Towards engineering increased Pantothenate (Vitamin B_5) levels in plants

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Abstract

Pantothenate (vitamin B_5) is the precursor of the 4'-phosphopantetheine moiety of coenzyme A and acyl-carrier protein. It is made by plants and microorganisms *de novo*, but is a dietary requirement for animals. The pantothenate biosynthetic pathway is well-established in bacteria, comprising four enzymic reactions catalysed by ketopantoate hydroxymethyltransferase (KPHMT), L-aspartate-α-decarboxylase (ADC), pantothenate synthetase (PS) and ketopantoate reductase (KPR) encoded by *panB*, *panD*, *panC* and *panE* genes, respectively. In higher plants the genes encoding the first (KPHMT) and last (PS) enzymes have been identified and characterized in several plant species. Commercially, pantothenate is chemically synthesised and used in vitamin supplements, feed additives and cosmetics. Biotransformation is an attractive alternative production system that would circumvent the expensive procedures of separating racemic intermediates. We explored the possibility of manipulating pantothenate biosynthesis in plants. Transgenic Oilseed rape (*Brassica napus*) lines were generated in which the *E. coli* KPHMT and PS genes were expressed under a strong constitutive CaMV 35SS promoter. No significant changes of pantothenate levels in PS transgenic lines was observed. In contrast plants expressing KPHMT had elevated pantothenate level in leaves, flowers siliques and seed in the range of 1.5 to 2.5 fold increase compared to the wild type plant. Seeds contained the highest vitamin content indicating that they might be the ideal target for production purposes. Our results also suggest that KPHMT might be the rate limiting step in pantothenate biosynthesis, and that cells and/or tissues make pantothenate just enough for emmediate metabolic needs.

Key words

Pantothenate, genetic engineering, *E. coli* pan genes, transgenic oilseed rape

Abbreviations

α-KIVA	α-ketoisoverate
AMP	adenosine monophosphate
ADC	L-aspartate-α-decarboxylase
GUS	β-glucuronidase
KPHMT	ketopantoate hydroxymethyltransferase
KPR	ketopantoate reductase
MUG	4-methylumbelliferyl-beta-galactosidase
PS	pantothenate synthetase

Introduction

Pantothenate, also known as vitamin B_5, is a water-soluble vitamin that plays an essential role in cellular metabolism as a precursor of the 4′-phosphopantetheine moiety of coenzyme A (Choudhry et al., 2003) and acyl carrier protein (Zhang et al., 2004). These are cofactors in many energy-yielding reactions such as fatty acid and carbohydrate metabolism, and other biological acetylations (Kleinkauf, 2000). Pantothenate biosynthesis *de novo* occurs in bacteria, fungi and plants, but it is a dietary requirement for animals. The pathway of biosynthesis is well-understood in *E. coli* consisting of four enzymes (*fig 1*) (Cronan et al., 1982). All the genes have been cloned, overexpressed and protein crystal structures solved (Lobley et al., 2003). The first step is the formation of ketopantoate from α-ketoisovalerate (α-KIVA; the oxo-acid of valine) using 5,10-methylene tetrahydrofolate as a cofactor, in a reaction catalysed by ketopantoate hydroxymethyltransferase (KPHMT; EC 2.1.2.11) (Teller et al., 1976). Ketopantoate is then reduced to D-pantoate by ketopantoate reductase (KPR; EC 1.1.1.169) (Shimizu et al., 1988) using NADPH as the hydrogen donor. In the second branch of the pathway, β-alanine is produced by the α-decarboxylation of L-aspartate in a reaction catalysed by the enzyme L-aspartate-α-decarboxylase (ADC; EC 4.1.1.15) (Ramjee et al., 1997). D-pantothenate is eventually formed from the condensation of D-pantoate and β-alanine, a reaction catalysed by pantothenate synthetase (PS; EC 6.3.2.1). This condensation proceeds via a pantoyl adenylate intermediate, where pantoate reacts with ATP to give pyrophosphate and pantoyl-AMP, which subsequently reacts with β-alanine to give pantothenate and AMP (Miyatake et al., 1979).

Knowledge from the bacterial pathway has been applied to study the pathway in plants (Chakauya et al., 2006; Coxon et al., 2005), and it is found to be broadly similar. The first direct evidence came from feeding of ^{14}C-valine to pea-leaf disks, when radiolabel was found in several of the intermediates, implicating the presence of KPHMT and KPR (Jones et al, 1994). This was followed by the isolation of a *panC* cDNA encoding PS from a *Lotus japonicus* cDNA library by functional complementation of the *E. coli* mutant (Genschel et al, 1999). cDNAs for *panC* and *panB* were subsequently identified in the *Arabidopsis* and rice genomes by sequence similarity (Ottenhof et al., 2004), and subsequently in many plant EST projects. In all cases plants appear to encode two *panB* genes for KPHMT, although their high sequence similarity suggests that they arose as a duplication within the plant lineage, rather than from two separate origins (Chakauya et al., 2006). Both KPHMTs are located in the mitochondria while PS is cytosolic (Ottenhof et al., 2004). Our knowledge of the rest of the pathway is less established. Currently no KPR has been specifically identified in plants, due in part to the presence of a great many short-chain oxidoreductases encoded by the plant genome, an estimated 138 in the Arabidopsis genome (Kallberg et al., 2002). β-alanine on the other hand appears to be made by a completely different route in eukaryotes, since genes for ADC are absent from all sequenced genomes. Instead pathways from uracil degradation and from spermine have been described in yeast and mammals respectively (Walsh et al., 2001; White et al., 2001), and plants encode putative genes for both routes (reviewed in Chakauya et al., 2006)

An estimated 4,000 tonnes of pantothenate is produced annually for cosmetic, pharmaceutical industries and feed additives (Vadamme, 1992). Current production is by bulk chemical synthesis but because the L-isomer is biologically inactive, the racemic intermediates have to be separated in an expensive process that require high optical resolution. As the demand for vitamins is increasing by about 4% per annum, several alternative production routes have been proposed including enzyme conversions in microorganisms and plants (Shimizu et al., 1992). However, the tight regulation and low flux of the plant pathway might limit the use of endogenous genes since the biosynthesis seems finely tuned to cellular requirements (Genschel et al., 1999), and indeed we were unable to modify levels of pantothenate by introduction of antisense *panC* or RNAi-panB constructs into Arabidopsis, suggesting that the plants were able to compensate for reduced enzyme activity in some way (Chakauya et al., 2006). The reconstitution of the complete bacterial pathway in plants is an alternative because unlike in plants, *E. coli* excretes excess pantothenate into the environment through a relatively unregulated pathway (Jackowski and Alix, 1990). It would therefore be reasonable to predict a significant increase of pantothenate by reconstituting the bacteria pathway in plants, and possible effects on CoA and other downstream metabolites. Indeed such manipulations in bacteria produce the expected increase in pantothenate (Sahm and Eggeling, 1999; Radmacher et al., 2002). Two recent studies have provided some preliminary data on the effects of such manipulations in plants. Fouad and Rathinasabapathi (2006) reported a 3-4 fold increase in pantothenate when *E. coli* ADC was expressed

