -

leaf taken from a wild-type plant cultured under the same conditions, wherein the leaf is harvested from the transgenic plant according to the eighth aspect, or produced by the method according to the tenth or eleventh aspect.

- 5 In a thirteenth aspect of the invention, there is provided a smoking article comprising nitrogen-reduced tobacco obtained from a mutant tobacco plant, which mutant is capable of decreasing the concentration of nitrogen in senescent leaves.
- 10 Nitrogen-reduced tobacco can include tobacco in which the nitrogen concentration is less than the corresponding concentration in a wild-type plant cultured under the same conditions. Such a smoking article may comprise tobacco obtained from a mutant tobacco plant, which may have been transformed with a genetic construct according to the first or second aspect of
- 15 the invention, or a vector according to the third aspect.

As used herein, the term “smoking article” can include smokeable products, such as rolling tobacco, cigarettes, cigars and cigarillos whether based on tobacco, tobacco derivatives, expanded tobacco, reconstituted tobacco or tobacco substitutes and also

20 heat-not-burn products.

It will be appreciated that the invention extends to any nucleic acid or peptide or variant, derivative or analogue thereof, which comprises substantially the amino acid or nucleic acid sequences of any of the sequences referred to herein, including functional

25 variants or functional fragments thereof. The terms “substantially the amino acid/polynucleotide/polypeptide sequence”, “functional variant” and “functional fragment”, can be a sequence that has at least 40% sequence identity with the amino acid/polynucleotide/polypeptide sequences of any one of the sequences referred to herein, for example 40% identity with the gene identified as SEQ ID No.17 (which

30 encodes one embodiment of PCK enzyme), or 40% identity with the polypeptide identified as SEQ ID No.19 (i.e. one embodiment of PCK enzyme), or 40% identity with the gene identified as SEQ ID No.21 (which encodes one embodiment of PPDK

- 27 -

enzyme), or 40% identity with the polypeptide identified as SEQ ID No.23 (i.e. one embodiment of PPDK enzyme).

Amino acid/polynucleotide/polypeptide sequences with a sequence identity which is greater than 65%, more preferably greater than 70%, even more preferably greater than 75%, and still more preferably greater than 80% sequence identity to any of the sequences referred to is also envisaged. Preferably, the amino acid/polynucleotide/polypeptide sequence has at least 85% identity with any of the sequences referred to, more preferably at least 90% identity, even more preferably at least 92% identity, even more preferably at least 95% identity, even more preferably at least 97% identity, even more preferably at least 98% identity and, most preferably at least 99% identity with any of the sequences referred to herein.

The skilled technician will appreciate how to calculate the percentage identity between two amino acid/polynucleotide/polypeptide sequences. In order to calculate the percentage identity between two amino acid/polynucleotide/polypeptide sequences, an alignment of the two sequences must first be prepared, followed by calculation of the sequence identity value. The percentage identity for two sequences may take different values depending on:- (i) the method used to align the sequences, for example, ClustalW, BLAST, FASTA, Smith-Waterman (implemented in different programs), or structural alignment from 3D comparison; and (ii) the parameters used by the alignment method, for example, local vs global alignment, the pair-score matrix used (e.g. BLOSUM62, PAM250, Gonnet etc.), and gap-penalty, e.g. functional form and constants.

Having made the alignment, there are many different ways of calculating percentage identity between the two sequences. For example, one may divide the number of identities by: (i) the length of shortest sequence; (ii) the length of alignment; (iii) the mean length of sequence; (iv) the number of non-gap positions; or (iv) the number of equivalenced positions excluding overhangs. Furthermore, it will be appreciated that percentage identity is also strongly length dependent. Therefore, the shorter a pair of sequences is, the higher the sequence identity one may expect to occur by chance.

Hence, it will be appreciated that the accurate alignment of protein or DNA sequences is a complex process. The popular multiple alignment program ClustalW (Thompson et al., 1994, Nucleic Acids Research, 22, 4673-4680; Thompson et al., 1997, Nucleic Acids Research, 24, 4876-4882) is a preferred way for generating multiple alignments of proteins or DNA in accordance with the invention. Suitable parameters for ClustalW may be as follows: For DNA alignments: Gap Open Penalty = 15.0, Gap Extension Penalty = 6.66, and Matrix = Identity. For protein alignments: Gap Open Penalty = 10.0, Gap Extension Penalty = 0.2, and Matrix = Gonnet. For DNA and Protein alignments: ENDGAP = -1, and GAPDIST = 4. Those skilled in the art will be aware that it may be necessary to vary these and other parameters for optimal sequence alignment.

Preferably, calculation of percentage identities between two amino acid/polynucleotide/polypeptide sequences is then calculated from such an alignment as $(N/T) \times 100$, where N is the number of positions at which the sequences share an identical residue, and T is the total number of positions compared including gaps but excluding overhangs. Hence, a most preferred method for calculating percentage identity between two sequences comprises (i) preparing a sequence alignment using the ClustalW program using a suitable set of parameters, for example, as set out above; and (ii) inserting the values of N and T into the following formula:- Sequence Identity = $(N/T) \times 100$.

Alternative methods for identifying similar sequences will be known to those skilled in the art. For example, a substantially similar nucleotide sequence will be encoded by a sequence which hybridizes to the sequences shown in SEQ ID Nos. 16, 17, 18, 20, 21, 22, or their complements under stringent conditions. By stringent conditions, we mean the nucleotide hybridises to filter-bound DNA or RNA in 3x sodium chloride/sodium citrate (SSC) at approximately 45°C followed by at least one wash in 0.2x SSC/0.1% SDS at approximately 20-65°C. Alternatively, a substantially similar polypeptide may differ by at least 1, but less than 5, 10, 20, 50 or 100 amino acids from the sequences shown in SEQ ID No's. 19, 23 or 24.

Due to the degeneracy of the genetic code, it is clear that any nucleic acid sequence could be varied or changed without substantially affecting the sequence of the protein encoded thereby, to provide a functional variant thereof. Suitable nucleotide variants are those having a sequence altered by the substitution of different codons that encode the same amino acid within the sequence, thus producing a silent change. Other suitable variants are those having homologous nucleotide sequences but comprising all, or portions of, sequence, which are altered by the substitution of different codons that encode an amino acid with a side chain of similar biophysical properties to the amino acid it substitutes, to produce a conservative change. For example small non-polar, hydrophobic amino acids include glycine, alanine, leucine, isoleucine, valine, proline, and methionine. Large non-polar, hydrophobic amino acids include phenylalanine, tryptophan and tyrosine. The polar neutral amino acids include serine, threonine, cysteine, asparagine and glutamine. The positively charged (basic) amino acids include lysine, arginine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. It will therefore be appreciated which amino acids may be replaced with an amino acid having similar biophysical properties, and the skilled technician will know the nucleotide sequences encoding these amino acids.

All of the features described herein (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined with any of the above aspects in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

For a better understanding of the invention, and to show how embodiments of the same may be carried into effect, reference will now be made, by way of example, to the accompanying Figures, in which:-

Figure 1 shows the protocol used for the cloning of the genetic constructs, BNP-PPDKcDNA and BNP-PPDKgDNA, in which: (a) PCR amplification of cDNA and genomic DNA forms of the cytosolic isoform of *Arabidopsis* PPDK; (b) Insertion into the cloning vector pCR 4Blunt-TOPO; (c) Restriction endonuclease digestion of the cloning vector (on left) to release PPDK, and the target SAG12-containing vector

- 30 -

pBNP, with *Avr*II and *Bam*HI (on right); (d) Ligation of PPDK into pBNP and an agarose gel showing DNA fragments produced by restriction endonuclease digestion of constructs with *Avr*II and *Bam*HI; and (e) Constructs introduced into *Arabidopsis* ecotype Columbia 0 by *Agrobacterium*-mediated transformation;

5 Figure 2a shows the plasmid pCR4 BLUNT-TOPO used for the construction of expression vectors according to the invention, and Figure 2b is a table summarising that PPDK-encoding cDNA or gDNA was inserted into pCR4 BLUNT-TOPO using an *Avr*II and *Bam*HI digest;

10 Figure 3a shows the plasmid pBNP containing the SAG12 promoter, and Figure 3b is a table summarising that PPDK inserts (cDNA or gDNA) were introduced into pBNP using *Avr*II and *Bam*HI digests, and that PCK inserts (cDNA or gDNA) were introduced into pBNP using *Xba*I and *Sac*I digests, resulting in vectors according to embodiments of the invention;

15 Figure 4 shows the selection of transgenic *SAG12-PPDK Arabidopsis thaliana* cell lines by Western blot. SAG12-PPDK cDNA cell lines are shown on top, and SAG12-PPDK gDNA cell lines are shown below;

Figure 5 shows the quantification of PPDK abundance in wild-type, Δ PPDK and five independent *SAG12-PPDKgDNA* lines from week five onwards;

20 Figure 6 shows the selection of *SAG12-PPDK* (cDNA and gDNA) in (b) K326 and in (c) Burley 21 tobacco lines;

Figure 7 shows the overexpression of PPDK in ripe leaves of K326 tobacco;

Figure 8 shows photos of rosettes of various cell lines of *Arabidopsis thaliana*, i.e. wild-type, Δ PPDK and *SAG12-PPDKgDNA* against time;

Figure 9 shows the *Arabidopsis* reproductive tissue mass at week nine;

25 Figure 10 shows the total plant fresh mass (i.e. rosette plus reproductive tissue) of wild-type and *SAG12-PPDKgDNA* plants from weeks three to nine after sowing;

Figure 11 shows the Nitrogen content in leaves of wild-type, Δ PPDK and *SAG12-PPDKgDNA* plants at week seven;

30 Figure 12 shows the Nitrogen content of individual seeds of wild-type, Δ PPDK and *SAG12-PPDK* plants;

Figure 13 shows the total free amino acid content in the leaves of wild-type, Δ PPDK and *SAG12-PPDKgDNA Arabidopsis* cell lines;

Figure 14 shows the seed mass of various tobacco cell lines, zero copy = wild-type, G4 (gDNA), G10, G8 and C10 (cDNA) are SAG12-PPDK lines;

Figure 15 shows the seed nitrogen content of various tobacco cell lines, zero copy = wild-type, G4 (gDNA), G10, G8 and C10 (cDNA) are SAG12-PPDK lines;

5 Figure 16 are the results of Western blotting for PCK/PPDK double inserts. All lines show higher levels of PCK especially in week 7 which correlates with activity of the SAG12 promoter;

Figure 17 shows that expression of both PCK and PPDK in transformed plants leads to an increase in rosette mass compared to control 7; and

10 Figure 18 shows a postulated biochemical pathway indicating that nitrogen remobilisation may occur through PCK- and PPDK-dependent formation of the transport amino acids asparagine and glutamine.

