Concurrent overexpression of amino acid permease *AAP1*(*3a*) and *SUT1* sucrose transporter in pea resulted in increased seed number and changed cytokinin and protein levels

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Abstract. Using pea as our model crop, we sought to understand the regulatory control over the import of sugars and amino acids into the developing seeds and its importance for seed yield and quality. Transgenic peas simultaneously overexpressing a sucrose transporter and an amino acid transporter were developed. Pod walls, seed coats, and cotyledons were analysed separately, as well as leaves subtending developing pods. Sucrose, starch, protein, free amino acids, and endogenous cytokinins were measured during development. Temporal gene expression analyses (RT-qPCR) of amino acid (*AAP*), sucrose (*SUT*), and *SWEET* transporter family members, and those from cell wall invertase, cytokinin biosynthetic (*IPT*) and degradation (*CKX*) gene families indicated a strong effect of the transgenes on gene expression. In seed coats of the double transgenics, increased content and prolonged presence of cytokinin was particularly noticeable. The transgenes effectively promoted transition of young sink leaves into source leaves. We suggest the increased flux of sucrose and amino acids from source to sink, along with increased interaction between cytokinin and cell wall invertase in developing seed coats led to enhanced sink activity, resulting in higher cotyledon sucrose at process pea harvest, and increased seed number and protein content at maturity.

Keywords: *Pisum sativum*, sucrose, legume, AAP, SUT, SWEET, cell wall invertase, IPT, CKX, transgenic, process peas, transgenics, seed coat, seed yield.

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Introduction

Devising strategies to meet the need for increased plant productivity in a world with increasing population and decreasing land availability for growing crops is a recognised challenge. Seeds provide a large proportion of human food and animal feed. Cereals provide bulk calories, but pulse crops including pea (*Pisum sativum* L.) have greater potential to contribute to protein security and, through their nitrogen fixing capabilities, to sustainable agriculture (Foyer *et al.* 2016; Considine *et al.* 2017). Breeding objectives for pea depend on the end use of the crop. 'Field peas' are harvested at maturity and the dry seeds are used primarily for production of pea protein powder and as animal feed. For 'process peas', seeds are harvested while immature, during a short window when sucrose content is high and starch concentration is still low. They are consumed fresh or frozen and as part of frozen vegetable mixtures.

Ensuring nutrient transport to seeds and plant metabolic processes are operating at optimal capacities is an essential approach to the challenge of enhancing seed yield and maintaining seed quality. Sugars and amino acids, the primary products of carbon (C) and nitrogen (N) metabolism, are

transported through the phloem from sites of synthesis (sources) to sites of utilisation (sinks) (Yadav *et al*. 2015). Plants acquire inorganic N from the soil through their roots or, as in legumes, additionally from the atmosphere through nitrogen-fixing *Rhizobia* in root nodules. Generally, amino acids are made that are either used in the roots or transported to photosynthetically active leaves that assimilate C (Tegeder 2014; Gojon 2017). Sucrose and amino acids are finally exported from the leaves and transported in the phloem to a variety of sink tissues (e.g. fruits and seeds).

Transporters that facilitate source to sink movement of amino acids include the amino acid permeases (*AAP*) (Sanders *et al*. 2009; Tegeder 2012). Physiological and molecular studies in *Arabidopsis thaliana* (L.) Heynh. have indicated that gene family members (GFMs) in Cluster 3A are predominantly involved in xylem-to-phloem transfer (Zhang *et al*. 2010). Those in Cluster 4B including *AtAAP1*, are necessary for phloem loading and/or seed development (Sanders *et al*. 2009; Santiago and Tegeder 2016), whereas the function of the GFMs in Cluster 1 has yet to be determined. Pea has multiple copies of *AAP*s, which are distributed in three gene clusters (1, 3A, and 4B) (Dhandapani *et al*. 2017), with expression throughout the plant (Tegeder *et al*. 2007; Jameson *et al*. 2016; Ninan *et al*. 2017, 2019).

Transporters involved in sucrose movement include the SUT/ SUC transporters (sucrose transporters) and the SWEET (sugar will eventually be exported) efflux carriers. *SUT/SUC* transporters belong to a small gene family with distinctive physiological roles associated with either loading sucrose into the phloem, or exporting from the vacuole into the cytoplasm (Ayre 2011; Jian *et al*. 2016). In pea, four *PsSUTs* are expressed in germinating seeds (Jameson *et al*. 2016), during early pod and seed development (Ninan *et al*. 2017), and in leaves (Ninan *et al*. 2019).

The *SWEETs* are a large gene family, with different members transporting hexoses (Clade I and II), sucrose (Clade III) and glucose and fructose (Clade IV) (Eom *et al*. 2015). Pea has multiple copies of the *SWEET* GFMs (Dhandapani *et al*. 2017; Ninan *et al*. 2019).