in tobacco leaves, confirming our earlier assertion that increased supply of β-alanine might elevate the levels of pantothenate *in vivo* (Chakauya et al., 2006). Recently, Jonczyk et al. (2008) showed that the *E. coli* PS gene could complement the mutant counterpart in *Arabidopsis.* However, increased PS activity in *Arabidopsis* leaves did not affect the steady state of pantothenate. They concluded that PS might be essential but not limiting for pantothenate production. The role of KPHMT in the regulation of the pantothenate biosynthetic pathway is not yet established, and so is the pantothenate level in different plant parts. In order to improve our understanding of pathway regulation and possible manipulations for increased vitamin production in plants, we expressed the *E. coli* KPHMT and PS genes in *B. napus* and analyzed several tissue parts. Our results showed marginal increase in pantothenate levels in leaves, flowers, siliques and seed of *E. coli panC* transgenic lines. However, we have obtained *panB* lines with 1.5 to 2.5 fold increase in the vitamin. We also found that plant tissues at germination had less pantothenate compared to rapidly expanding leaves.

Materials and Methods

Materials

All reagents used in the study were analytical grade and were obtained from BDH Laboratory Supplies (Poole, UK) or Fisons (Suffolk, UK) unless otherwise stated. Calcium-D-pantothenate was from Sigma Chemical Company (Poole, UK). Restriction endonucleases and *Taq* DNA polymerase were obtained from New England Biolabs (Hitchin, UK) and oligonucleotide

synthesis was performed by Invitrogen (Paisley, UK). DNase 1 and PVDF membrane were from Amersham Pharmacia Biotechnology (Buckinghamshire, UK).

General methods

General DNA manipulation and standard molecular biology procedures, unless otherwise stated, were carried out as described by Sambrook et al. (1989). PCR conditions were as recommended by the manufacturer of the polymerase enzyme. *E. coli* DH5α was the general host for cloning and plasmid propagation.

Generation of overexpression constructs for plant transformation

The *E. coli panB* and *panC* genes were expressed under a tandemly duplicated CaMV 35SS promoter (Kay et al., 1987) in pGreen0029:35SS vector generated by inserting the CaMV 35SS cassette from pJIT60 (Guerineau and Mullineaux, 1993) into the *Xho*1-*Sal*1 restriction sites of pGreen0029 (Hellens et al., 2000). To generate plasmid pGEB the *E. coli panB* gene, originally cloned from *E. coli* K12 and inserted in the *Acc*1-*Sal*1 restriction site of pUC19 to form plasmid pAL01 (von Delft et al., 2003), was digested from the plasmid with *Hind*III and *Bam*H1 and the resulting 1.1 kb fragment was ligated into the same sites of pGreen0029:35SS. The *E. coli panC* gene was originally cloned into the *Sma*1-*Eco*R1 sites of pUC19 to form pEC (von Delft et al., 2001). Plasmid pGEC was then generated by cleaving a 875 bp *Hin*dIII-*Bam*H1 fragment from pEC and ligating it into the

corresponding sites of pGreen0029:35SS. As a positive control for transformation, the *uidA* gene encoding β-glucuronidase (GUS) was expressed from the construct pGUS (Biogemma UK Ltd, unpublished).

Plant germplasm and transformation

Oilseed rape cultivar Westar was used for transformation. Seeds were washed in 70% ethanol, surface sterilised with 15% (w/v) bleach for 20 mins and then washed four times in sterile distilled water. They were then planted at a density of 20 seeds per pot on germination media and germinated at 24℃ and 16-hour light and 8-hour dark photoperiod at a light intensity of 60-80 $\mu Em^{-2}s^{-1}$. For transformation cotyledons from five-day old seedlings were excised in such a way that they included approximately 2 mm petiole at the base and immersed onto the agar plates with *Agrobacterium tumefaciens* strain pGV3101::pSoup containing either pGEB, pGEC or pGUS constructs. Regeneration and selection was as described by Moloney et al. (1989). Putative transformants were then placed into shoot elongation media and finally rooting media. As soon as the plantlets developed a small root mass they were transferred to potting mix (osmocote exact slow release fertiliser granules mixed with Standard M2 compost 750 g per 75 litres of compost) before taken to the greenhouse and grown under the same light and temperature growth conditions in as described above. Leaf samples for PCR analysis and GUS assays were collected at this stage.

GUS activity assay

Quantitative GUS analysis of transgenic plants expressing the *uidA* reporter gene encoding β-glucuronidase, was carried out by measuring fluorescence as 4-methylumbelliferyl-beta-galactoside (MUG) is converted to methylumbelliferone (MU) (Jefferson et al., 1987). Leaf tissue (50 mg) from young leaf plants was homogenised in 500 µl GUS lysis buffer (GLB) and assayed in a Perkin Elmer LS-50 fluorimeter connected to a microtiter plate reader. The assay procedure was as described by Singh et al (2002). Activity, calculated by reference to standards, was expressed as pmol (MU).mg^{-1} protein.min^{-1}.

DNA extraction and PCR analysis

Genomic DNA was extracted from approximately 200 mg of leaf tissue using a modification of the method by Dellaporta et al. (1983), and used for PCR. The standard PCR programme had 35 cycles and temperature cycling was performed on a Stratagene Robocycler using the following amplification protocol: 4 mins 95℃ 94℃ 45 sec, 45℃ 60 sec, 72° C 2½ mins and 4 mins final extension time.

To analyse GUS reporter plants PCR analysis was run first with NPTII primers 5′-CACGACGGGCGTTCCTTGC-3′ and 5′-GGTGGTCGAATGGGCAGGTAGC-3′, and then the GUS primers 5′-TGCTGTCGGCTTTAACCTCT-3′ and 5′-GGCACAGCACATCAAAGAGA-3′ for sense and antisense, respectively. Analysis of the *E. coli panB* and *panC* transgenic plants was carried out in two stages. Firstly, the vector specific

primers, 5′-TGACGCACAATCCCACTATCCTTC-3′ and 5′-CTATGGAAAAACGCCAGCAACGC-3′ annealing in the CaMV 35SS promoter and CaMV terminator were used. This was followed by a pair of CaMV35SS promoter primer 5′-TGACGCACAATCCCACTATCCTTC-3′ and the reverse primer 5′-CCCAGCAGCTTCTAAGGCTA-3′ for the *panB* gene or 5′-TTTCATTCAGTTCTTGCCCC-3′ for *E. coli panC*. Amplified products were separated on 1.5% agarose gel stained with ethidium bromide.