Examples

15

Example 1 - The generation of SAG12 promoter *Arabidopsis* PPDK plant transformation constructs

A series of SAG12 PPDK and PCK expression vectors were generated, as shown in Figure 3, and then analysed for their ability to increase the expression of PPDK and PCK in transformed plants. In one embodiment, the constructs may encode PCK or a functional variant or fragment thereof, and not PPDK. In another embodiment, the constructs may encode PPDK or a functional variant or fragment thereof, and not PCK. However, in yet another embodiment, the constructs may encode PCK or a functional variant or fragment thereof, and PPDK or a functional variant or fragment thereof. Firstly, genomic DNA for the *Arabidopsis thaliana* PPDK gene was isolated, as described below.

25

Isolation of genomic DNA

Genomic DNA (gDNA) from *Arabidopsis thaliana* ecotype Columbia 0 was extracted from leaves using the DNeasy Plant Mini Kit (Qiagen) according to the recommended protocol. Genomic DNA was used as a template for PCR reactions as described below using primer sequences summarized in Table 1.

30

Table 1: Primer sequences

Primer	Sequence	SEQ ID No.
AtCytFWD-AvrII	AAT CCT AGG ATG ATG CAG CGA GTA TTC ACC	1
AtCytREV-BamHI	AAT GGA TCC TCA TGC AAC AAC TAC TTG AGC AGC	2
AtPPDKexon15FWD	CCT CGC CAA AGG AAT CTT AC	7
AtPPDKSeqF1	CTT GGC TTG AAC GAC CAA GTC	3
AtPPDKSeqF2	GGT TGC AGG GAT AGG AAC ACC	4
AtPPDKSeqR1	CGT CTG ATT GCA TCA GCC CAG	5
AtPPDKSeqR2	CCT GAG GAG TTC CTA CAA GTG	6
BNP-1271F	TGC CTG CTT GCC GAA TAT C	10
BNP-1291TV	CAG AAA AGC GGC CAT TTT CCA CCA	12
BNP-1334R	CCG GCC CAC AGT CGA TGA	11
BNP-nostREV	CAA GAC CGG CAA CAG GAT TCA	8
BNP-SAG12FWD	ACC CCA TCT CAG TAC CCT TCT G	9
NtCyc-184F	CTC AAC CTT CCA CCG TGT GAT	13
NtCyc-267T	TCT ACG GTG CCA AAT TCG CCG A	15
NtCyc-316R	ACC GGT GTG CTT CCT CTT GAA	14
AtPCK-XbaI-FOR	ATTCTCTAGAATGTCTGGCCGGTAACGGAAATG	25
AtPCK-SacI-REV	ATTGAGCTCCTAAAAGATAGGACCAGCAGCG	26

Isolation of RNA and synthesis of cDNA

- 5 Total RNA was extracted from 7 day old *Arabidopsis* cotyledons. RNA extractions were performed on ice using RNase-free equipment, and solutions were made using water treated with diethyl pyrocarbonate (DEPC, Sigma-Aldrich). 1 ml DEPC was added per 1 litre water, the mixture was stirred overnight in a fume hood, and then autoclaved. RNA was extracted using TriPure Isolation Reagent (Roche). 200 mg tissue was ground
- 10 in liquid nitrogen using a mortar and pestle, 1 ml TriPure Isolation Reagent added and the recommended protocol followed. The RNA pellet was resuspended in 20 µl RNA Secure (Ambion) preheated to 60 °C for 10 minutes. The sample was centrifuged at 4°C for 5 minutes at 13,000 rpm and the supernatant transferred to a clean 1.5 ml microcentrifuge tube to remove contaminating debris.

- 33 -

Quantity and purity of the RNA were determined spectrophotometrically by taking readings at 260 and 280 nm (Maniatis et al., 1982, Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory) using an Eppendorf Biophotometer spectrophotometer. The RNA was also examined using agarose gel electrophoresis using 1.5 % (w/v) agarose (Melford Laboratories) in 0.5 x TBE. Samples were suspended in 1x RNA sample buffer, and subjected to electrophoresis at 80 V using 0.5 x TBE as running buffer. RNA sample buffer at 4x working concentration contained 0.002 % (w/v) ethidium bromide (Sigma-Aldrich), 2x TBE (2x Tris-Borate-EDTA Buffer (2 x TBE) contained 180 mM Tris-HCl, 180 mM boric acid and 8 mM EDTA pH 8.3, 13 % Ficoll 400 (Sigma-Aldrich), 0.01 % bromophenol blue and 7 M urea (Fischer Scientific).

Reverse transcription of RNA to synthesise cDNA was performed using 2 µg RNA, 1 µg Oligo dT(15) primer (Roche), 1 x Moloney Murine Leukaemia Virus (MMLV) buffer (Promega), 0.4 mM dNTPs (Bioline), 40 units Recombinant RNasin Ribonuclease Inhibitor (Promega), 200 units MMLV Reverse Transcriptase (Promega) and nuclease-free water to a final volume of 25 µl. The sample was incubated at 42 °C for 1 hour.

Amplification of *Arabidopsis* PPDK

The genomic and cDNA versions of the gene encoding the cytosolic isoform of PPDK were amplified from the genomic or cDNA templates by PCR using a forward primer (AtCytFWD-*Avr*II, SEQ ID No. 1) containing the *Avr*II restriction site and a reverse primer (AtCytREV-*Bam*HI, SEQ ID No. 2) containing the *Bam*HI restriction site, which are shown in Table 1. The PCR reaction mixture contained 1 x HF buffer (NEB), 2 mM magnesium chloride (MgCl₂, NEB), 0.5 mM dNTPs (Bioline), 100 ng template (cDNA or genomic DNA), 0.5 µM each primer and 1 unit Phusion High-Fidelity DNA Polymerase (NEB). Thermal cycling was performed using a Techne Thermal Cycler with an initial denaturation step of 98 °C for 30 seconds, followed by 30 cycles of 98 °C for 10 seconds, 55 °C for 30 seconds and 72 °C for 2 minutes, with a final extension step of 72 °C for 5 minutes.

- 34 -

Once the cDNA and gDNA of *Arabidopsis* PPDK had been isolated/prepared, it was then used to generate various constructs, using the protocol that is summarized in Figure 1. The cDNA and genomic DNA forms of the cytosolic isoform of *Arabidopsis* PPDK were fused to the senescence-induced *SAG12* promoter in a pBNP vector, in order to overexpress PPDK during senescence.

(a) PCR amplification of cDNA and genomic DNA forms of cytosolic isoform of *Arabidopsis* PPDK using primers containing *Avr*II and *Bam*HI restriction sites, generating 2.4 and 4.3 kb products respectively.

(b) Insertion into cloning vector pCR 4Blunt-TOPO. The PPDK inserts were sequenced in their entirety, and found to be identical to expected sequences.

(c) Restriction endonuclease digestion of cloning vector and destination vector pBNP with *Avr*II and *Bam*HI.

(d) Ligation into BNP and agarose gel showing DNA fragments produced by restriction endonuclease digestion of constructs with *Avr*II and *Bam*HI. Expected band sizes were 14.8 kb and 2.4 kb for the cDNA construct, and 14.8 kb and 4.4 kb for the gDNA construct.

(e) Constructs introduced into *Arabidopsis* ecotype Columbia 0 by *Agrobacterium*-mediated transformation. Constructs were sequenced across ligation sites. The gene *nptII* encodes neomycin phosphotransferase, conferring kanamycin resistance in plants.

LB and RB: left and right borders respectively of T-DNA; *nos* Pro: *nopaline synthase* promoter; *nos* t: *nopaline synthase* terminator; *SAG12* Pro: promoter of *Arabidopsis* *SAG12* gene.

Referring to Figure 1, the generation of the constructs will now be described in detail below:

Cloning into the pCR4 Blunt-TOPO vector

PCR products were examined using agarose gel electrophoresis using 1 % (w/v) agarose, 0.5 µg ml⁻¹ ethidium bromide (Sigma-Aldrich) in 0.5 x TBE. Samples were suspended in 1 x DNA sample buffer (DNA Sample Buffer at 6x working concentration contained 50 mM Tris(hydroxymethyl) aminomethane (Tris-HCl,

- 35 -

Melford Laboratories), 60 % glycerol and 0.25 % (w/v) bromophenol blue (Sigma-Aldrich) and subjected to electrophoresis at 80V using 0.5 x TBE as running buffer.

The PPKK cDNA band of 2.6 kb and the PPKK genomic DNA band of 4.4 kb were purified using the QIAQuick PCR Purification Kit (Qiagen) according to the recommended protocol, and eluted using 30 µl molecular biology grade water (BDH Laboratory Supplies). PCR products were ligated (blunt-end) into the pCR4 Blunt-TOPO vector (Invitrogen) according to the recommended protocol. The cloning reaction (2 µl) was transformed into 50 µl sub-cloning efficiency DH5a *E. coli* (Invitrogen) following the recommended procedure. Transformed *E. coli* cells were grown at 37 °C overnight on Luria-Bertani (LB) agar (Luria Bertani Broth (LB broth) contained 10 g l⁻¹ Bacto-Tryptone (BD), 5 g l⁻¹ Bacto-Yeast Extract (Oxoid) and 85 mM sodium chloride (Fisher Scientific). The pH was adjusted to 7.0 with 10 M sodium hydroxide prior to autoclaving. LB agar was made by adding 1.5 % (w/v) agar (BD) to LB broth containing 50 µg ml⁻¹ kanamycin (Melford Laboratories).