Cell wall invertases (CWINV) convert sucrose into fructose and glucose and, as such, are an integral component of the movement of sucrose between sources and sinks (Ruan *et al*. 2010; Yu *et al*. 2015). They play a key role in the early stages of seed development by creating a high sugar environment (Weber *et al*. 2005). In embryos, Wang and Ruan (2013) suggested the transition from cell division and expansion to storage activities is usually associated with a decrease in CWINV expression. Cross-talk between cytokinin and CWINV is suggested to enhance phloem unloading and sugar import into endosperm (Rijavec *et al*. 2009).

Cytokinins are implicated in regulation of cell division and establishment of sink activity in fruits and seeds (Jameson and Song 2016). The content of cytokinin in plants is a balance between biosynthesis by isopentenyl transferase (IPT), destruction by cytokinin dehydrogenase (CKX) and inactivation by glucosylation (Jameson and Song 2020). Strong homeostatic controls operate via destruction and/or glucosylation to regulate the levels of active cytokinins during developmental processes such as seed development (Jameson and Song 2016; Chen *et al*. 2020).

Genetic modification has been used to understand the contributory role of transporter genes in increasing the C and N nutrition of seeds. Transporter genes for sucrose and amino acid supply to seed sinks, have been overexpressed individually in a range of species. Often the genes and their products were upregulated but, since their products also act as signals (Sang *et al*. 2012), understanding their actions is complex. C, N or S partitioning to seeds has been altered via manipulation of C transporters in barley (Weichert *et al*. 2010), rice (Wang *et al*. 2015), pea (Rosche *et al*. 2002; Weigelt *et al*. 2008; Lu *et al*. 2020) and in *Arabidopsis* (Dasgupta *et al*. 2014); N transporters in *Vicia narbonensis* L. (Rolletschek *et al*. 2005; Götz *et al*. 2007) and in pea (Zhang *et al*. 2015); and S transporters in pea (Tan *et al*. 2010).

Seeds of transgenic lines of pea and *V. narbonensis* plants that overexpressed *AAP1* (GFM in Cluster 3A) showed increased amino acid uptake and higher protein content. Seed yield was increased in pea (Weigelt *et al*. 2008; Zhang *et al*. 2015) but not in *V. narbonensis* (Rolletschek *et al*. 2005). These studies with *AAP1* in pea (Weigelt *et al*. 2008; Zhang *et al*. 2015) and *V. narbonensis* Rolletschek *et al*. (2005) generally concluded that seed storage protein amounts are limited by the import of amino acids into cotyledons and that utilisation of enhanced amino acids in the seed may also be limited by the need for C to provide the carbon skeleton for protein synthesis. In addition, Götz *et al*. (2007) found that concentrations of cytokinins were significantly increased in transgenic *AAP1 V. narbonensis* lines and suggested that cytokinin plays a role in N-mediated growth.

Transgenic barley overexpressing *HvSUT1* (Weichert *et al*. 2010) and pea overexpressing *PsSUT1* (J. Grant, unpubl. data; Lu *et al*. 2020), had increased seed yield and the seeds showed an increase in storage protein accumulation, suggesting the increased sucrose delivered to the embryo was used for protein as well as for carbohydrate synthesis. Further, in the transgenic pea plants, amino acid movement from source to sink was also improved (Lu *et al*. 2020). In both barley and pea, overexpression of the *SUT1* gene led to increased seed yield.

Little is known about the interrelationship of C and N transport processes and their impact on cytokinin dynamics for seed performance. Our goal was to reveal these relationships by manipulating both sucrose and amino acid transport to pea seeds and to analyse the consequence for seed metabolism and nutritional quality.

To investigate the regulatory control of transporters over N and C uptake and metabolism in seeds, homozygous transgenic process pea lines were produced overexpressing both a sucrose transporter (*AtAAP1::PsSUT1*) and an amino acid permease transporter (*35S::PsAAP1(3a)*). The *AtAAP1* promoter was used to target the SUT1 transporter to the phloem and seed (Tegeder *et al*. 2007; Lu *et al*. 2020), whereas the 35S promoter was used to drive cellular amino acid import throughout the plant (Holtorf *et al*. 1995). To gain insights into the control mechanism between elevated N and C transport and hormonal regulation, we analysed a developmental series of seed components for (i) sucrose, starch and protein content, (ii) expression of the transgenes and associated genes involved in source-sink relationships, and (iii) cytokinins as known regulators of yield components (Jameson and Song 2016). Our approach presents a

successful strategy to maximise sink development for protein, starch and sucrose content in seed.

Experimental procedures

Development of transgenic Pisum sativum

Pea cultivars 'Bolero' (process pea, with wrinkled seeds and green cotyledons) and 'Bohatyr' (field pea, with round seeds and yellow cotyledons) were transformed according to the methods of Grant *et al*. (1995); Grant *et al*. (1998), using *Agrobacterium tumefaciens* strain AGL1 with constructs cloned either into pTKan with the coding sequence of the *SUT1* gene (U76190) from *Pisum sativum* L. and the *AAP1* promoter from *Arabidopsis thaliana* (L.) Heynh. (Hirner *et al*. 1998) or into pBINAR with the coding sequence of *AAP1*(*3a*) gene (AY956395) from *P. sativum* and the 35S cauliflower mosaic virus promoter. Single copy lines were determined by Southern analyses from these transformations, and homozygous progeny lines were selected.