RT-PCR

Total RNA was extracted from leaf tissues as described by Vorwoerd et al. (1989), and all samples were treated with DNase 1 to eliminate DNA contamination. In each case the RT reactions were performed according to the manufacturer directions for Superscript II Kit (Invitrogen, UK). For *panB* the primers 5′-GATGGCCATATGAAGCTGGT-3′ and 5′-CCCAGCAGCTTCTAAGGCTA-3′ were used to amplify a 550 bp fragment with an *Eco*RV restriction site. For *panC* the primers 5′-GATGGCCATATGAAGCTAGC-3′ and 5′-CGGAAGCCTTTTTCATTCAG-3′ were used. vitaminThe PCR reactions were performed using the following thermal profile: 4 mins at 94°C followed by 28 cycles of 45 sec 94°C, 45 sec 55°C and 2.5 min 72°C. The final extension for the PCR product was at 72°C for 5 min.

Western blot analysis

Soluble plant protein was extracted from fresh plant material or tissue from the -80℃ freezer. Approximately 100 mg of leaf tissue w as homogenised in 300 µl of Tricine buffer pH 8 in a microfuge tube, followed by centrifugation, and protein quantified as described by Bradford (1976). Proteins were separated by SDS-PAGE (Laemmli, 1970), and then transferred to PVDF membrane. Membranes were probed with rabbit made antibodies raised against *E. coli* KPHMT (Jones et al., 1993). Immunoreactive proteins were detected with a secondary antibody, goat anti-rabbit IgG (horseradish peroxidase; Sigma).

Extraction and assay of pantothenate from plant tissues

The pantothenate extraction method was adapted from Rychlik (2000). Plant tissues (0.03 g for seed, 0.1g for all other tissues) were ground in liquid nitrogen and extracted with 1.2 ml sodium acetate buffer (0.02 M; pH 6.8) at room temperature for 10 mins. One ml chloroform was added, vortexed, and then incubated for 10 mins at 75°C. Thereafter it was centrifuged at 5500 rpm for 10 min, 4°C , and the supernatant was filtered into 1.5 ml eppendorf tube with 0.2 um syringe filter, and stored frozen at -80°C until required. Free pantothenate was determined using a microbiological assay described by Wyse et al. (1985) with minor modifications. An overnight culture of *E. coli panC* mutant AT1371 (Cronan et al., 1982) was washed three times in 1xGB1 buffer [0.1 M KH_2PO_4, 0.15 M $(NH_4)_2SO_4$; pH 7.0] and re-suspended in GB1 minimum media containing 1xGB1 buffer and nutrient solution [0.4% (w/v) D-glucose, 0.025% $MgSO_4.7H_2O$, 0.25 ng/ml $FeSO_4.7H_2O$ and 5 ng/ml Thiamine] and supplemented with 1.35% (w/v in 0.1 N HCl) Adenine, 2.53%

(w/v) L-arginine, 0.31% (w/v) L-histidine and 4.6% (w/v) L-proline. All assays were carried out in equal volumes of diluted plant sample and bacteria culture in 200 µl-volume 96-well microtiter plates in GB1 minimal medium. The cells were incubated at 37°C with shaking (180 rpm) for 16 hours and growth was measured as the turbidity at 605 nm using a plate reader (Anthos Labtech instruments) connected to a computer. Standard curves were constructed using known amounts of calcium pantothenate in GB1 (0-30 ng/l), and the appropriate dilutions of experimental samples that gave values in the linear range were used to determine the pantothenate concentration in the plant material. Mean measurements were tested for significant differences by t-test ($p \leq 0.05$) or ANOVA using MINITAB software.

Germination assay

Samples of thirty seeds replicated twice, were sown on ½ MS media and percentage germination calculated after 24 and 48 hours. In this case, the emergence of the radical marked the end of germination and beginning of plant establishment.

Fatty acid analysis

Fatty acid compositions of whole tissues were determined by gas chromatography as previously described by Wilmer et al. (1996).

Results

Expression of E. coli panB and panC genes in oilseed rape

We explored the possibility of utilizing prokaryotic genes to manipulate pantothenate biosynthesis in plants. The *E. coli* genes *panB* and *panC*, were cloned under the control of the CaMV35SS promoter and transformed into oilseed rape. The *uidA* gene encoding GUS was used as an internal control for testing our transformation system and to investigate the effectiveness of the CaMV35SS-pA35S promoter-terminator combination (Jefferson et al., 1987; Sweetman et al., 2002) in driving transgene expression under the experimental conditions. For the GUS lines, five out of nine were PCR positive for both the GUS and NPTII genes. We then assayed for GUS activity in the leaves of the transgenic plants. GUS activity ranging 0.8 to 1.6 $pmol.min^{-1}.mg^{-1}$ protein was observed in the five plants (*fig 2)* that had been shown by PCR analysis to contain the insert. Three lines had 0.05 $pmol.min^{-1}.mg^{-1}$ protein which is the same as GUS-like activity in the wild type control. These results demonstrated that the CaMV35SS-pA35S cassette was effective in driving transgene expression in oilseed rape using the NPTII as the selection marker.

We then analysed the plants transformed with the *panB* and *panC* constructs by PCR. Out of the 12 *panB* kanamycin resistant plants six were PCR positive for the transgene but only 3 independent lines (B3, B4 and B5) showed the expected 1 kb fragment in both T0 and T1 generations while no amplification product was observed in the non-transgenic control as expected (*fig 3b*). These lines were selected for further analysis. For the plants transformed with *E. coli panC,* five plants were PCR positive at T0 generation and three at the T1 generation (C3, C4 and C7; *fig 3f*) and these were analysed further. In order to determine the segregation pattern of the

transgenes, progeny from the self-pollinated primary transformants were planted and the offsprings analysed by PCR. Lines B3, C3 and C7 showed normal Mendelian inheritance of single insert (3:1), while B4, B5 and C4 showed a 2:1 ratio. The unusual ratios may be due to the fact that *Brassica napus* is an amphidiploid with an unpredictable segregation pattern.

As shown in *fig 3c*, RT-PCR analysis revealed that all three *E. coli panB* transgenic lines had detectable mRNA transcript levels, appearing as a single 550 bp band with the same size as the positive control. Similarly, the *E. coli panC* plants had detectable mRNA transcript in all the lines analysed (C3, C4 and C7 (*fig 3g*). The antibodies we had against Lotus PS were tested for their ability to detect *E. coli* PS, but although they cross-reacted with purified recombinant enzyme there was no reproducible cross-reaction with the transgenic plants (data not shown). However, antibodies against *E. coli* KPHMT were available (Jones et al., 1993), and these were used to screen the *panB* transgenic plants by immunoblotting. Plant soluble protein (100 µg) was fractionated on SDS-PAGE, blotted onto PVDF membrane and challenged with anti-KPHMT antiserum (Jones et al., 1993). A 28 kDa protein band was detected in the three transgenic lines analysed (*fig 3d*), which corresponded to the KPHMT band in the extract of *E. coli* overexpressing *panB*::pAL01 plasmid (von Delft et al., 2003). This band was absent in the non-transgenic plant. Furthermore, no cross-reaction was observed when an identical membrane was probed with pre-immune antisera (data not shown) indicating that the antibodies were specific to the recombinant *E. coli* KPHMT and that the *panB* gene was successfully translated in the transgenic plants.