E. coli colonies were screened by PCR using 1 x NH₄ buffer (Bioline), 2.5 mM MgCl₂ (Bioline), 0.5 mM dNTPs (Bioline), 0.3 µM each primer (AtCytFWD-AvrII and AtCytREV-BamHI), i.e. SEQ ID No's 1 & 2, and 0.5 units BioTaq NA Polymerase (Bioline). Thermal cycling was performed with an initial denaturation step of 95 °C for 5 minutes, followed by 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 4 minutes 30 seconds, with a final extension step of 72 °C for 5 minutes. PCR products were examined using 1 % (w/v) agarose gel electrophoresis as above.

Colonies containing the desired insert were grown overnight in a shaking incubator at 37 °C in 5 ml LB broth containing 50 µg ml⁻¹ kanamycin. Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen) according to the recommended protocol. DNA was digested with 10 units *Bam*HI (NEB1) in 1 x *Bam*HI Buffer at 37 °C for 1 hour. Digests were examined using 1 % (w/v) agarose gel electrophoresis as above. Inserts in plasmids showing the correct restriction enzyme digest pattern were sequenced using the primers AtCytFWD-AvrII (SEQ ID No.1) , AtPPDKSeqF1 (SEQ ID No. 3), AtPPDKSeqF2 (SEQ ID No. 4), AtPPDKSeqR1 (SEQ ID No. 5), AtPPDKSeqR2 (SEQ ID No. 6) and AtCytREV-BamHI (SEQ ID No.2), shown in

- 36 -

Table 1, using a 3730 DNA Analyzer (Applied Biosystems). Sequences were analysed in BioEdit (Ibis Biosciences).

These constructs were named TOPO-PPDKcDNA and TOPO-PPDKgDNA, and are
5 illustrated in Figures 2a and 2b.

Cloning of pBNP constructs

PPDK was ligated into the BNP1380000001 binary vector under the control of the senescence-induced promoter SAG12. The SAG12-containing backbone plasmid,
10 BNP1380000001, is based on pBINPLUS (F. A. van Engelen et al. Transgenic Research (1995) 4, 288-290), and is illustrated in Figure 3a. First, *E. coli* colonies carrying TOPO-cDNA or TOPO-gDNA were used to inoculate 25 ml LB broth containing 50 µg ml⁻¹ kanamycin. These cultures were incubated overnight in a shaking 37 °C incubator and plasmid DNA was extracted using the Plasmid Midi Kit (Qiagen)
15 according to the recommended protocol. The pBNP1380000001 vector was purified from a 100 ml culture containing 50 µg ml⁻¹ kanamycin using the Plasmid Midi Kit.

These plasmids were then subjected to digestion by the restriction enzymes *AvrII* and *BamHI*. Digest reactions were incubated overnight at 37 °C and contained either 2 µg
20 DNA (BNP) or 4 µg DNA (TOPO-PPDKcDNA and TOPO-PPDKgDNA), 1 x Buffer 2, 10 units *AvrII* and 10 units *BamHI*. Samples were separated by crystal violet agarose gel electrophoresis (Rand, 1996, Crystal violet can be used to visualize DNA bands during gel electrophoresis and to improve cloning efficiency. Elsevier Trends Journals Technical Tips Online.) using 0.8 % (w/v) agarose, 0.5 x TBE and 25 µM
25 crystal violet (Hopkin and Williams) subjected to electrophoresis at 50 V for approximately 2 hours using crystal violet sample buffer (250 µM crystal violet (Hopkin and Williams) and 30 % (w/v) sucrose (Fisher Scientific)), and 0.5 x TBE containing 25 µM crystal violet as running buffer. Gel band extraction using the QIAQuick Gel Extraction Kit (Qiagen) according to the recommended protocol was used to extract
30 the 14.4 kb BNP, 2.6 kb PPDK cDNA and 4.4 kb PPDK genomic DNA fragments. Fragments were inspected and quantified relative to HyperLadder I (Biolone) using 1 % agarose gel electrophoresis using ethidium bromide.

- 37 -

Due to the similar sizes of the genomic DNA fragment (4.4 kb) and the TOPO backbone (3.9 kb), the gel-extracted genomic DNA fragment was phosphatase-treated to prevent ligation to any contaminating TOPO backbone. Shrimp Alkaline

5 Phosphatase (SAP, 1 unit, Roche) was added to 1 µg gel-extracted genomic DNA fragment in 1 x Dephosphorylation Buffer (Roche) and the reaction incubated at 37 °C for 30 minutes then inactivated at 65 °C for 10 minutes. Ligation reactions were performed using either the cDNA or the genomic DNA fragment with the digested BNP in a 10:1 molar ratio, 1 x ligation buffer (NEB) and 1 unit T4 DNA ligase (NEB).
10 Ligation reactions were incubated overnight at 16 °C and 2 µl used to transform library-efficiency DH5_ *E. coli* (Invitrogen) according to the recommended protocol.

Transformed *E. coli* were transferred onto LB agar containing 50 µg ml⁻¹ kanamycin and incubated overnight at 37 °C. *E. coli* colonies were screened by PCR using 1 x NH₄
15 buffer, 2.5 mM MgCl₂, 0.5 mM dNTPs, 0.3 µM each primer (AtPPDKexon15FWD (SEQ ID No. 7) and BNPnostREV (SEQ ID No. 8), shown in Table 1, and 0.5 units BioTaq DNA Polymerase. Thermal cycling was performed with an initial denaturation step of 95 °C for 5 minutes, followed by 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 3 minutes, with a final extension step of 72 °C for 5 minutes.
20 PCR products were examined using 1 % (w/v) agarose gel electrophoresis as above. Colonies containing the desired insert were grown overnight in a shaking incubator at 37 °C in 5 ml LB broth containing 50 µg ml⁻¹ kanamycin. Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit according to the recommended protocol. DNA was digested with 10 units *Bam*HI and 10 units *Sst*I in 1 x *Bam*HI Buffer and at 37 °C
25 for 1 hour before being subjected to 1 % (w/v) agarose gel electrophoresis as above. One colony each resulting from the cDNA and genomic DNA ligations showing the correct restriction enzyme digest pattern were selected and sequenced using the primer BNP-SAG12FWD (SEQ ID No. 9), shown in Table 1, as described above.

30 These constructs were named BNP-PPDKcDNA and BNP-PPDKgDNA, and are illustrated in Figures 3a and 3b.

Example 2 - Transformation of *Arabidopsis thaliana* with BNP-PPDKcDNA and BNP-PPDKgDNA

Agrobacterium tumefaciens strain GV3101-R was transformed by electroporation using the following plasmids: BNP-PPDKcDNA, and BNP-PPDKgDNA, shown in Figure 3, the preparation of which has been described in Example 1.

Electrocompetent *Agrobacterium* was made from cultures of LB broth containing 25 mg l⁻¹ rifampicin (Sigma-Aldrich), grown at 30 °C and with an optical density at 600 nm of 0.4 to 0.6, measured using an Eppendorf Biophotometer spectrophotometer. Cultures of 500 ml were centrifuged at 4000 g for 15 minutes, the supernatant discarded and the cells resuspended in 500 ml cold 10 % glycerol (Fisher Scientific). Centrifugation and resuspension were repeated using 250 ml glycerol, followed by 10 ml and finally 2 ml glycerol. Cells were aliquoted into 50 µl aliquots, flash frozen in liquid nitrogen and stored at -80 °C. Electroporation was performed using a BioRad Gene Pulser. Plasmid DNA (200 ng) was added to 50 µl *Agrobacterium* cells as above in a pre-chilled electroporation cuvette (Gene Pulser Cuvette, BioRad) and the cells were incubated on ice for 5 minutes. The cuvette was subjected to electroporation using a pulse of 2.5 mV, 400 ohm resistance and 25 µF capacitance and 1 ml SOC (Super Optimal Broth, Catabolite Repression contained 20 g/l Bacto-Tryptone, 5 g/l Bacto-Yeast Extract, 85 mM sodium chloride, and 250 mM potassium chloride. 10 M sodium hydroxide was used to adjust to pH 7.0 prior to autoclaving. Before use, sterile magnesium chloride was added to a final concentration of 10 mM and sterile glucose (Fisher Scientific) was added to a final concentration of 20 mM. The cells were incubated in a shaking incubator at 30 °C for 2 hours before being transferred onto LB agar containing 50 µg ml⁻¹ kanamycin and 50 µg ml⁻¹ rifampicin.

After incubation at 30 °C for 2 days, colonies were screened by PCR and then by restriction enzyme digestion. Colonies positive for the PCR screen and showing the expected restriction digest pattern were inoculated into 5 ml LB broth containing 50 µl ml⁻¹ kanamycin and 50 µl ml⁻¹ rifampicin and incubated in a shaking incubator at 30 °C overnight. The following day, 600 µl of this culture was used to inoculate 500 ml

LB broth as above, and incubated in a shaking incubator at 30 °C for 30 hours before being used for floral dipping.

Arabidopsis plants were used for floral dipping at 4 weeks old. All constructs were transformed into wild-type ecotype Columbia 0. *Agrobacterium* cultures prepared as described above were centrifuged at 4 °C for 15 minutes at 5,000 g and the cell pellets resuspended in 250 ml sterile 5 % sucrose (Fisher Scientific) solution (w/v) and 0.05 % Silwett L-77 (OSi Specialties). Plants were immersed in the cell suspension for approximately 10 seconds with gentle agitation, before being covered in clingfilm and kept away from direct light for 24 hours. Following this, plants were returned to the normal growth regime and seed was harvested.

Selection of transgenic *Arabidopsis thaliana*

Seed from transformed plants was selected on 50 µg ml⁻¹ kanamycin (Dufecha Biochemie) according to Harrison et al. (2006), Plant Methods, 2, 19. T1 plants showing antibiotic resistance were grown to seed and self-pollinated. The seed was selected as above and T2 lines showing a 3:1 resistant:non-resistant ratio were chosen as carrying a single copy of the transgene. These plants were again self-pollinated and selected as above. T2 lines that produced 100 % resistant offspring (T3) were chosen as being homozygous and were used for all experiments. Mendelian genetics was used to select T2 generation plants that were homozygous for the transgene, and contained a single transgene copy. Five independent single-insert homozygous lines were selected each for plants transformed with *SAG12-PPDKcDNA* and *SAG12-PPDKgDNA*.

Example 3 - Transformation of *Nicotiana tabacum* with BNP-PPDKcDNA and BNP-PPDKgDNA.