Production of double homozygous transgenic lines

Transgenic 'Bolero' line 104 (104-SUT1) homozygous for the *SUT1* gene was used as the recurrent parent for backcrossing with the donor parent transgenic 'Bohatyr' line 1/55 (1/55-AAP1(3a)) homozygous for the *PsAAP1*(*3a*) gene for four generations, to obtain lines with 96.9% similarity to the recurrent process pea parent (see Supplementary material Fig. S1 for schematic). The presence of both transgenes was confirmed at each generation using PCR. After four backcrosses the lines were selfed and grown for two generations to obtain the double homozygous lines. PCR was used to confirm the presence of both transgenes using samples from pea seed cotyledons before planting and in leaf samples of the germinated plants (Grant and Cooper 2006). Primers used for confirming the presence of the promoter-gene of interest for *AtAAP*1::*PsSUT*1 were 5′-CGTCCCTCCATAT GTGGTCT-3′, 5′-GTTAGCGACGTCGAGAATCCAG-3′ and for *35S*::*PsAAP1* were 5′-CATTCCTTCGCAAGACCC-3′, 5′-ACCGTGTATCCAATCGCGACC-3′. For the presence of the AtAAP1 promoter the primers were 5′-CGTCCCTCC ATATGTGGTCT-3′, 5′-CAACACAGAAGGACACAACCA-3′. Other primers for the presence of the introduced genes are as in Grant *et al*. (1998) and Zhang *et al*. (2015).

Plant production

Plants were grown in pots (PB3) in a controlled environment at the Lincoln University Biotron Facility, programmed for 16 h 23°C days, 8 h 16°C nights. The pots were fertilised regularly with Hoagland solution from flower initiation. Flowers were tagged just as the standard petal opened, at which time pollination will have occurred. Pods were harvested at 12, 14, 16, 18, 20, 23, 30, 37 days after pollination (DAP) for the seed and pod developmental series. At 14 DAP the water content of the cotyledons was between 85.5–85%, at 18 DAP it was 81.5–79.7%, at 20 DAP it was 77.8–76.3%, at 23 DAP it was 74.2–71.6%, at 30 DAP it was 62.1–59.9% and at 37 DAP it was 50.4–33%.

Individual pods were collected (at least four per time point per line), opened and the middle 3–4 seeds were taken. Seed coats and embryos from the middle seeds were dissected and pooled into seed coats and embryos, while the cotyledons were cut up,

mixed, divided into four aliquots, and weighed. The aliquots were then used for dry weight, sucrose, protein, and RNA analyses. The latter three aliquots were flash frozen in liquid nitrogen and stored at –80°C until required.

Yield components were determined on four plants chosen at random and these plants were not used for any other analyses. A further 10 plants per line were grown for phenotyping only. Characters assessed included date of first flower, pod number, seeds per pod, seed number, and seed weight.

Sucrose, starch and free amino acid analyses

One hundred milligrams of frozen cotyledons or seed coats were extracted in 1 mL 80% (v/v) ethanol using a tissue lyser (Tissue Lyser II, www.retsch.com). Sucrose analysis was as described in Revanna *et al*. (2013) using the reconstituted supernatant. This reconstituted supernatant was also used for free amino acid analysis (Gosden 1979). For the free amino acid analysis, 60 μL of the supernatant was pipetted into the well of a microtitre plate and 60 μL of ninhydrin reagent added, the plate covered with plastic foil and incubated at 85°C for 25 min. Once cooled, 90 μL of 50% ethanol was added. The samples were in triplicate and the plate read at 570 nm. The precipitate from the alcohol extraction was used for starch measurement using the Megazyme total starch kit (Ireland, www.megazyme.com).

Protein analyses

Total protein was extracted from 100 mg frozen cotyledons using 400 μL of Hou buffer (Hou *et al*. 2005) with 2 mM dithiothreitol instead of mercaptoethanol. Extracts were mixed at 100°C for 5 min, centrifuged for 2 min at 5000*g*, and the supernatant used for analysis with Coomassie protein assay reagent (www.ThermoFischer.com) and NanoOrange (Invitrogen) according to the manufacturers' instructions.

Expression analyses

Samples of ovaries (–1 DAP), entire pods including ovules (with petals removed) from 0 to 3 DAP, pod walls and seed were separated 5, 7, and 10 DAP and all flash frozen in liquid nitrogen. From 12 DAP the pod walls, seed coats and cotyledons were collected separately, and are the same samples as described in 'Plant production'. Leaves subtending pods at 5 DAP (5 days) and 20 DAP (20 days) were similarly collected.