Pantothenate level and plant phenotype

To assess the effect of the transgene on plant phenotype, we measured extractable pantothenate, seed viability and oil profiles in the different transgenic lines. Since pantothenate levels in oilseed rape had not been measured before, we initially assayed the leaves, flowers, siliques and seed of the control plants to establish the levels in different tissues. As shown in *fig 4*, leaves and siliques had similar vitamin contents (10.5 ± 0.8 nmol.g^{-1} FW), while the flowers and seeds had on average 15 and 40 nmol.g^{-1} FW respectively. This compares well with 29.6 nmol.g^{-1} FW reported for cauliflower (USDA nutrient database Release 18). A similar pattern in vitamin distribution in different tissues is observed with the transgenic lines whichever the transgene used (*fig 4*). For the plants expressing *E. coli panB*, a comparison of the control against the transgenic populations showed that B3 and B4 had significantly more pantothenate in all tissues measured, while B5 had significantly increased pantothenate in the seed. In contrast, there was no significant difference (P>0.05) in the vitamin levels of the *panC* lines and wild type in all tissues measured, with the exception of C7, which had an extremely high pantothenate level in the seed (120 ± 38 nmol.g^{-1} FW). When we measured the seed viability, line C7 had a significantly low germination percentage of 41.6 ± 3.3 % (*Table 1*) compared to other transgenic lines and wild type populations. Interestingly, C7 also showed an abnormal oil profile with very low total oil (*fig 5*) consisting of high C16:0, C18:0 and low, C18:1 and C18:3 (*Table 2)*. Whether this phenotype was a transgenic or tissue culture effect (Larkin and Scowcroft, 1981) is not clear, but it seems the low

germination percentage was a result of the high saturated fatty acids (Murphy, 2006).

Pantothenate levels during seedling establishment.

After establishing that seeds contained the highest pantothenate levels in the plant, we investigated in more detail what happens to pantothenate during early seedling germination and establishment. Extractable pantothenate in seedlings of wild type control, B3, B4, C3 and C4 was normalized by either fresh weight or unit tissues. On a fresh weight basis, pantothenate levels in the control plants was highest in dry seeds decreasing significantly by about 60% at 2 days after planting (dap; *fig 6*) and then becoming constant until 6 dap. The same pattern was observed with both *panB* and *panC* transgenic lines. However, pantothenate level in B3 and B4 was higher than the control in the first two days after planting. The change in pantothenate per day was higher during seed germination (first two days) and slowed during leaf emergence. When the vitamin level is expressed per unit tissue it increases linearly within the first two days and then exponentially thereafter, and this was observed for both transgenic and non-transgenic lines. Most significantly, a positive correlation between pantothenate per tissue and fresh weight ($y=0.0029x+0.0498$; $R^2=0.99$) was observed for all the plant populations. When put together, these results show that plant tissues had less pantothenate at germination compared to rapidly expanding leaves.

Discussion

There is a growing interest in finding alternative methods to produce pantothenate to complement the current chemical synthesis (Sahm and Eggelling, 1999), and plants offer an attractive possibility. Although an earlier study by Jonczyk et al. (2008) showed no change in pantothenate level as a result of overexpressing PS in the leaves of Arabidopsis, little is known about the effect on such manipulations on other parts of the plant such as seed, siliques and flowers. We utilised our knowledge of the plant and bacterial pantothenate and ectopically expressed the *E. coli panB* and *panC* pantothenate biosynthesis genes in oilseed rape. We managed to detect the mRNA transcript of the two transgenes in their respective transgenic plants while recombinant KPHMT protein was detectable in *panB* lines. We then measured pantothenate level in these plants. We then measured pantothenate levels in different tissues. The trend in pantothenate level in mature tissues of wild type plant was, seeds>flowers>leaves>siliques (42.6, 16.1, 10.5 and 8.71 nmol.g^{-1} FW; *fig 4*). There is no obvious explanation for this trend simply because pantothenate biosynthesis seem be developmentally regulated. The photosynthetic state of the green siliques might explain the similar level of pantothenate to leaves of oilseed rape and cabbage (6.4 nmol.g^{-1} FW; USDA national nutrient database Release 6). Brassicaceae in the form of cauliflower florets (29.6 nmol.g^{-1} FW) and broccoli florets (24.2 nmol.g^{-1} FW) are the major sources of dietary vitamin B_5. It is therefore not surprising that pantothenate levels for flowers. It is plausible that free pantothenate is the most abundant form in seed compared to other forms. This is supported by the fact that sunflower kernels have the highest

pantothenate level measured to date in plants (301 nmol.g^{-1} FW, USDA national nutrient database Release 6). Moreover, we have observed an upregulation of the endogenous *panB* and *panC* genes in the flowers of Arabidopsis plants compared to leaves (Coxon and Smith, unpublished observations). Transgenic plants expressing *E. coli panC* gene had marginal increases in the vitamin levels suggesting that PS activity might not be limiting production of the vitamin, which agrees with Jonczyk et al. (2008) observations with Arabidopsis overexpressing *E. coli* PS. The poor germination of line C7 could be related to the elevated saturated stearic acid which reduces membrane fluidity and thus impairment of function (Murphy, 2006; Millar et al., 2000) This phenomenon has been observed with transgenic oilseed rape produced for use in the manufacture of edible spreads.

In comparison the *E. coli* KPHMT lines B3 and B4 showed consistently high pantothenate levels in all tissues (1.5 to 2-fold) compared to the wild type control (*fig 4*). There are two interlinked possible explanations for the resultant increase of the vitamin in *panB* transgenic lines. Firstly, overexpression of the unregulated *E. coli* KPHMT might have increased the flux of α-KIVA away from valine and isoleucine into pantothenate biosynthesis pathway. This is supported studies in *Corynebacterium glutamicum* by Chassagnole et al. (2003) which suggested that metabolic engineering of pantothenate biosynthetic pathway could begin with increasing flux of α-KIVA to pantothenate possibly by upregulating *panB*. Secondly, plant KPHMTs are localised in the mitochondria while PS is cytosolic (Ottenhof et al., 2004). This

subcellular localisation of the pathway probably may well be important for the tight control of pantothenate biosynthesis in plants. Expressing the unregulated *E. coli* KPHMT in the cytosol may have circumvented any regulatory controls thereby increasing carbon flux into pantothenate biosynthesis. Of course in order for this to occur, activity of the next enzyme, ketopantoate reductase must be present in the cytosol. Currently, there is no information about this enzyme in any plant except that it might be a non-specific reductases. It is therefore reasonable to infer that the increased pantothenate was because of expression of *E. coli panB*.

We also measured the changes in pantothenate levels during early seedling establishment. Extractable pantothenate increased with an increase in FW. The linear increase during the first two days coincided with normal physiological germination suggesting a high turnover rate between synthesis and utilisation resulting in a small pool of free pantothenate. An alternative explanation is that there is rapid remobilisation of pantothenate from bound sources to cater for the high rate of metabolism during germination meaning little or no embryonic synthesis of pantothenate. However, observations from microarray work show upregulation of *panB* and *panC* genes in embryos of *Arabidopsis* (Zimmermann et al., 2004) supporting the idea of high turnover. The exponential increase in rapidly expanding leaves may only be a result of synthesis. The changes in pantothenate observed here seem to follow the same pattern with fatty acid and acetyl-CoA levels (Elborough et al., 1994) suggesting that individual cells make enough pantothenate for their specific needs with little or no requirement for export to compensate for deficits

elsewhere in the plant (Jonczyk et al., 2008). We are currently assaying the pantothenate level throughout the life cycle of the plant to investigate this further.