Electrocompetent *Agrobacterium tumefaciens* strain LBA4404 was transformed by electroporation as described in Example 2 using the plasmids BNP, BNP-PPDKcDNA and BNPPDKgDNA. Following electroporation, 1 ml LB broth was added to electroporated cells, which were then incubated in a shaking incubator at 28 °C for 2 hours before being transferred onto LB agar containing 50 µg ml⁻¹ kanamycin and 100 µg ml⁻¹ spectinomycin (Sigma-Aldrich). After incubation at 28 °C for 2 days one

- 40 -

colony was used to inoculate 50 ml LB broth containing 50 $\mu\text{g ml}^{-1}$ kanamycin and 100 $\mu\text{g ml}^{-1}$ spectinomycin. This culture was incubated in a shaking incubator at 28 °C for 3 days. Plasmid DNA was extracted and analysed by restriction enzyme digest, and 50 μl culture was used to inoculate 50 ml LB broth containing 50 $\mu\text{g ml}^{-1}$ kanamycin and 100 $\mu\text{g ml}^{-1}$ spectinomycin. This culture was incubated in a shaking incubator at 28 °C overnight.

Nicotiana tabacum cultivars Burley 21 and K326 were grown from seed and the youngest leaves were excised from 8 week old plants and sterilised in 8 % (v/v) Domestos thick bleach (Domestos) for 10 minutes before being rinsed in sterile distilled water. A number 6 cork borer was used to punch leaf discs, which were then placed into 25 ml of *Agrobacterium* culture for 2 minutes. Leaf discs were then placed underside down onto MS medium containing 2.2 μM 6-benzylaminopurine (BAP) and 0.27 μM α -naphthaleneacetic acid (NAA). These were placed in a growth room at 22 °C for two days. Leaf discs were then transferred to selective MS medium as above containing 500 $\mu\text{g ml}^{-1}$ clarofan (Roussel Laboratories) (non-cocultivated controls) or 500 $\mu\text{g ml}^{-1}$ clarofan and 100 $\mu\text{g ml}^{-1}$ kanamycin. Leaf discs were transferred to fresh selective MS medium as above every 14 days for 6 weeks. Callus and shoot clumps were then removed from discs and placed onto LS medium containing 0.5 μM BAP, 500 $\mu\text{g ml}^{-1}$ clarofan and 100 $\mu\text{g ml}^{-1}$ kanamycin. After 2 weeks shoots were transferred to 150 ml jars with LS medium containing 0.5 μM BAP, 500 $\mu\text{g ml}^{-1}$ clarofan and no kanamycin.

After a subsequent 3 weeks, the dominant shoot was transferred to LS medium containing 250 $\mu\text{g ml}^{-1}$ clarofan and no BAP or kanamycin. After another 3 weeks shoots were further cleaned by transferring the shoot tip to LS medium with no antibiotics or BAP. When sufficient roots had generated, plants were transferred to soil in greenhouses.

Selection of transgenic *Nicotiana tabacum*

Quantitative PCR (Q-PCR) was used to quantify transgene copy number in T0 and T1 plants. Where possible, single insert T0 and homozygous T1 plants were chosen for analysis. For transgene detection, primers BNP-1271F (SEQ ID No. 10) and BNP-

1334R (SEQ ID No. 11) were used, as shown in Table 1, and the Vic/TAMARA-labelled probe BNP1291TV (SEQ ID No. 12) was used, annealing to the *nptII* transgene. For internal quantitation, primers NtCyc-184F (SEQ ID No. 13) and NtCyc-316R (SEQ ID No. 14) were used, and the FAM/TAMARA-labelled probe NtCyc-267T (SEQ ID No. 15) was used, annealing to the endogenous gene encoding cyclophilin. Quantitation was by the Comparative Ct method ($\Delta\Delta C_t$ method, Bubner & Baldwin (2004), Plant Cell Reports, 23, 263–271). Reaction mixtures contained 1 x Universal Master Mix (ABI), 0.9 μ M each primer, 0.2 μ M each probe (using separate reactions for BNP and NtCyc primers and probes) and approximately 500 ng genomic DNA template extracted from leaf tissue using the Dneasy Plant Mini Kit (Qiagen) according to the recommended protocol. Thermal cycling was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems) with an initial denaturation step of 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Data were analysed using the supplied SDS2.2 software (Applied Biosystems).

Following regeneration in tissue culture of transformed T0 generation K326 and Burley 21 tobacco, quantitative PCR (Q-PCR) was used to select plants carrying a single copy of the transgene. Single-insert plants were selected to reduce the possibility of transgene silencing. Q-PCR was performed using oligonucleotides complementary to the *nptII* transgene, encoding neomycin phosphotransferase. This gene was present between the left and right borders of the T-DNA transferred into the plant genome in constructs pBNP, BNP-PPDKcDNA and BNP-PPDKgDNA. Plants transformed with pBNP only served as empty vector controls, to ensure that regeneration through tissue culture was consistent between plants transformed with the empty vector and those transformed with the vectors containing PPDK coding sequence. Following regeneration and Q-PCR checks to confirm that successful transformation had occurred, these plants were discarded.

For each construct (*SAG12-PPDKcDNA* and *SAG12-PPDKgDNA*) and each cultivar (K326 and Burley 21), six plants were selected. It was not possible to select only single-insert plants, so where six single-insert plants were not available, plants with the lowest

- 42 -

copy number possible were selected. These plants were self-pollinated and the seed was harvested.

For each selected T0 plant, 14 offspring were grown and Q-PCR was repeated to select homozygous plants. Use of homozygous plants should ensure stability of the transgene in future generations. For each T0 parent, four offspring carrying the transgene were selected. However, it was not always possible to select homozygous offspring for every T0 parent. Where parental copy number was higher than two, plants with lower copy numbers were selected to reduce the possibility of transgene silencing, and also to simplify the selection of homozygotes from the T2 generation, if necessary. In this way, for each construct and each cultivar four biological replicates (siblings) from five independent lines were selected with low transgene copy numbers for all further experiments.

Example 4 - Detection and quantification of PPKK protein in transformed *Arabidopsis* plants

Protein extraction

Arabidopsis leaf tissue (100 mg) was ground under liquid nitrogen in a 1.5 ml microcentrifuge tube using a micropestle and 400 µl extraction buffer (Potassium Phosphate Buffer (plus Protease Inhibitor Cocktail (PIC, Sigma))) was added. Protein concentration was determined by Bradford assay (Jones et al., 1989, Journal of Chemical Ecology, 15, 979–992) using Bio-Rad Protein Assay Reagent (Bio-Rad) according to the recommended protocol.

Polyacrylamide gel electrophoresis

Proteins were separated by polyacrylamide gel electrophoresis (PAGE) in a resolving gel containing 10 % (v/v) acrylamide (37.5:1 acryl:bis acryl, Severn Biotech Ltd), 50 % (v/v) Immunoblot Resolving Buffer (see Section 2.10), 0.05 % (w/v) ammonium peroxodisulphate (APS, AnalaR) and 0.05 % (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED, Severn Biotech Ltd). The stacking gel contained 5 % acrylamide as above, 50 % (v/v) Immunoblot Stacking Buffer, 0.06 %

- 43 -

(w/v) APS and 0.1 % (v/v) TEMED. Electrophoresis was performed on 20 µg total protein extracted using 1 x Immunoblot Sample Buffer (66 mM Tris-HCl, 10 % (v/v) glycerol, 0.7 mM SDS, 0.7 M β-mercaptoethanol (BDH Laboratory Supplies) and 0.05 % (w/v) bromophenol blue and Immunoblot Running buffer (25 mM Tris-HCl, 0.29 mM glycine (Fisher Scientific) and 3.5 mM SDS at a current of 70 mA for 1 hour 30 minutes.

Duplicate gels were run simultaneously. One was used for immunoblot analysis (described hereinafter) and the second stained using GelCode Blue Safe Protein Stain (Thermo Scientific) according to the recommended protocol. Gel Drying Film (Promega) was used to dry stained gels.

Immunoblot analysis

Proteins from the gel for immunoblot analysis were transferred onto a Protan BA83 Cellulose Nitrate Membrane (Schleicher and Schuell) using Protean Extra-Thick Blot Paper (Bio-Rad) and Immunoblot Transfer Buffer (48 mM Tris-HCl, 39 mM glycine, 1.3 mM SDS and 20 % (v/v) methanol (Fisher Scientific)) in a Semi-Dry Blotter (Bio-Rad) at 15 V for 1 hour. Ponceau Stain (0.5 % (w/v) Ponceau S (Fluka) and 1 % (w/v) glacial acetic acid (Fisher Scientific)) was applied to the membrane after blotting to verify protein transfer, and the membrane was then rinsed in distilled water.

Blocking buffer for PPK immunoblot analysis was made fresh each day using 1 % (w/v) dried skimmed milk powder (Marvel) and 0.1 % (v/v) polyoxyethylene sorbitan monolaurate (TWEEN 20, Sigma-Aldrich) in phosphate buffered saline (PBS: 1.5 mM potassium dihydrogen orthophosphate (KH₂PO₄, AnalaR), 8.1 mM disodium hydrogen orthophosphate, 2.7 mM potassium chloride and 137 mM sodium chloride. Hydrochloric acid was used to adjust to pH 7.4. Membranes were incubated in blocking buffer at room temperature on a shaker for 1 hour. Primary hybridisation was performed using a 1:10,000 dilution of rabbit anti-PPDK antibody (Chris Chastain, Minnesota State University) followed by 3 washes of 5 minutes each in blocking buffer. Secondary hybridisation was performed using a 1:1000 dilution of Donkey Anti-Rabbit Biotinylated Whole Antibody (GE Healthcare) followed by 3 washes as above. Tertiary

hybridisation was performed using a 1:1000 dilution of Streptavidin-Biotinylated Horse Radish Peroxidase Complex (GE Healthcare) followed by 3 washes of 5 minutes each in PBS containing 0.1 % (v/v) TWEEN 20.

- 5 Membranes were then rinsed 3 times in distilled water. Detection was performed using Western Lightning Chemiluminescence Reagent (Enhanced Luminol, Perkin Elmer) according to the recommended protocol.