Target gene sequences were isolated as reported in Dhandapani *et al*. (2017) and Ninan *et al*. (2019) from a pea transcriptome of mixed tissues. RNA isolation, cDNA synthesis and reverse transcription quantitative PCR (RT-qPCR) were as described in Dhandapani *et al*. (2017), using their *PsELONGATION FACTOR* (*eEF-1α*), *PsGAPDH* and *PsACTIN* as reference genes. Briefly, the reference genes were selected after first comparing their expression stability in cDNA samples from different tissues at different developmental stages. The average mean of the reference genes was then used as the internal control to normalise the data by correcting for differences in the quantity of cDNA used as templates (Song *et al*. 2012; Dhandapani *et al*. 2017). An inter-run calibrator was also used in each RT-qPCR run, as all the samples could not be tested at one time. The calibrator was a mixture of all cDNAs of

all tissue types. The relative expression (fold change) of each target gene was then calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001) using a constant ΔCt of 30 minus the average correction factor derived from the reference genes as the value against which all samples were calculated.

For ease of comparison, the data are presented as heat maps, with values for the double transgenics (and 104-SUT1 for cotyledons) calculated in fold-change relative to the non-transgenic control 'Bolero'. The two independent double transgenic lines provide the biological replication. Additionally, datasets for the field pea single transgenic donor parent 1/55-AAP1(3a) and its wild-type 'Bohatyr' control are provided in the Supplementary material (Fig. S4).

Endogenous cytokinin analyses

Three biological replicates of whole pods (1 and 3 DAP), pod walls (7, 12, 14, and 16 DAP), seed coats (10, 12, 14, 16, and 20 DAP) and cotyledons (7, 10, 12, 14, 16, and 20 DAP) were extracted from 'Bolero' and double transgenic line 562 in 1.0 mL of modified Bieleski buffer (60% MeOH, 10% HCOOH and 30% H_2O) together with a cocktail of stable isotope-labelled internal standards (0.25 pmol of each of the cytokinin bases, ribosides, *N*-glucosides, and 0.5 pmol of cytokinin *O*-glucosides and nucleotides added per sample). All samples were purified using the method published previously by Dobrev *et al.* (2002) with some minor modifications (Antoniadi *et al.* 2015). The samples were analysed by the LC-MS/MS system consisting of an ACQUITY UPLC® I-Class System (Waters) and Xevo® TQ-S (Waters) triple quadrupole mass spectrometer scanning in a multiple reaction-monitoring (MRM) mode of selected precursor and product ions (Svačinová *et al.* 2012). Quantification was obtained using a standard isotope dilution method (Rittenberg and Foster 1940).

Statistical analyses

Statistical analyses were carried out using GENSTAT 18th edn. Samples were compared with each other using two sample *t*-tests. For sucrose at 23 DAP and protein at 30 DAP and 37 DAP there was a minimum of four biological replicates. For seed size and seed weight there was a minimum of 12 biological replicates. The means of the double transgenic lines were compared with the means of 'Bolero' and to the means of 104-SUT1. In addition, the means of 'Bolero' were compared with the means of 104-SUT1. Further details are provided in figure legends.

Results

Phenotype, yield and cotyledon component analyses

The double homozygous (*SUT1* + *AAP1*(*3a*)) transgenic pea lines ('562' and '382'), were phenotypically similar to the single *SUT1* homozygous line (104-SUT1) and to the process pea cultivar 'Bolero' from which they were developed. All lines were determinate, grew to a similar height, were white flowered, and had wrinkled seeds at dry pea harvest. Line 562 generally flowered 1 week later than 382, 104-SUT1 and 'Bolero'. Line 562 also segregated for yellow/green cotyledons, whereas line 382 always produced green cotyledons at dry pea harvest. During seed development, sucrose, starch and protein content in cotyledons each showed similar trends for all lines although

there were quantity differences between them (Fig. 1). 'Bolero' showed a high amount of sucrose at the early stages and a much more rapid decline at later stages than any of the transgenic lines. At 23 DAP line 382 showed a significantly greater amount of sucrose in the cotyledons compared with either 104-SUT1 ($P <$ 0.001) or 'Bolero' ($P <$ 0.001) (Fig. 1*a*). As well, line 562 and 104-SUT1 both had significantly more sucrose than 'Bolero' ($P =$ 0.03 and $P =$ 0.01 respectively) but not more than each other. The accumulation of starch was similar for all lines (Fig. 1*b*). The sucrose-starch crossover point, which is when the cotyledons have similar amounts of starch and sucrose, occurs ~2 days before peas are normally harvested for processing. In the double transgenic lines, this crossover point was delayed. For line 562 the crossover point was ~1 day later, and for 382, 2 days later than in 'Bolero' (Fig. 1*c*) and in 104-SUT1 (data not shown). By 30 DAP, the sucrose content of all lines was similar.