The implications of the current study are two-fold. Firstly, we provide direct evidence that *panB* might be a regulatory step in the biosynthetic pathway. As such manipulations for increased vitamin levels might have to target α-KIVA and β-alanine supply. Secondly, seeds might be the most ideal target for increasing vitamin level from a production perspective. There are several advantages of seed specific expression of pharmaceuticals including stability and presence of well-characterised seed specific promoters. Moreover, there are documented success stories of vitamin overproduction in seeds including provitamin A (Ye et al., 2000) and vitamin E (Savidge et al., 2002). Interestingly, the two-fold increase in pantothenate in transgenic seeds overexpressing *panB* did not affect seed viability or show any obvious phenotypic abnormalities under normal growth conditions.

In conclusion, we have demonstrated that bacterial genes might be the best option to engineer the plant pathway, especially by increasing carbon flux into the pantothenate biosynthesis pathway. We managed to double pantothenate levels in seeds of oilseed rape by expressing *E. coli* KPHMT and there is active pantothanate biosynthesis during early seedling establishment of plants.

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Table Legends

Table 1. Seed viability of transgenic oilseed rape lines expressing *E. coli panB* and *panC* genes measured as seed weight and seed germination. The errors represent standard error of the mean (n=3).

Table 2. Fatty acid composition of TAG (mol % per 20 seeds) in transgenic oilseed rape lines.

686 **Figure Legends**

687 Fig. 1 Pantothenate biosynthesis pathway in *E. coli*.

688 Four enzymes are involved in the synthesis of D-pantothenate from α-ketoisovalerate
689 (α-KIVA) and L-aspartate, that is, ketopantoate hydroxymethyltransferase (KPHMT),
690 ketopantoate reductase (KPR), L-aspartate-α-decarboxylase (ADC) and
691 pantothenate synthetase (PS). Enzyme names are given in red with the
692 corresponding genes in blue. The enzyme structures are KPHMT, KPR, ADC
693 and PS and encoded by the genes *panB*, *panE*, *panD* and *panC* respectively.

694

695 Fig. 2 GUS activity in transgenes in leaves of transgenic oilseed rape. WT-
696 wild type plant. Transgenic lines numbered represent different transformation
697 events. Error bars show the standard error of the mean (n=3).

698

699 Fig. 3 Expression analysis of oilseed rape transgenic lines transformed with *E.*
700 *coli panB* (a-d) and *panC* (e-g) genes. (a) Schematic diagram of the *panB*
701 construct, (b) PCR analysis of T1 *panB* lines (c) RT-PCR analysis of
702 transgenic lines with RNA from leaves of T1 plants, and (d) Western blot
703 analysis for recombinant KPHMT. (e and f) Schematic diagram of *panC*
704 construct and PCR analysis, and (g) RT-PCR of *panC* transgenic lines. pGEB
705 = *panB* plasmid DNA, pGEC = *panC* plasmid DNA, KPHMT = extract from *E.*
706 *coli* transformed with plasmid *panB*::pAL01, WT = non-transgenic control.

707

708 Fig 4 Extractable pantothenate in mature leaves, flowers, siliques and seeds
709 of transgenic oilseed rape transformed with *E. coli panB* (B3-B5) or *panC*
710 constructs (C3-C7). Error bars represent standard error of the mean (n≥20).

711

Fig 5 Total TGA in oilseed rape (T1 seed) transformed with the *E. coli panB* and *panC* genes. The statistics is the mean of 20 seeds expressed as percentage of the seed weight.

Fig 6 Changes in extractable pantothenate during early seedling establishment. Pantothenate was measured using microbiological assay and normalised by either (A) fresh weight or unit seedling (or seed) (B). Error bars represent standard error of the mean (n=3).

Table 1

Transgenic line	Seed weight (g/1000 seeds)	% Germination (n=3)
WT	4.5	95.8 ± 0.9
B3	4.5	100
B4	2.8	100
B5	3.7	89 ± 6.2
C3	5.1	85
C4	5.1	100
C7	4.1	41.6 ± 3.3