Referring to Figure 4, there is shown the selection of transgenic *SAG12-PPDK*
10 *Arabidopsis thaliana* cell lines by Western blot. Protein was extracted from leaf tissue of eight week old *Arabidopsis* wild-type, Δ PPDK *SAG12-PPDKcDNA* and *SAG12-PPDKgDNA* plants, and subjected to immunoblot analysis to select lines expressing higher levels of PPDK. Recombinant maize PPDK (30 μ g) was used as a positive control. PPDK was not detected in wild-type or PPDK mutant plants (Δ PPDK).
15 *SAG12-PPDKgDNA* plants were expressing higher levels of PPDK than *SAG12-PPDKcDNA*. All subsequent analysis was performed on the five *SAG12-PPDKgDNA* lines.

PPDK was undetectable in wild-type and Δ PPDK plants, was detectable at low levels
20 in some *SAG12-PPDKcDNA* plants, and was detectable in all *SAG12-PPDKgDNA* lines. In three of these lines, much higher amounts of PPDK were detected compared to wild-type or *SAG12-PPDKcDNA* lines. Therefore, the presence of introns appears to have a positive effect on PPDK expression levels, and the *SAG12-PPDKgDNA* lines were selected for use in all further experiments.

25 Immunoblots against PPDK were performed on wild-type, Δ PPDK and *SAG12-PPDKgDNA* plants in order to determine the onset and extent of PPDK accumulation in *SAG12-PPDKgDNA* plants.

30 Referring to Figure 5, there is shown the quantification of PPDK abundance in wild-type, Δ PPDK and five independent *SAG12-PPDKgDNA* lines from week five onwards. PPDK abundance was calculated as a percentage of wild-type for each time-

point from weeks five to nine. Data are shown as mean of these five time-points. Error bars show one SEM. *SAG12-PPDKgDNA* lines are arranged in order of increasing PPDK abundance. The differences between genotypes were tested using ANOVA ($F = 4.775$, $df = 6$, $p = 0.001$). Lines significantly different to wild-type were G12.3 ($p = 0.005$) and G16.1 ($p = 0.004$).

Proteins were extracted from leaf tissue harvested at weekly intervals from three weeks to nine weeks after sowing. PPDK was detected in low amounts in wild-type plants from week five onwards, confirming that the observed increase in transcripts results in an increase in protein abundance. In *SAG12-PPDKgDNA* plants, onset of PPDK accumulation was also observed at week five, but PPDK abundance is higher than in the wild-type. No PPDK protein was detected at any stage in Δ PPDK plants.

A method to allow quantification of PPDK in leaf protein extracts was also optimized. Different amounts of recombinant maize PPDK protein were subjected to SDS-PAGE and immunoblot analysis. Band intensity was calculated using AlphaEase imaging software and a standard curve constructed. The regression line was calculated using SigmaPlot software which was subsequently used to calculate PPDK amounts in plant leaf samples. Since immunoblot detection levels can vary, it was necessary to include at least three standards on every immunoblot to allow true comparison between different immunoblots. This technique for quantification of PPDK abundance in different extracts was used to select and compare different transgenic lines.

PPDK protein abundance was calculated by reference to the known standards of recombinant maize PPDK protein for at least four biological replicates for each line at each time-point. The analysis showed that PPDK abundance in *SAG12-PPDKgDNA* was higher than in wild-type from week five onwards. PPDK abundance was calculated as a percentage of wild-type at each time-point from week five onwards for each *SAG12-PPDKgDNA* line, and the mean taken of these values to quantify PPDK accumulation over the senescence period in each *SAG12-PPDKgDNA* line. This allowed the *SAG12-PPDKgDNA* lines to be ordered according to increasing PPDK abundance, as shown in Figure 5.

Since proteins which accumulate in high amounts in transgenic plants may be subjected to inactivation, the increased protein abundance does not necessarily imply increased enzyme activity. PPDK is reversibly phosphorylated in the dark, resulting in its inactivation. An antibody has been raised against the phosphorylated version of PPDK (Phospho-PPDK), allowing specific detection of the inactive form of PPDK, whereas the antibody used previously (anti-PPDK) detects PPDK regardless of phosphorylation state (Chastain et al., 2002, Plant Physiology, 128, 1368–1378). The anti-PhosphoPPDK antibody was used to detect inactivated PPDK over a time-course from three weeks to nine weeks after sowing, on immunoblots probed initially with anti-PPDK antibody and then stripped and reprobed.

Surprisingly, inactivated PPDK (phosphorylated) was detectable only at very low levels in wild-type plants in a number of immunoblots, and only during the later stages of senescence. In *SAG12-PPDK*gDNA plants, depending on the time-point inactivated, PPDK was detected at higher levels. However, at time-points soon after onset of increased PPDK abundance, very little or no inactivated PPDK was detected despite high abundance of total PPDK. These results suggest that while some PPDK inactivation could occur in *SAG12-PPDK*gDNA plants, increased abundance of enzymatically active PPDK is present in *SAG12-PPDK* plants at least during early stages of senescence.

Example 5 - Detection and quantification of PPDK protein in transformed *N.tabacum* plants

N.tabacum leaf tissue (100 mg) was ground under liquid nitrogen in a 1.5 ml microcentrifuge tube using a micropestle and 400 µl extraction buffer (Overcoat Buffer (PIC)) was added. Protein concentration was determined by Bradford assay (Jones et al., 1989) using Bio-Rad Protein Assay Reagent (Bio-Rad) according to the recommended protocol. Proteins were separated by polyacrylamide gel electrophoresis (PAGE) as described in Example 4. Proteins were analysed and PPDK protein quantified by immunoblot as described for Example 4.

- 47 -

Immunoblots against PPDK were performed on leaf protein extracted from K326 and Burley 21 T1 generation tobacco leaves induced to senesce by detachment and incubation in the dark at 30 °C. Senescence was induced in this way so that transgenic lines with highest PPDK abundance could be identified, and other lines discarded, before plants reached maturity. In K326 plants, PPDK was detected in high abundance in three *SAG12-PPDKgDNA* lines (G4, G8 and G10) and in one *SAG12-PPDKcDNA* line (C10). In Burley 21 plants, PPDK was abundant in four *SAG12-PPDKgDNA* lines (G3, G7, G15 and G23), but was not present in as high abundance as in K326 lines. The four lines each for K326 and Burley 21 that had highest PPDK abundance were used for all further analysis. Since highest PPDK abundance was observed almost exclusively in *SAG12-PPDKgDNA* lines, it appears that introns have a positive effect on PPDK expression.

Referring to Figure 6, there is shown the selection of *SAG12-PPDK* K326 and Burley 21 tobacco lines, as follows:

(b) PPDK immunoblot to select transgenic K326 lines. Senescence was induced in green leaves of six week old plants by detachment and dark incubation at 30 C for three days. Protein was extracted and subjected to immunoblot analysis to select lines expressing higher levels of PPDK. Recombinant maize PPDK (50 µg) was used as a positive control. One representative immunoblot is shown, with one offspring from each parental T0 plant. Plants from parental lines *SAG12-PPDKgDNA* G4, G8 and G10 and from *SAG12-PPDKcDNA* line C10 were used for all subsequent analysis.

(c) PPDK immunoblot to select transgenic Burley 21 lines, as for K326 in section (b). Plants from parental lines *SAG12-PPDKgDNA* G3, G7, G15 and G23 were used for all subsequent analysis.

Immunoblots against PPDK were performed on wild-type, zero copy (negative segregant), *SAG12-PPDKgDNA* K326 *SAG12-PPDKcDNA* K326 and *SAG12-PPDKgDNA* Burley 21 tobacco to determine the onset of expression of PPDK and to quantify the degree of PPDK overexpression in the transformed plants. Proteins were extracted from K326 wild-type and transformed plant leaves one (oldest) to eight (young) of three month old plants and immunoblots against PPDK were performed.

Referring to Figure 7, there is shown the overexpression of PPDK in ripe leaves of K326 tobacco. PPDK abundance was calculated from immunoblots. Data are shown as mean of 10 biological replicates for zero copy plants, and four biological replicates for each *SAG12-PPDK* line. Error bars show one SEM. *SAG12-PPDK* lines are arranged in order of increasing PPDK abundance. The difference between genotypes were tested using ANOVA ($F = 6.995$, $df = 4$, $p = 0.001$). Lines significantly different to zero copy plants were G10 ($p = 0.006$), G8 ($p = 0.003$) and C10 ($p = 0.000$).

PPDK abundance was higher in older leaves of wild-type plants, indicating that PPDK is naturally upregulated during senescence in tobacco as well as in *Arabidopsis*. In the transformed K326 plants, PPDK abundance was also higher in older leaves, and was much higher than in wild-type plants. There was also higher abundance of PPDK in younger leaves of transformed lines with highest PPDK abundance. Immunoblots were performed on protein extracted from 'ripe' leaves of zero copy plants (negative segregants) and transformed plants. Ripe leaves are those at a harvestable stage, and are in the later stages of senescence. PPDK abundance was quantified. PPDK abundance was higher in ripe leaves of all four independent transformed lines compared to zero copy plants. Overexpression of PPDK during senescence using the *SAG12* promoter was successful for K326 tobacco.

Example 6 - Phenotypic analysis of transformed *Arabidopsis* plants

Analysis of *Arabidopsis* plant growth

Referring to Figure 8, it is clearly shown that the overexpression of PPDKgDNA in *Arabidopsis thaliana* generates plants with larger rosettes. Wild-type, Δ PPDK and *SAG12-PPDKgDNA* plants were photographed at three, five, seven and nine weeks after sowing. *SAG12-PPDKgDNA* plants were larger than wild-type. There was no discernable difference between PPDK plants and wild-type plants.

Fresh masses of rosette and reproductive tissue were determined using a Mettler Toledo AB104-S balance to the nearest 0.1 mg. Dry mass of rosette tissue was

determined from samples freeze dried overnight using an Edwards Super Modulyo Freeze Dryer to the nearest 0.1 mg.