For protein content, the double transgenic lines showed a higher concentration between 23 and 30 DAP than 'Bolero' and 104-SUT1 (Fig. 1*d*) and by 30 DAP both double transgenic lines had significantly more protein than either 'Bolero' or 104-SUT1 (Fig. 1*d*). Accumulation of protein then continued at a slower rate until 37 DAP where the double transgenic lines had an average of 252.8 mg protein g^{-1} DM (line 382) and 247.03 mg g^{-1} DM (line 562). At 37 DAP 'Bolero' cotyledons contained 181.5 mg protein g^{-1} DM, whereas the single transgenic line 104-SUT1, showed an elevated protein content of 221.4 mg g^{-1} DM. The double transgenic lines had significantly more protein at 37 DAP than wild-type 'Bolero' ($P =$ 0.02) although not significantly more than 104-SUT1.

Further, the free amino acid content was similar for all lines, except for line 382, which contained more free seed amino acids at an early developmental stage (14 DAP) (Fig. 1*e*).

In the field pea donor parent 1/55-AAP1(3a) and in its wild-type parent 'Bohatyr' sucrose content was high at 14 DAP and already low at 18 DAP whereas starch and protein content was increasing. Fig. S2 highlights some of these differences between field peas and process peas.

For the yield components, although the seed number per plant was quite variable, the double transgenic lines had significantly more seed per plant than the wild-type 'Bolero' (for line 562 $P =$ 0.008; for line 382 $P =$ 0.03). In addition, the double transgenic line 562 had more seed than the single *PsSUT1* transgenic line, 104-SUT1, $P =$ 0.05, but not significantly more seed than the double transgenic line 382, $P =$ 0.08 (Fig. 2*a*). The first four trusses on the main branch of a process pea indicate the yield at green pea harvest (H. Stace, pers. comm.). Line 562 had more pods and, therefore, more seed than 'Bolero' or line 104-SUT1, while line 382 generally had an extra pea per pod compared with 'Bolero' or line 104-SUT1 (Fig. S3).

For seed weight, the single *PsSUT1* transgenic line (104-SUT1) and the double transgenic line 382 produced heavier seed than 'Bolero' ($P =$ 0.01 and $P =$ 0.03 respectively); however, double transgenic line 562 produced seed with weights between those of 'Bolero' and line 104-SUT1 (Fig. 2*b*).

By combining seed number and seed weight the total seed yield per plant for the double transgenic line 562 is 89% greater than 'Bolero' and 33% greater than 104-SUT1. Line 382 and 104-SUT1 yields are 42 and 41%, respectively, greater than 'Bolero'.
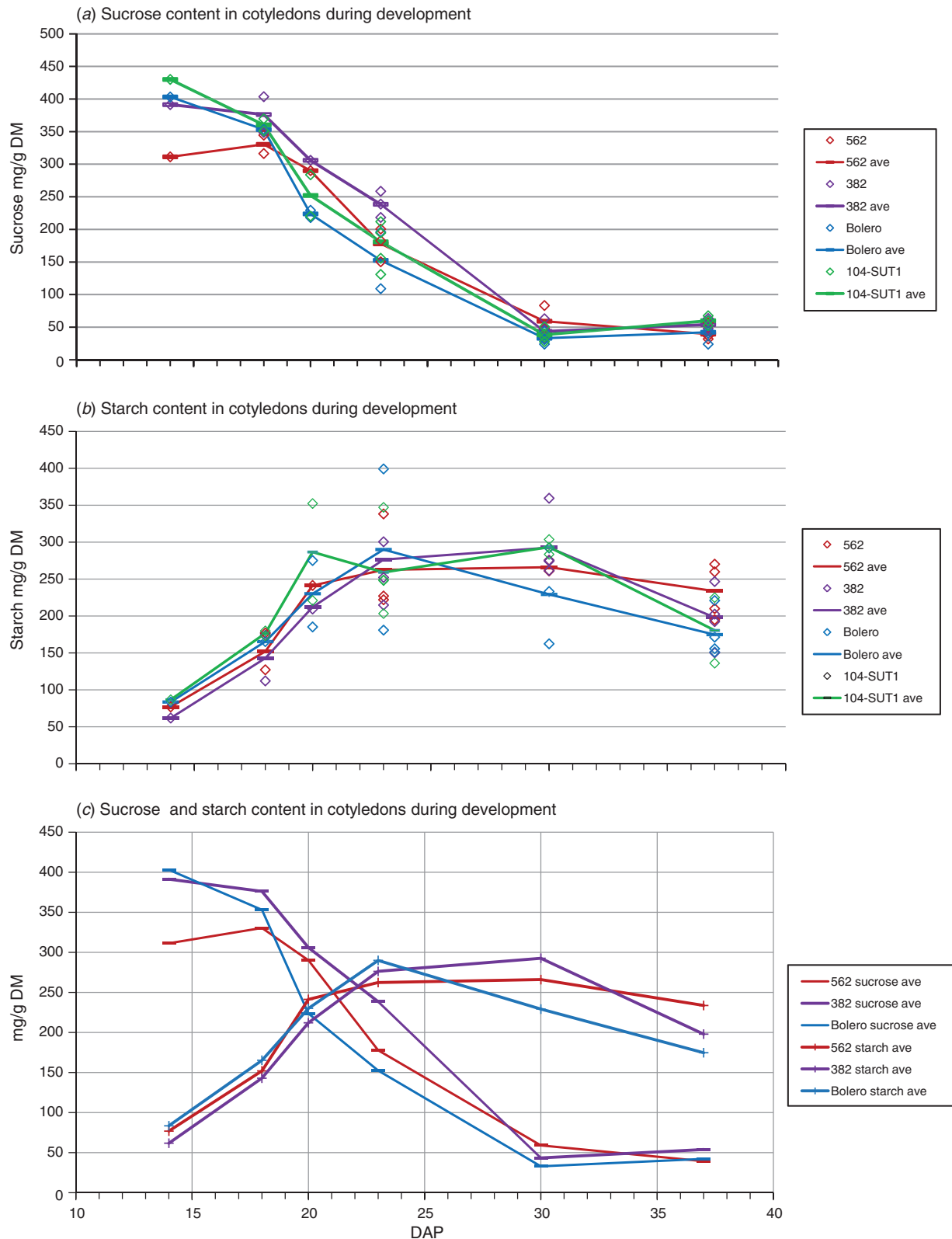
Fig. 1. (*a*) Sucrose content in pea cotyledons during development. Double transgenic line 382 showed a significant increase in sucrose at 23 days after pollination (DAP) compared with that in the recurrent parent 104-SUT1 ($P = 0.06$) and wild-type 'Bolero' ($P = 0.03$). (*b*) Starch content in cotyledons during development. The high variability meant that there were no significant differences. (*c*) Sucrose and starch content during development showing the crossover point for starch and sucrose was delayed in the double transgenics by 1–2 days. (*d*) Protein content in cotyledons during seed development. Double transgenics showed greater amounts of protein at 30 DAP than the wild-type 'Bolero' (382 $P = 0.01$; 562 $P = 0.06$) and 104-SUT1 (382 $P < 0.001$; 562 $P = 0.01$). At 37 DAP the double transgenics had significantly greater amounts of protein than 'Bolero' ($P = 0.02$). (*e*) Free amino acids in cotyledons measured as micrograms per mL of glutamine equivalent.