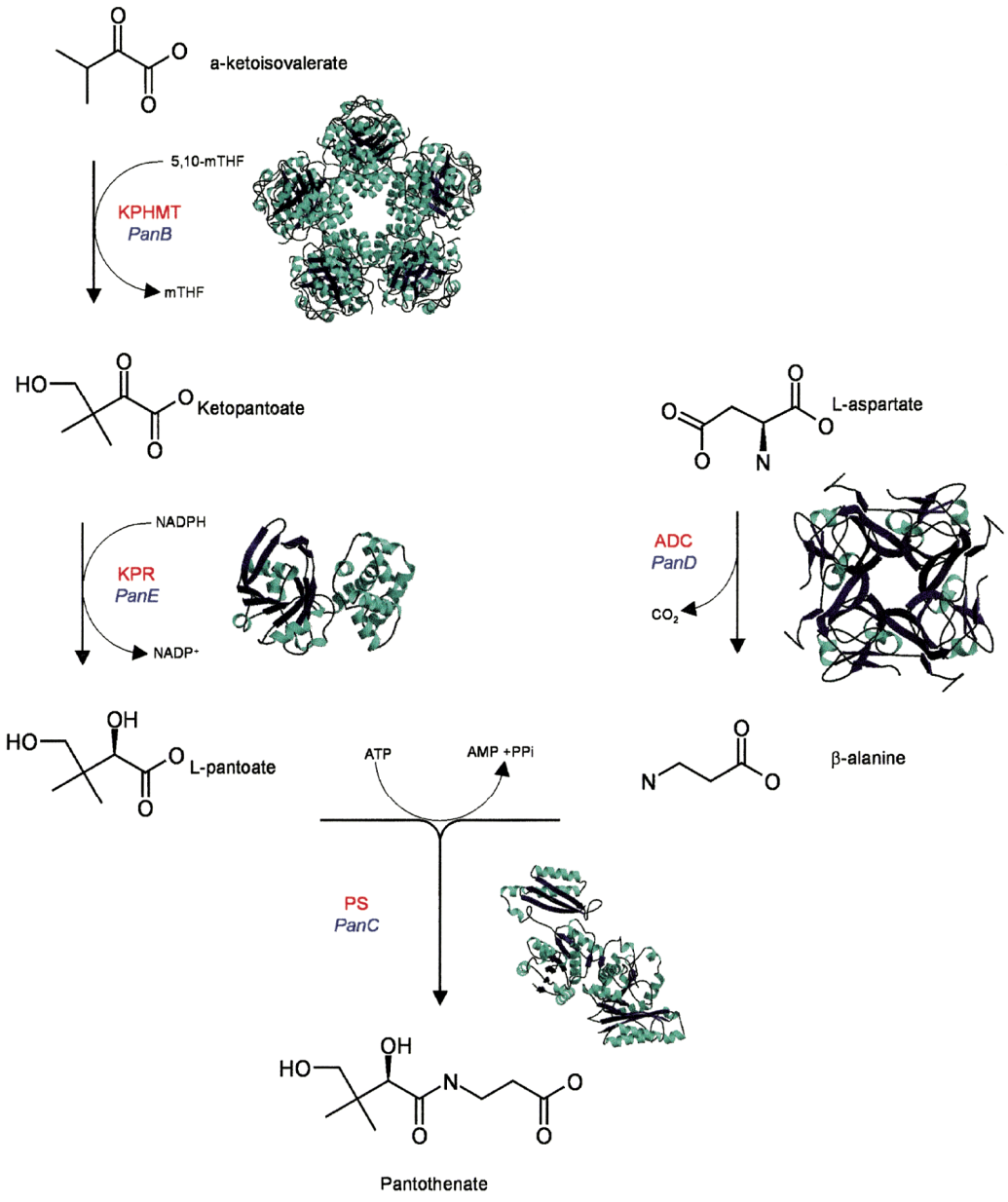
Table 2

Sample	Mol% fatty acids					
	C16:0	C18:0	C18:1	C18:2	C18:3	C20:1
WT	6.3	2.6	64.3	16.3	9.1	1.4
B3	6.9	5.5	59.6	15.3	10.6	2.0
B4	7.5	5.1	66.9	14.4	5.1	1.1
B5	8.0	5.4	64.5	11.5	8.9	1.6
C3	6.8	4.6	66.1	15.6	6.2	0.8
C4	6.5	3.3	67.7	16.0	6.3	0.2
C7	11.1	9.6	64.4	9.1	3.3	2.6

741 Fig 1

742

743



744

745

Fig 2

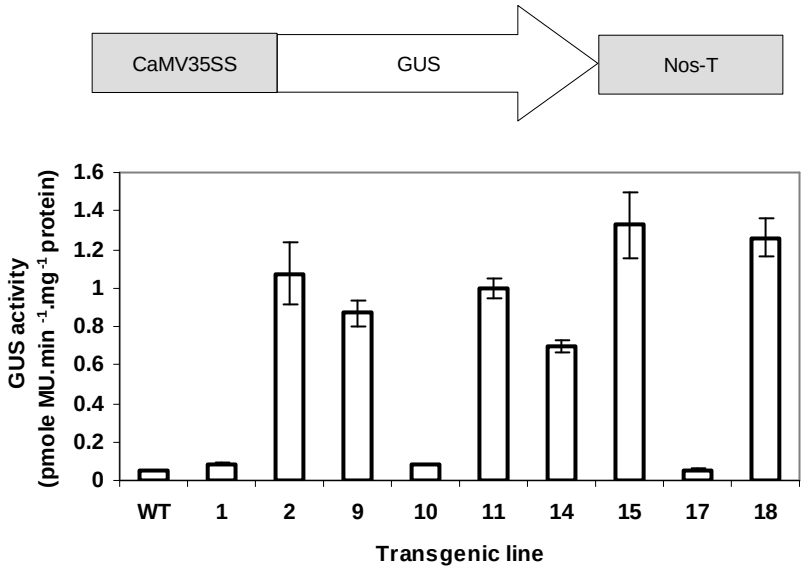

CaMV35SS
GUS
Nos-T
GUS activity
(pmole MU.min -1.mg-1 protein)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
1
2
9
10
11
14
15
17
18
Transgenic line