Referring to Figure 9, there is shown the reproductive tissue mass at week nine. A dose response to PPDK was observed, with lines with higher PPDK abundance having higher reproductive tissue mass. Total reproductive tissue (stems, cauline leaves, flowers and siliques) was weighed. Values are means of three biological replicates. Error bars show one SEM. Transgenic *SAG12-PPDKgDNA* lines are arranged in order of increasing PPDK abundance. Difference between genotypes was tested using ANOVA (F = 8.062, df = 6, p = 0.001). All *SAG12-PPDKgDNA* lines were significantly different to wild type (G9.4 p = 0.031, G5.4 p = 0.000, G19.2 p = 0.000, G12.3 p = 0.001, G16.1 p = 0.001). While the trend is not absolute, lines with higher PPDK abundance tended to have higher reproductive tissue mass. Reproductive tissue as percentage of total plant fresh mass was also calculated, but was not significantly different for wild-type, Δ PPDK or *SAG12-PPDKgDNA* plants when tested using ANOVA (F = 0.544, df = 6, p = 0.767, data not shown).

However, the proportion of reproductive tissue as a percentage of total plant mass was unchanged, suggesting that the plant as a whole was bigger and that resource allocation between vegetative and reproductive tissue was unchanged. Total plant fresh mass and reproductive tissue mass were also measured in Δ PPDK plants, and were not significantly different to wild-type. Rosette dry mass was also measured, and as for total plant fresh mass and reproductive tissue mass, *SAG12-PPDKgDNA* plants were found to have significantly higher mass than wild-type.

Referring to Figure 10, there is shown total plant fresh mass (rosette plus reproductive tissue) of wild-type and *SAG12-PPDKgDNA* plants from weeks three to nine after sowing. Data are shown as mean of three biological replicates for wild-type, and 15 biological replicates (three plants each for five independent lines) for *SAG12-PPDKgDNA*. Error bars show one SEM. Wild-type and *SAG12-PPDKgDNA* total plant masses were compared using a student's t-test at each timepoint. Rosette mass was significantly higher in *SAG12-PPDKgDNA* plants at week nine (p = 0.032).

The difference was not significant prior to onset of PPDK overexpression due to the *SAG12* promoter at week five. All five independent *SAG12-PPDKgDNA* lines had increased rosette dry mass. A dose response to PPDK abundance was observed as for reproductive tissue mass, with lines with higher PPDK content having higher mass. Rosette dry mass was also measured in Δ PPDK plants, and was not significantly different to wild-type plants.

Determination of *Arabidopsis* leaf surface area

Surface area of the rosette was determined by sampling a single mature leaf, usually leaf 10, determining the mass of the leaf and of the whole rosette as above and photographing the leaf alongside a ruler. The surface area of the leaf was measured using ImageJ software (National Institutes of Health), and the surface area of the whole rosette calculated from this.

Rosette surface area was increased in *SAG12-PPDKgDNA* plants following onset of PPDK overexpression. While surface area was significantly larger in *SAG12-PPDKgDNA* plants at weeks six and seven, by weeks eight and nine surface area was not significantly different from wild-type. This large decrease in surface area could be due to increased remobilisation of nutrients in *SAG12-PPDKgDNA* plants compared to wild-type. Surface area in Δ PPDK plants was not significantly different to wild-type plants.

Determination of Chlorophyll concentration

Chlorophyll content was measured in relative units using a CCM-200 hand-held chlorophyll meter (Opti-Sciences).

Fluorescence measurement of *Arabidopsis*

The FV / FM ratio was measured using a Hansatech FMS2 fluorometer. Leaves were dark adapted using leaf clips, provided by the manufacturer, overnight prior to measurement. To determine whether onset of senescence was altered in *SAG12-PPDKgDNA* or Δ PPDK plants relative to wild-type plants, FV / FM ratios were

measured. FV / FM is an estimate of the quantum efficiency of Photosystem II, and declines when photoinhibition occurs. It is a useful measure of the onset of senescence, since decline in photosynthesis occurs early in senescence, before a decline in chlorophyll content is detected. FV / FM was measured over a timecourse from four weeks (when leaves become large enough to measure FV / FM) to nine weeks after sowing. In wild-type, *SAG12-PPDKgDNA* and Δ PPDK plants, FV / FM was maximal at week seven and subsequently declined, indicating that timing of onset of senescence was the same in all three genotypes. However, in *SAG12-PPDKgDNA* plants FV / FM was significantly higher than wild-type at week eight, indicating prolonged photosynthetic activity in the later stages of senescence.

Nitrogen content

Tissue for nitrogen analysis was freeze dried using an Edwards Super Modulyo Freeze Dryer. *Arabidopsis* leaf tissue (25 mg) or tobacco leaf tissue (100 mg) was packaged in nitrogen-free weighing paper (Elementar Analysensysteme GmbH). A Rapid N III Nitrogen Analyzer (Elementar Analysensysteme GmbH) was used to measure nitrogen content as a percentage of dry weight, using the recommended settings. Aspartic acid (Sigma-Aldrich) was used as a standard. Leaf nitrogen content was measured in *Arabidopsis* wild-type, *SAG12-PPDKgDNA* and Δ PPDK plants over a timecourse from three to nine weeks after sowing.

Referring to Figure 11, there is shown the nitrogen content in leaves of wild-type, Δ PPDK and *SAG12-PPDKgDNA* plants at week seven. Data are shown as mean of eight biological replicates for wild-type and Δ PPDK and four biological replicates for each *SAG12-PPDKgDNA* line. Error bars are one SEM. *SAG12-PPDKgDNA* lines are arranged in order of increasing PPDK abundance. The difference between genotypes was tested using ANOVA ($F = 6.047$, $df = 6$, $p = 0.000$). Δ PPDK plants and all *SAG12-PPDKgDNA* lines were significantly different to wild type (Δ PPDK $p = 0.004$, G9.4 $p = 0.000$, G5.4 $p = 0.000$, G19.2 $p = 0.001$, G12.3 $p = 0.007$, G16.1 $p = 0.006$).

Leaf nitrogen was significantly lower in all five independent *SAG12-PPDKgDNA* lines compared to wild-type from week seven onwards, supporting the hypothesis that

- 52 -

increasing PPDK abundance during senescence could enhance efficiency of nitrogen remobilization. PPDK expression and activity peaked at week six in *SAG12-PPDKgDNA* plants, which suggests that a time delay occurred between increased PPDK abundance and a measurable decrease in leaf nitrogen, which could be attributed to the time taken to convert protein amino acids to transport amino acids (asparagine and glutamine) and transport them out of the leaf.

Analysis of *Arabidopsis* seed

Individual seed mass and nitrogen content were measured in seed of wild-type, *SAG12-PPDKgDNA* and Δ PPDK *A.thaliana* plants.

Referring to Figure 12, there is shown the nitrogen content of individual seed of wild-type, Δ PPDK and *SAG12-PPDK* plants. There were significant increases in seed nitrogen content of the *SAG12-PPDKgDNA* plants. Data are shown as mean of eight biological replicates for wild-type and Δ PPDK and four biological replicates for each *SAG12-PPDKgDNA* line. Error bars are one SEM. *SAG12-PPDKgDNA* lines are arranged in order of increasing PPDK abundance. Difference between genotypes was tested using ANOVA ($F = 6.704$, $df = 6$, $p = 0.000$). Lines significantly different to wild-type were G19.2 ($p = 0.000$), G12.3 ($p = 0.005$) and G16.1 ($p = 0.002$). Lines with higher PPDK abundance tend to have higher seed nitrogen mass. Hence, individual seed mass was increased in all five independent *SAG12-PPDKgDNA* lines relative to the wild-type, and a dose response to PPDK abundance was observed, with plants with higher PPDK abundance having higher seed mass. Total seed harvested from each plant was also weighed, and there was no significant difference between wild-type and *SAG12-PPDKgDNA* plants, so increased seed size in *SAG12-PPDK* plants did not compromise total seed harvest.

Free Amino Acid content of *Arabidopsis* leaf tissue

Leaf tissue (100 mg) was ground under liquid nitrogen in a 1.5 ml microcentrifuge tube using a micropestle (Eppendorf) and 300 μ l sterile deionised water was added. Samples were subjected to centrifugation at 13,000 rpm at 4 °C for 5 minutes. Protein

- 53 -

concentration was determined by Bradford assay (Jones et al., 1989) using Bio-Rad Protein Assay Reagent (Bio-Rad) according to the recommended protocol. Samples were prepared for amino acid analysis using the EZfaast Amino Acid Sample Testing Kit (Phenomenex) according to the recommended protocol. Samples were resuspended
5 in 10 mM ammonium formate (BDH laboratory supplies) in 50 % sterile distilled water and 50 % ultra grade methanol (Romil).

Samples were then subjected to liquid chromatography-mass spectrometry (LC-MS) using a Q Trap LC/MS/MS (Applied Biosystems/MDS SCIEX) with an EZfaast 250 x
10 3.0 mm AAA-MS column (Phenomenex) and 10 mM ammonium formate in 50 % (v/v) sterile distilled water and 50 % (v/v) ultra grade methanol as the mobile phase. The mass spectrometer was used in positive ion mode with conditions as recommended in the EZfaast Kit (Phenomenex). Results were analysed in the supplied Analyst software (Applied Biosystems/MDS SCIEX).

15 Total free amino acid content was measured in leaves of wild-type, *SAG12-PPDKgDNA* and Δ PPDK *Arabidopsis* plants over a timecourse from weeks three to nine after sowing.

20 Referring to Figure 13, there is shown the total free amino acid content is increased in leaves of *Arabidopsis* overexpressing PPDK. Data are shown as mean of six biological replicates for wild-type and Δ PPDK and four biological replicates for each *SAG12-PPDKgDNA* line. Error bars are one SEM. *SAG12-PPDKgDNA* lines are arranged in order of increasing PPDK abundance. All five *SAG12-PPDKgDNA* lines have higher
25 total amino acid content than the wild-type, but variation is high and the difference was not significant when tested using ANOVA ($F = 1.314$, $df = 6$, $p = 0.289$).

In *SAG12-PPDKgDNA* plants, total free amino acid content was significantly higher than in wild-type plants at week seven, the same time at which leaf nitrogen content
30 becomes significantly lower than wild-type, and one week after maximum PPDK abundance in leaves of *SAG12-PPDKgDNA* plants. The increase occurred in all five independent *SAG12-PPDKgDNA* lines, although variation was high and the differences

between individual lines and the wild-type were not significant. This increase suggests that at this time-point amino acid production occurs at a greater rate than amino acid export can occur, and so amino acids accumulate during the leaf. Total free amino acids were not significantly different in Δ PPDK plants compared to wild-type.