(*d*) Protein content in cotyledons during development

(*e*) Free amino acids in cotyledons during development
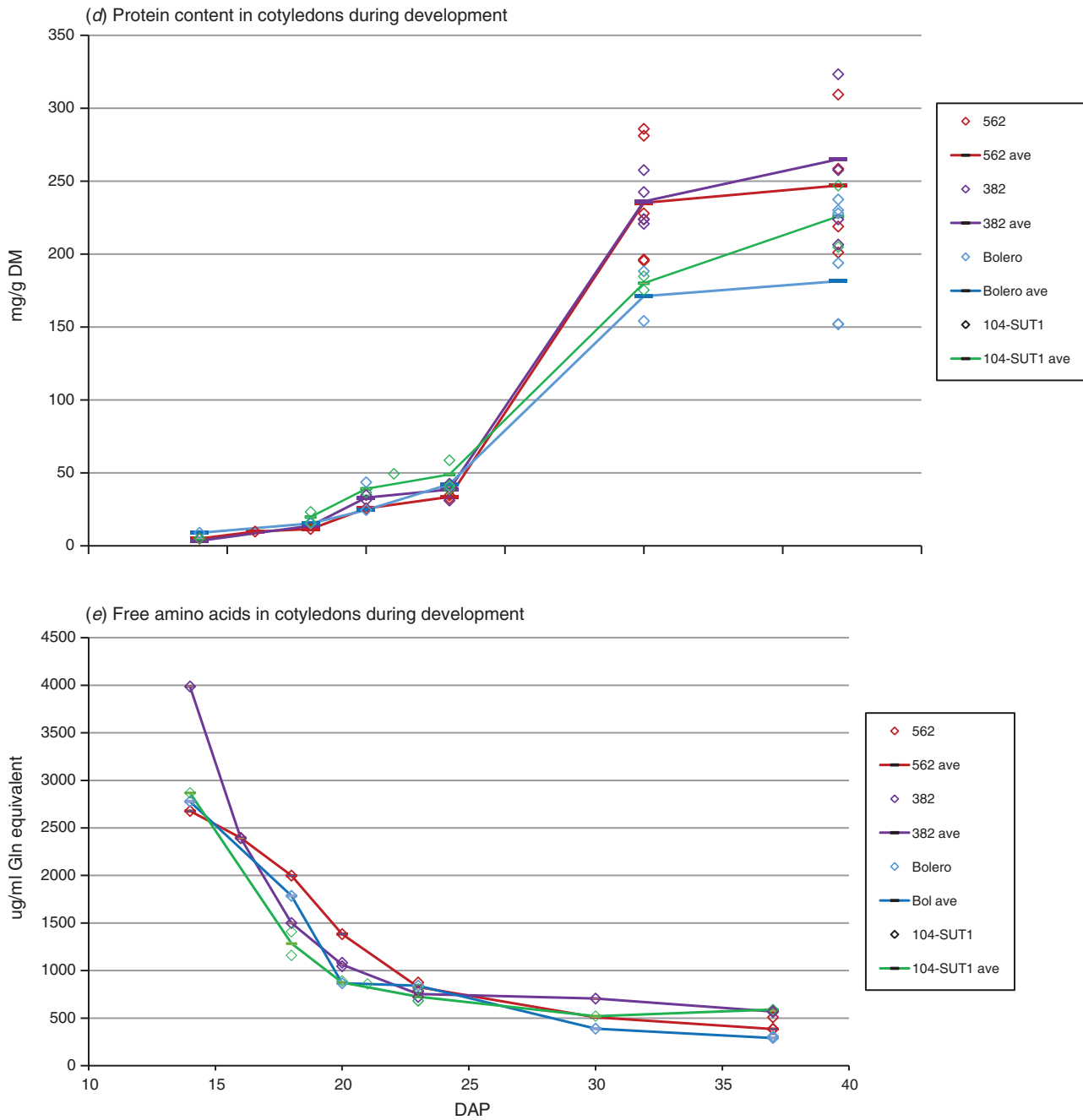
Fig. 1. (*Continued*).