Fig 3

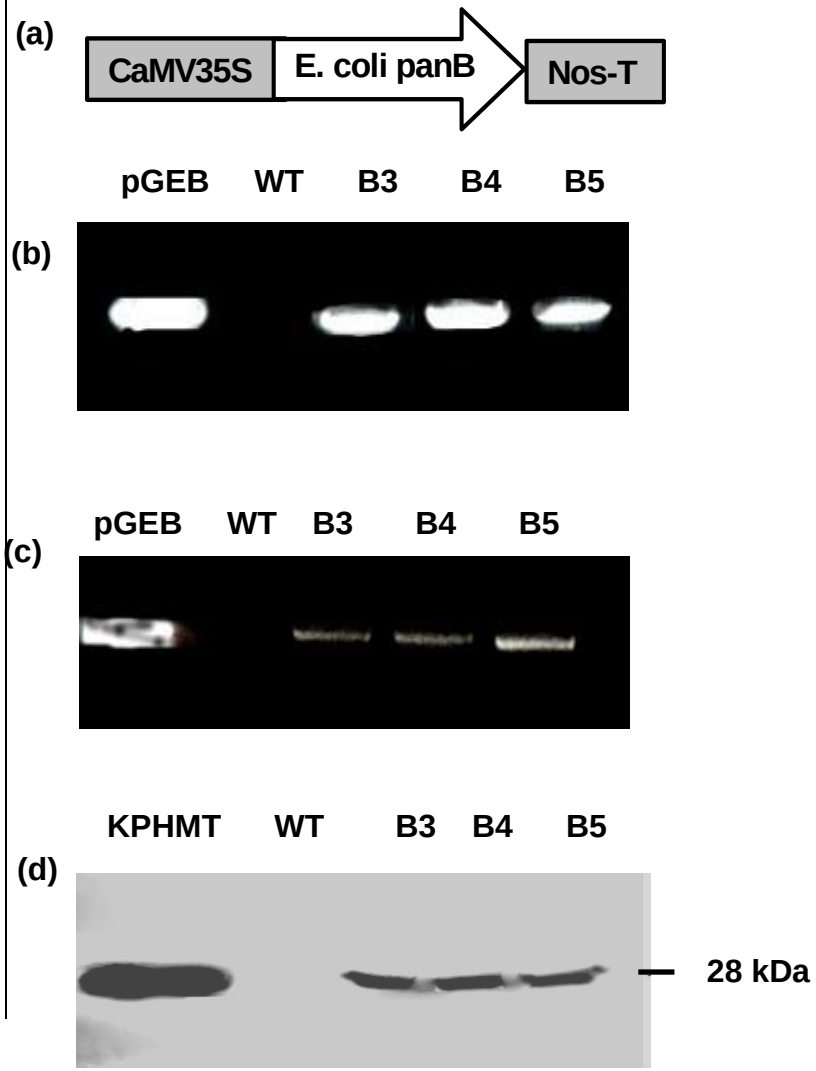
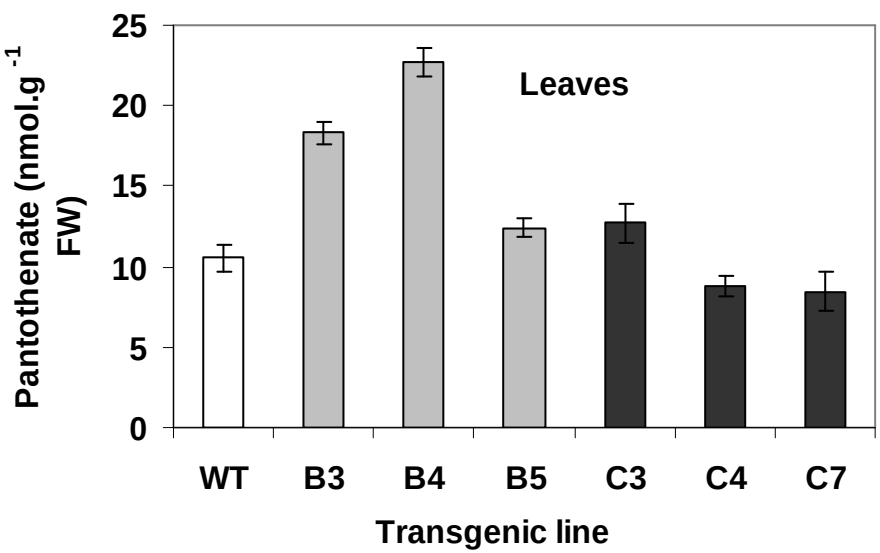
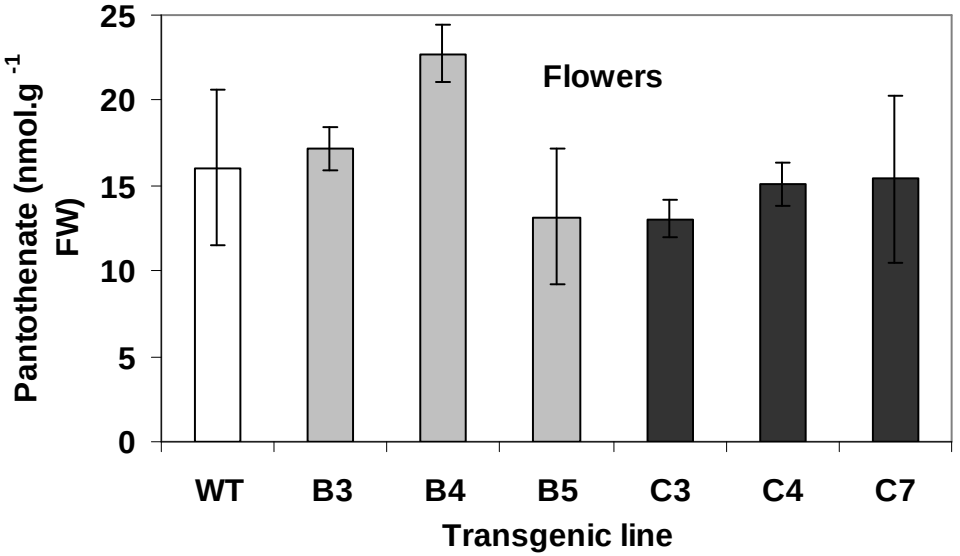
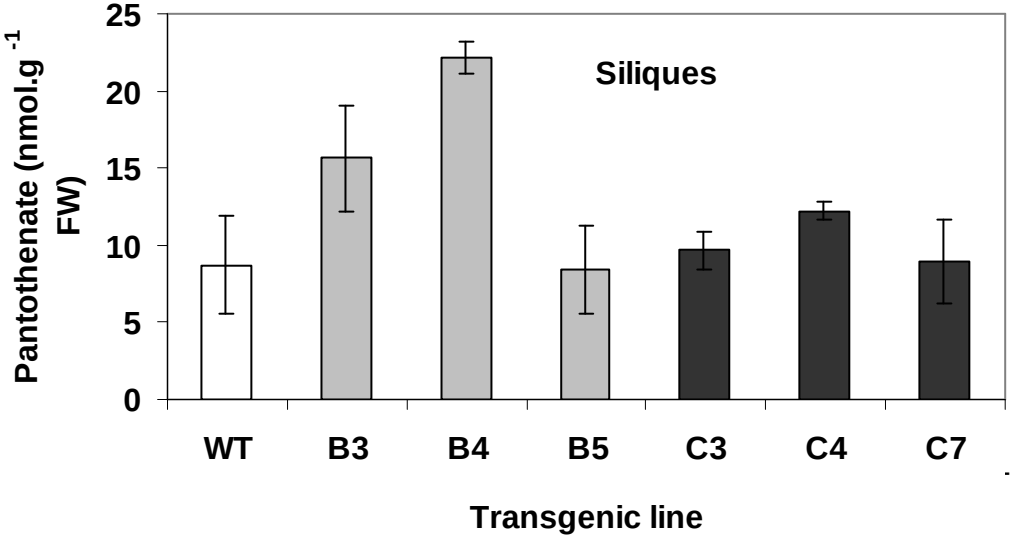

(a)
CaMV35S
E. coli panB
Nos-T
pGEB WT B3 B4 B5
(b)
(c)
pGEB WT B3 B4 B5
KPHMT WT B3 B4 B5
(d)
28 kDa
(e)
CaMV35S
E. coli panC
Nos-T
pGEC WT C3 C4 C7
(f)
pGEC WT C3 C4 C7
(g)

Fig 4


Leaves
Pantothenate (nmol.g -1 FW)
25
20
15
10
5
0
WT
B3
B4
B5
C3
C4
C7
Transgenic line


Flowers
Pantothenate (nmol.g -1 FW)
25
20
15
10
5
0
WT
B3
B4
B5
C3
C4
C7
Transgenic line


Siliques
Pantothenate (nmol.g -1 FW)
25
20
15
10
5
0
WT
B3
B4
B5
C3
C4
C7
Transgenic line

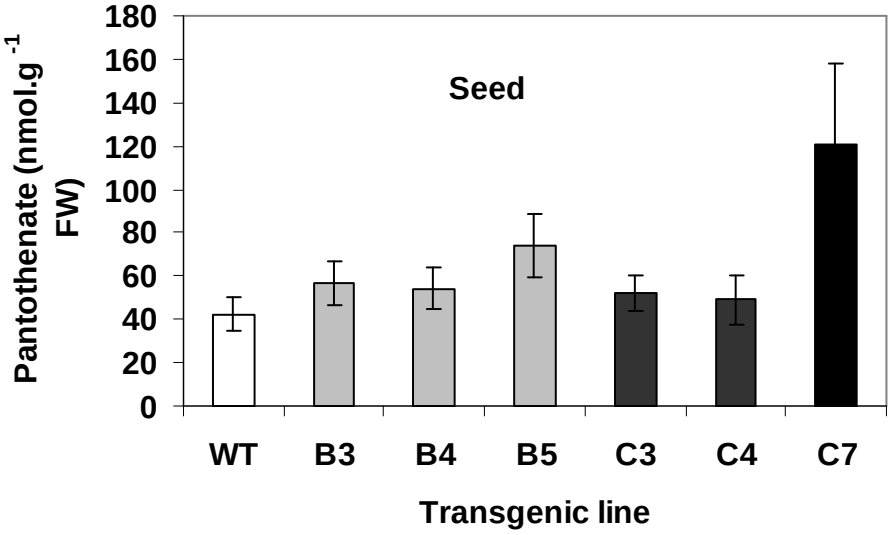

Seed
Pantothenate (nmol.g -1 FW)
180
160
140
120
100
80
60
40
20
0
WT
B3
B4
B5
C3
C4
C7
Transgenic line