5

Transport amino acids (glutamine and asparagine) were also measured and expressed as a percentage of total free amino acids. In *SAG12-PPDKgDNA* plants, transport amino acid content was significantly higher than in wild-type plants at weeks seven and eight. The increase occurred in all five independent *SAG12-PPDKgDNA* lines, although
10 variation was high and the differences between individual lines and the wild-type were not significant. Therefore, in addition to an increase in total free amino acid content in *SAG12-PPDKgDNA* plants, the content of transport amino acids increases as a proportion of the total. Again, this suggests that glutamine and asparagine production exceed the export capacity of the leaf at these timepoints, supporting the hypothesis
15 that increased PPDK abundance during senescence increases the efficiency of amino acid inter-conversions leading to the formation of transport amino acids. In Δ PPDK plants, no significant difference to the wild-type was observed.

Example 7 - Phenotypic analysis of transformed tobacco plants

20

Analysis of tobacco leaf nitrogen content

Leaf nitrogen content was measured in ripe leaves of negative segregant and *SAG12-PPDK* K326 tobacco. Ripe leaves are those ready for harvesting for tobacco production. Leaf nitrogen content of *SAG12-PPDK* plants was lower than that of
25 negative segregant plants, for some of the four independent *SAG12-PPDK* lines.

Ripe leaves were used to measure leaf nitrogen content since these leaves are at the stage at which they would be harvested for tobacco production. However, the fact that there was little difference in leaf nitrogen content in ripe leaves does not necessarily
30 imply that nitrogen remobilisation was not increased. In time-courses of senescing *SAG12-PPDKgDNA Arabidopsis* plants, leaf nitrogen content was significantly lower than wild-type, but by late senescence the difference was much smaller. It is therefore

possible that a difference in leaf nitrogen content in tobacco occurs earlier in senescence, and that the difference decreases by the time leaf nitrogen content was measured.

5 Analysis of tobacco leaf amino acid content

Amino acid content was measured in mature leaves of K326 tobacco, induced to senesce by detachment and incubation in darkness at 30 °C for three days. This allowed comparisons to be made between the same leaves before and after induction of senescence. Induction of senescence by leaf detachment and dark incubation shows
10 greatest overlap of gene expression patterns with age-related senescence.

In K326 tobacco, total amino acid content increased following induction of senescence in negative segregant plants and in *SAG12-PPDK* plants. The increase was calculated as a percentage of total amino acid content before induction of senescence. For *SAG12-PPDK* line C10, the increase was significantly smaller than that in negative segregant
15 plants, but no other *SAG12-PPDK* line showed a significant difference to negative segregant plants. Overexpression of PPDK during senescence therefore appeared to have little effect on total amino acid content following induction of dark-induced senescence. Transport amino acid (glutamine and asparagine) content also increased in
20 K326 tobacco following induction of senescence, but the increase occurred in both negative segregant and *SAG12-PPDK* lines, and there was no significant difference between genotypes in the degree of increase following induction of senescence.

Analysis of tobacco seed

25 Individual seed mass was measured in K326 tobacco, and was significantly higher in *SAG12-PPDKgDNA* and *SAG12-PPDKcDNA* plants compared to negative segregant plants, for all four independent transgenic lines. Mass of seed per seed pod was also measured, and was significantly higher in *SAG12-PPDKgDNA* and *SAG12-PPDKcDNA* plants.

30

Referring to Figure 14, there is shown that the seed size is increased in *SAG12-PPDK* K326 tobacco. Individual seed mass in K326 zero copy (plants which are negative

- 56 -

segregants for the *SAG12-PPDK* insert) and *SAG12-PPDK* plants, was calculated by photographing, counting and weighing approximately 1000 seed. Data are shown as mean of 10 biological replicates for zero copy plants, and four biological replicates for each *SAG12-PPDK* line. Error bars are one SEM. *SAG12-PPDK* lines are arranged in order of increasing PPDK abundance in ripe leaves. Seed mass is higher in *SAG12-PPDK* plants. The difference between genotypes were tested using ANOVA ($F = 4.870$, $df = 4$, $p = 0.006$). Lines significantly different to zero copy plants were G8 ($p = 0.005$) and C10 ($p = 0.002$).

The percentage nitrogen content of seed was higher for all four independent *SAG12-PPDKgDNA* and *SAG12-PPDKcDNA* lines, but the difference was not significant. However, mass of nitrogen per seed was significantly higher in *SAG12-PPDKgDNA* and *SAG12-PPDKcDNA* plants.

Referring to Figure 15, it is clearly shown that seed nitrogen content is increased in *SAG12-PPDK* K326 tobacco. The mass of nitrogen in individual seed of K326 zero copy and *SAG12-PPDK* plants, was calculated from seed mass and seed nitrogen content data. Data are shown as mean of 10 biological replicates for zero copy plants, and four biological replicates for each *SAG12-PPDK* line. Error bars are one SEM. *SAG12-PPDK* lines are arranged in order of increasing PPDK abundance in ripe leaves. Mass of nitrogen in individual seed is higher in *SAG12-PPDK* plants. The differences between genotypes were tested using ANOVA ($F = 7.807$, $df = 4$, $p = 0.001$). Lines significantly different to zero copy plants were G8 ($p = 0.000$) and C10 ($p = 0.000$).

This suggests that nitrogen supply to seeds was increased in *SAG12-PPDKgDNA* and *SAG12-PPDKcDNA* K326 tobacco plants. In K326 plants, a dose response to PPDK was observed. Plants with higher PPDK abundance in ripe leaves had larger seed individual seed mass, higher mass of seed per seed pod, and increased mass of nitrogen in individual seed. This strongly supports a role for PPDK in nitrogen remobilisation, since increased PPDK content appears to be connected with increased nitrogen supply to seed.

- 57 -

In summary, in *SAG12-PPDK* K326 tobacco individual seed mass (Figure 14) and nitrogen mass per seed (Figure 15) were both increased, suggesting that nitrogen remobilisation was increased by overexpression of PPDK. An increase in transport amino acid content of senescing leaves would therefore be expected, as for *Arabidopsis* *SAG12-PPDKgDNA* plants. However, in *Arabidopsis* amino acid content was measured in naturally senescent leaves, whereas in tobacco, senescence was induced by leaf detachment and incubation in darkness. Since the processes that occur in dark-induced and age-related senescence differ, the processes occurring in the tobacco leaves were unlikely to be analogous to those in the *Arabidopsis* leaves.

Example 8 - Generation of *Arabidopsis* plants over-expressing PCK and PPDK

Over-expression of *Arabidopsis* PCK (At4g37870.1) during senescence was achieved by fusing the coding region and genomic clone of PCK to the senescence-associated gene 12 (*SAG12*) promoter within the BNP1380000001 binary vector, which was transformed into *Arabidopsis thaliana*, as described in Examples 1 and 2.

The At PCK coding sequence was first isolated from *Arabidopsis* cDNA, and the genomic sequence was isolated from *Arabidopsis* genomic DNA using PCR, as described in Example 1 in relation to PPDK. However, PCR primers were designed including the start and stop codons of the *Arabidopsis thaliana* (At) PCK gene, and also included an *Xba*I restriction site in the forward primer, AtPCK-Xba IFOR (SEQ ID No.25), and a *Sac*I restriction site in the reverse primer, AtPCK-Sac IREV (SEQ ID No.26), as shown in Table 1, in order to facilitate the subsequent ligation of the gene into the BNP1380000001 vector, which is illustrated in Figure 3a.

The cDNA and genomic DNA templates used for each PCR reaction were prepared according to Example 1. The PCR reaction mixture contained 1 x HF buffer (NEB), 2 mM magnesium chloride (NEB), 0.5 mM dNTPs (Bioline), 100 ng of template (cDNA or genomic DNA), 0.5 μ M each primer and 1 unit Phusion High-Fidelity DNA Polymerase (NEB). Thermal cycling was performed using a 'Techne' Thermal Cycler with an initial denaturation step of 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 60°C for 30 s and an extension time of 72°C for 2 min 30 s for the coding region

- 58 -

and 4 min 30 s for the genomic clone. The last step involved a final extension at 72°C for 10 min.

These PCR products resulted in a 2 kb band for the coding sequence (cDNA) and a 3.5 kb band for the genomic sequence of PCK, and were PEG-precipitated. The amplified DNA was ligated (blunt-end) into the pCR4 Blunt-TOPO vector (Invitrogen) according to the recommended protocol, as for PPK described in Example 1. The plasmid was then transformed into Library Efficiency DH5 α *E.coli* cells. Kanamycin (50 μ g ml⁻¹) was used as the selective antibiotic. Positive colonies were selected by colony PCR using the primers AtPCK-Xba IFOR (SEQ ID No.25) and AtPCK-Sac IREV (SEQ ID No.26) for both the coding region and the genomic clone. Positive colonies were grown overnight in a shaking incubator at 37°C in 5 ml of LB broth containing 50 μ g ml⁻¹ kanamycin.

Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen Ltd) and insert sizes were analysed by a sequential enzyme restriction digest. This was carried out by digesting 1 μ g of DNA with 10 units of *Xba*I and 1 x *Xba*I buffer at 37°C for 2 h and then adding 10 units of *Sac*I and 1 x *Sac*I Buffer at 37°C overnight. Plasmid DNA containing the correct inserts was sequenced using AtPCK-Xba IFOR/AtPCK-Sac IREV. Sequence was analyzed using BioEdit and the amplified AtPCK coding sequence and genomic sequence were verified using BLASTX.