Gene expression analyses

Leaves subtending pods

The *AAP1*(*3a*) transgene was strongly expressed in the double transgenic lines in leaves subtending to pods at 5 DAP and 20 DAP (Fig. 3*a*). However, relative to wild-type 'Bolero', there was no difference in gene expression at 20 DAP, whereas a strong effect was observed for leaves 5 DAP. Generally, an upregulation of *SWEET* (with the exception of *PsSW17*) and *SUT* GFMs was observed.

Further, Cluster 3A *PsAAP* GFMs were upregulated, whereas two cluster 4B members were strongly downregulated. Variable effects were found for *CWINV* GFMs. There was a weak downregulation in the expression of *PsIPT* GFMs, strong downregulation of *PsCKX2*, but consistent upregulation of *PsCKX7* in the transgenic younger leaves relative to control. A very similar pattern of gene expression for the donor parent (*35S::PsAAP1*(*3a*) single transgenic line 1/55-AAP1(3a)) was seen in leaves 5 DAP, although expression of the transgene itself was much reduced in the leaves 20 DAP (Fig. S4).

(a) Seed number - grown twice (minimum of 12 plants per line)

(b) Seed weight - grown twice (minimum 12 plants per line)

Legend: ■ Bolero ■ 104-SUT1 ■ 382 ■ 562

Fig. 2.   Seed yield in the wild-type pea 'Bolero', the recurrent parent line 104-SUT1 and the double transgenic lines 382 and 562. All lines were grown twice in the Lincoln University Biotron with a minimum of 12 plants for each line. (*a*) Seed number: the double transgenic lines had significantly more seeds per plant than the wild-type 'Bolero' (562 $P = 0.008$; 382 $P = 0.03$). Line 562 had significantly more seed than 104-SUT1 ($P = 0.05$). (*b*) Seed weight: the single *Ps*SUT1 transgenic line (104-SUT1) and the double transgenic line 382 produced heavier seed than 'Bolero' ($P = 0.01$ and $P = 0.03$ respectively).
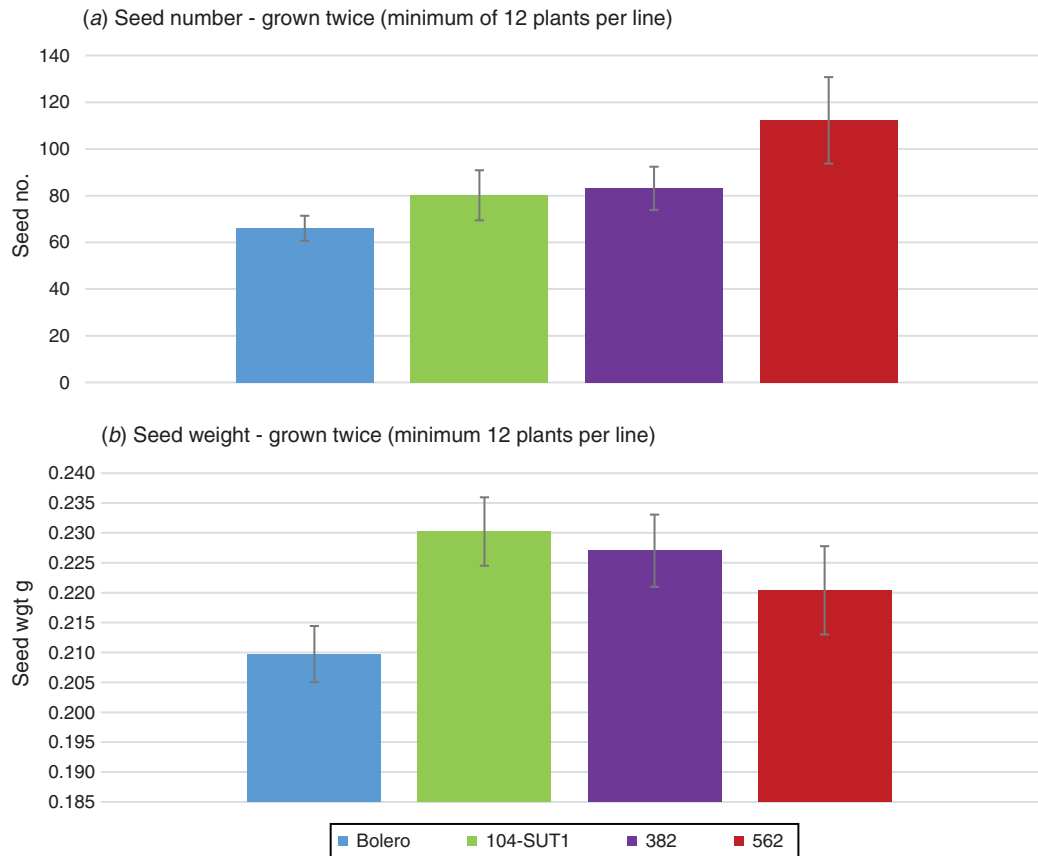
Pod walls