Fig 5

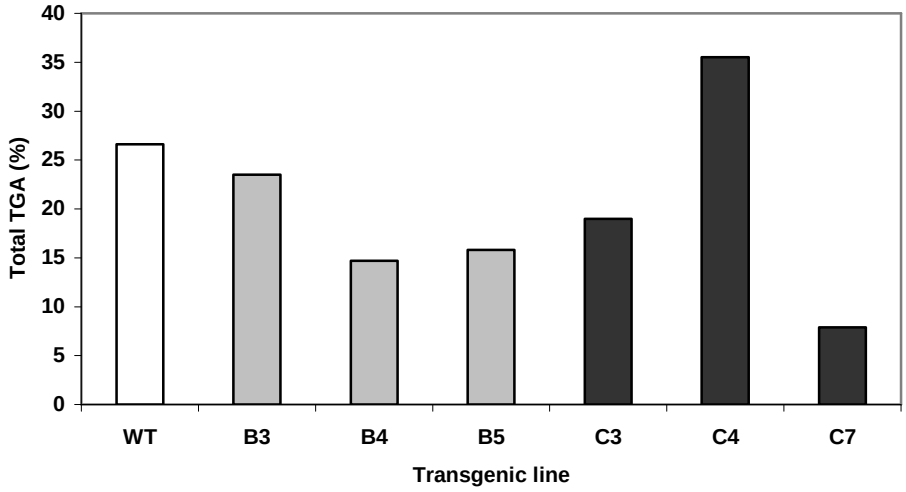
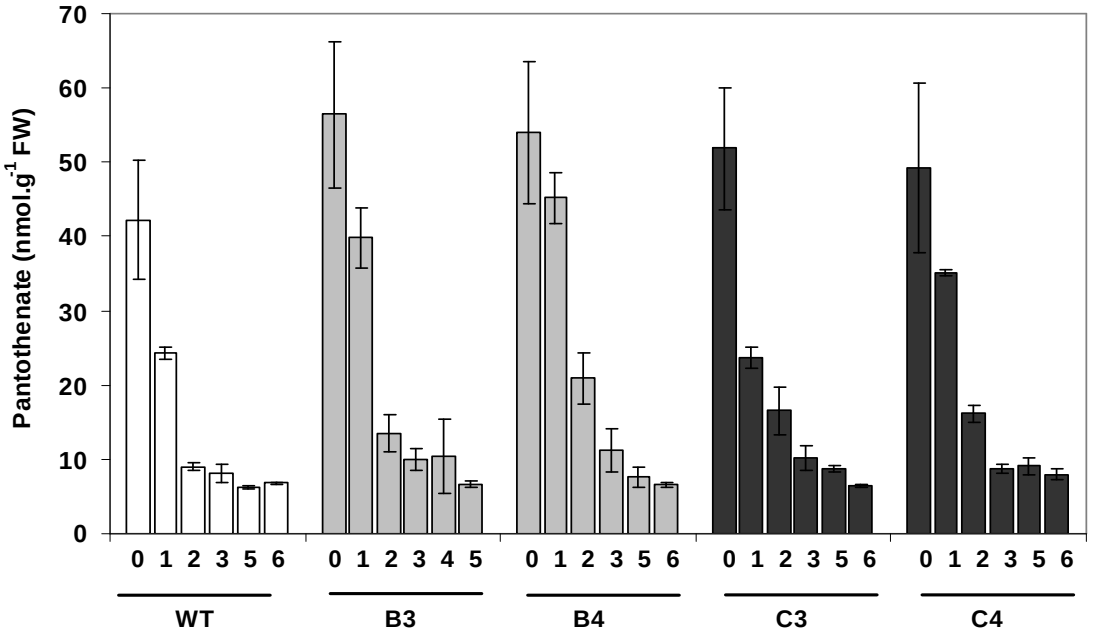
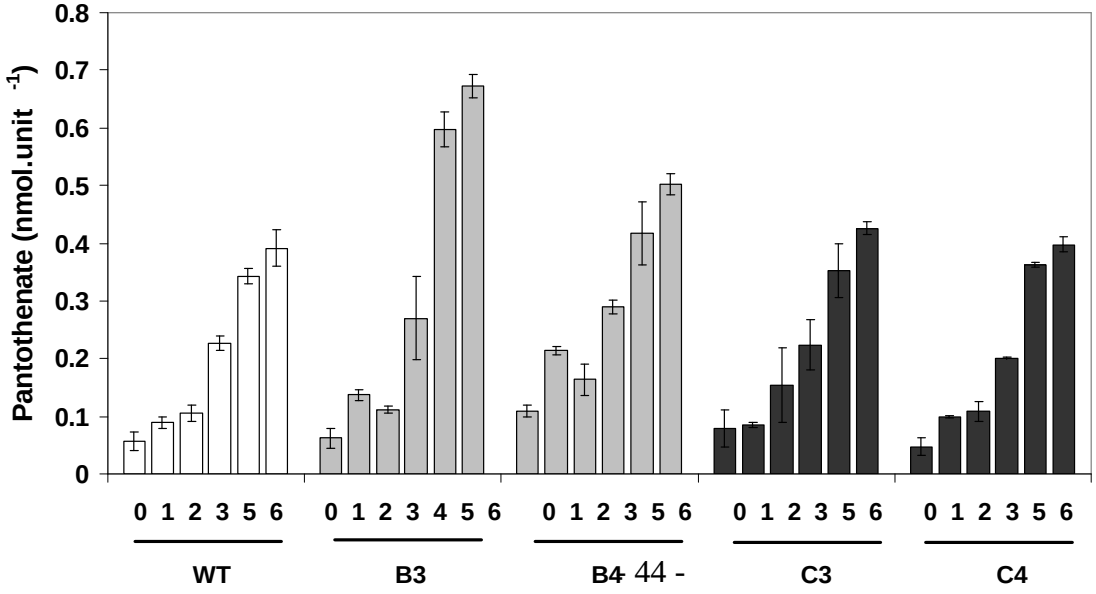

40
35
30
25
20
15
10
5
0
Total TGA (%)
WT
B3
B4
B5
C3
C4
C7
Transgenic line

Fig 6

(A)


Pantothenate (nmol.g-1 FW)
70
60
50
40
30
20
10
0
0 1 2 3 5 6
0 1 2 3 4 5
0 1 2 3 5 6
0 1 2 3 5 6
0 1 2 3 5 6
WT
B3
B4
C3
C4

(B)


Pantothenate (nmol.unit -1)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 5 6
0 1 2 3 4 5 6
0 1 2 3 5 6
0 1 2 3 5 6
0 1 2 3 5 6
WT
B3
B4
C3
C4