In order to ligate the AtPCK coding region and genomic clone into the pBNP vector shown in Figure 3a, *E. coli* colonies representing each of the two plasmids were used to inoculate 25 ml of LB broth containing 50 μ g ml⁻¹ kanamycin. The cultures were left shaking overnight at 37 °C and the plasmid DNA was purified using the QIAfilter plasmid midi kit (Qiagen Ltd). The pBNP vector was purified in an identical fashion but from 100 ml of culture containing 50 μ g ml⁻¹ kanamycin. The same sequential enzyme digest as described above was performed on all purified plasmid DNA. 3 μ g of DNA was digested for the plasmids containing the coding region and genomic clone and 1 μ g of DNA for the pBNP vector. Samples were separated by crystal violet gel electrophoresis and the products were purified using the Qiaquick Gel Extraction kit

(Qiagen Ltd). The 14.4 kb pBNP vector product was treated with alkaline phosphatase to prevent self-ligation and the coding region and genomic clone inserts were ligated into the pBNP vector using *Xba*I/*Sac*I digests. Library efficiency DH5 α *E.coli* cells were transformed with 2 μ l of the ligation reaction and positive colonies were screened by PCR using AtPCK-Xba IFOR and AtPCK-Sac IREV.

Plasmid DNA was extracted from the colonies containing the desired insert using the QIAprep Spin Miniprep kit (Qiagen Ltd) and DNA was subsequently digested with 10 units of *Xba*I, 15 units of *Sac*I and 1 x *Xba*I Buffer at 37° C for 2 h. Two separate colonies each of the coding sequence and the genomic sequence insert in the pBNP vector, which showed the correct expected restriction enzyme digest pattern, were selected and sequenced using the BNP-SAG12FWD primer (SEQ ID No.9). The sequence was analyzed using the BioEdit program. The constructs generated were named pALBNP1 (coding sequence) and pALBNP2 (genomic sequence), as shown in Figure 3b.

Five homozygous single insert lines were generated and over-expression of PCK was verified through immunoblotting as described in Example 3, using a PCK specific antibody. A polyclonal antiserum was raised in rabbit using synthetic peptides designed against the PCK amino acid sequence. The sequences were (i) DEHCWTETGVSNI EG (SEQ ID No.27), and (ii) CVDLSREKEPDIWNA (SEQ ID No.28), which were chemically synthesised and then coupled to keyhole limpet haemocyanin before being co-injected into rabbits. The results obtained using the antibody are illustrated in Figure 16, which shows that transformed plants had elevated levels of PCK protein.

SAG12-PCK-PPDK cell lines were generated by crossing single SAG12-PCK transformants (3)1 and (19)4 with a strong SAG12-PPDK over-expressing line. Analysis of the plants having elevated levels of both PCK and PPDK showed increased rosette mass, as shown in Figure 17.

- 60 -

Finally, although the inventors do not wish to be bound by hypothesis, Figure 18 shows a speculative biochemical pathway illustrating how PCK and PPDK may affect Nitrogen remobilisation.

5 PCK/PPDK double constructs

The inventors observed that, when transformed into a plant, the PCK and PPDK single constructs shown in Figure 3 resulted in increased rates of nitrogen remobilisation from senescent leaves, and also an increase in the amount of vegetative plant growth (which corresponds to an increase in crop yield), when
10 these enzymes were over-expressed in senescent leaves. The inventors therefore decided to produce two double constructs in which the genes encoding PCK and PPDK were both inserted into pBNP130000001 under the control of the SAG12 promoter.

15 The first double construct was made by ligating the gDNA encoding PCK downstream (i.e. the 3' end) of the PPDK-encoding gDNA fragment of BNP-PPDKgDNA using an *Xba*I/*Sac*I digestion. Thus, the SAG12 promoter in the plasmid was responsible for expression of both the PPDK gene and the PCK gene.

20

The second double construct was made by ligating the gDNA encoding PPDK immediately downstream of the PCK-encoding gDNA fragment of pALBNP2 using an *Avr*II/*Bam*HI digest. Again, the SAG12 was responsible for expression of both the PPDK gene and the PCK gene.

25

Claims

1. A genetic construct comprising a senescence-specific promoter operably linked to at least one coding sequence, which encodes at least one polypeptide
5 having phosphoenolpyruvate carboxykinase (PCK) activity and/or pyruvate orthophosphate dikinase (PPDK) activity.
2. A genetic construct comprising a promoter operably linked to at least one coding sequence, which encodes at least one polypeptide having
10 phosphoenolpyruvate carboxykinase (PCK) activity and pyruvate orthophosphate dikinase (PPDK) activity.
3. A genetic construct according to claim 2, wherein the promoter is a senescence-specific promoter.
15
4. A genetic construct according to any preceding claim, wherein the promoter is isolated from a senescence-associated gene in *Arabidopsis*.
5. A genetic construct according to any preceding claim, wherein the
20 promoter is selected from a group consisting of: SAG12, SAG13, SAG101, SAG21 and SAG18, or a functional variant or fragment thereof.
6. A genetic construct according to any preceding claim, wherein the promoter is a SAG12 promoter, or a functional variant or fragment thereof.
25
7. A genetic construct according to any preceding claim, wherein the promoter comprises a nucleotide sequence substantially as set out in SEQ ID No.16, or a functional variant or fragment thereof.
- 30 8. A genetic construct according to claim 1, wherein the at least one coding sequence encodes (i) a phosphoenolpyruvate carboxykinase (PCK), or a

- 62 -

functional variant or fragment thereof, and/or (ii) a pyruvate orthophosphate dikinase (PPDK), or a functional variant or fragment thereof.

9. A genetic construct according to any preceding claim, wherein the coding
5 sequence, which encodes the polypeptide having PCK activity, is derived from *Arabidopsis*.

10. A genetic construct according to any preceding claim, wherein the coding
10 sequence, which encodes the polypeptide having PPDK activity, is derived from *Arabidopsis spp.*, *Zea spp.*, *Flaveria spp.*, or *Cleome spp.*.

11. A genetic construct according to any preceding claim, wherein the coding
sequence, which encodes the polypeptide having PCK activity, comprises a
nucleic acid sequence substantially as set out in SEQ ID No:17 or SEQ ID
15 No:18, or a functional variant or fragment thereof.

12. A genetic construct according to any preceding claim, wherein the
polypeptide having PCK activity comprises an amino acid sequence substantially
as set out in SEQ ID No:19, or a functional variant or fragment thereof.

13. A genetic construct according to any preceding claim, wherein the coding
sequence, which encodes a polypeptide having PPDK activity, comprises a
nucleic acid sequence substantially as set out in SEQ ID No:20, SEQ ID No:21,
or SEQ ID No:22, or a functional variant or fragment thereof.

14. A genetic construct according to any preceding claim, wherein the
polypeptide having PPDK activity comprises an amino acid sequence
substantially as set out in SEQ ID No:23 or SEQ ID No:24, or a functional
variant or fragment thereof.

15. A genetic construct according to claim 1, wherein the construct encodes
PCK or a functional variant or fragment thereof, and not PPDK.

16. A genetic construct according to claim 1, wherein the construct encodes PPDK or a functional variant or fragment thereof, and not PCK.

5 17. A genetic construct according to any one of claims 1-14, wherein the construct encodes PCK or a functional variant or fragment thereof, and PPDK or a functional variant or fragment thereof.

10 18. A recombinant vector comprising the genetic construct according to any one of claims 1 to 17.

19. A recombinant vector substantially as illustrated in Figure 3.

15 20. A method of increasing the concentration of PCK and/or PPDK in a leaf of a test plant to above that of the corresponding concentration of PCK and/or PPDK in a wild-type plant cultured under the same conditions, the method comprising altering plant metabolism in the test plant to achieve increased levels of PCK and/or PPDK in plant leaves after the initiation of leaf senescence.

20 21. A method of decreasing the concentration of nitrogen in the leaves of a test plant to below that of the corresponding concentration of nitrogen in a wild-type plant cultured under the same conditions, the method comprising altering plant metabolism in the test plant to achieve increased levels of PCK and/or PPDK in plant leaves after the initiation of leaf senescence.

25 22. A method of increasing the growth rate of a test plant compared to the corresponding growth rate of a wild-type plant cultured under the same conditions, the method comprising altering plant metabolism in the test plant to achieve increased levels of PCK and/or PPDK in plant leaves after the initiation
30 of leaf senescence.

- 64 -

23. A method according to any one of claims 20-22, wherein the method comprises transforming a test plant cell with a genetic construct according to any one of claims 1-17, or a vector according to either claim 18 or claim 19.

5 24. A method according to any one of claims 20-23, wherein the method comprises increasing the concentration of PCK and PPDK in the plant leaves to above that of the corresponding concentration of PCK and PPDK in the wild-type plant cultured under the same conditions.

10 25. A cell comprising the genetic construct according to any one of claims 1 to 17, or the recombinant vector according to either claim 18 or claim 19.

26. A transgenic plant comprising the genetic construct according to any one of claims 1 to 17, or the recombinant vector according to either claim 18 or
15 claim 19.

27. A transgenic plant according to claim 26, wherein the plant is from the Brassicaceae family.

20 28. A transgenic plant according to claim 26, wherein the plant is from the Poales family.

29. A transgenic plant according to claim 26, wherein the plant is from the Solanaceae family.

25 30. A plant propagation product obtainable from the transgenic plant according to any one of claims 26 to 29.

31. A plant propagation product according to claim 30, wherein the plant
30 propagation product is a seed.

32. A method of producing a transgenic plant which remobilises nitrogen at a higher rate than a corresponding wild-type plant cultured under the same conditions, the method comprising the steps of:-

- i) transforming a plant cell with the genetic construct according to any one of claims 1-17, or the vector according to either claims 18 or 19; and
- ii) regenerating a plant from the transformed cell.

33. A method of producing a transgenic plant having a higher growth rate than a corresponding wild-type plant cultured under the same conditions, the method comprising the steps of:-

- i) transforming a plant cell with the genetic construct according to any one of claims 1-17, or the vector according to either claims 18 or 19; and
- ii) regenerating a plant from the transformed cell.

34. A harvested leaf containing a lower level of nitrogen than the corresponding level of nitrogen in a harvested leaf taken from a wild-type plant cultured under the same conditions, wherein the leaf is harvested from the transgenic plant according to any one of claims 26-29, or produced by the method according to either claim 32 or claim 33.

35. A smoking article comprising nitrogen-reduced tobacco obtained from a mutant tobacco plant, which mutant is capable of decreasing the concentration of nitrogen in senescent leaves.

36. A smoking article according to claim 35, wherein the smoking article is made from tobacco obtained from a mutant tobacco plant which has been transformed with the genetic construct according to any one of claims 1 to 17, or the vector according to either claim 18 or claim 19.