The *AAP1(3a)* transgene was strongly expressed in pod walls of the double transgenics lines relative to the 'Bolero' wild-type (Fig. 3*b*). Elevated expression of the *SUT1* transgene was also apparent compared with other *SUT* GFMs. However, compared with the seed coat and cotyledon, fewer of the tested genes were upregulated in the pod wall, although at 16 DAP, *CWINV* and most *SWEETS* were upregulated relative to those in the 'Bolero' control. With the exception of *IPT4* at 1 DAP, there was little change in the expression of the cytokinin *IPT* or the *CKX* GFMs in pod walls relative to that in the 'Bolero' control.

Ovules

Strong expression of *AAP1(3a)* in the double transgenic lines could be detected in the undissected pods immediately post fertilisation, and in the dissected pod walls and seeds (Fig. 4). *SUT1* expression was only modestly elevated relative to the 'Bolero' control, as were other *SUT* GFMs. There was little change in expression of Clade I *SWEETs* relative to 'Bolero', but enhanced expression in both Clade II and III SWEETs was detected. Particularly noticeable was the reduced expression

of the Clade III *SWEET15b* in the dissected pod walls compared with the strong expression in the whole seed (embryo plus seed coat) relative to 'Bolero'. Enhanced *CWINV* expression was noticeably greater in the combined tissues at 3 DAP, and in whole seed at 5 and 7 DAP but less so in the separated pod walls, indicating the importance of CWINV to the developing seed.

PsIPT1, *2* and *4* showed modest increases in expression relative to control at 1 DAP, and in the young pod wall, but generally reduced expression in the whole seed. Expression of *PsCKX2* was strongly elevated relative to the 'Bolero' control (Fig. 4).

Seed coats

AAP1(3a) was strongly and consistently expressed in the seed coats of the double transgenics relative to the 'Bolero' control (Fig. 5*a*). Several *PsAAPs* belonging to Clusters 1 and 3A were also upregulated, whereas this was less so for Cluster 4B AAPs. *PsSUT1* was not consistently upregulated relative to the control 'Bolero', although *PsSUT2* and *5* were. There was a general upregulation of expression shown for the *CWINV* GFMs. Several

(a) Seedline 382,562 vs Bolero (LEAVES)

Target genes	Stages			
	382 5d	562 5d	382 20d	562 20d
PsIPT 1 (604)	1.4	−1.8	−1.1	−1.1
PsIPT 2 (605)	−2.6	−4.2	1.0	−1.1
PsIPT 4 (421)	−3.4	−8.5	−1.0	−1.1
PsCKX 1 (930)	1.4	4.6	16.9	10.9
PsCKX 2 (627)	−45.7	−17.1	−1.3	−1.6
PsCKX 5 (942)	−2.4	−2.1	1.3	1.3
PsCKX 7 (910)	5.2	6.0	−1.1	1.8
PsSUT 1 (366 Transgene)	16.6	12.2	1.2	1.3
PsSUT 2 (948)	61.5	22.2	−1.7	1.6
PsSUT 3 (674)	6.5	3.7	−1.2	1.2
PsSUT 5 (666)	−5.2	−12.7	1.2	1.1
PsAAP 1(3a) (532 Transgene)	20.6	59.7	65.9	20.4
PsAAP 7a (498)(Cluster 1)	−13.0	−26.1	−1.5	−1.2
PsAAP 7b (9261)	2.3	−1.3	−1.9	1.1
PsAAP 2a (675)(Cluster 3A)	42.1	49.6	1.9	3.3
PsAAP 2c (4401)	4.0	5.0	−3.7	−1.7
PsAAP 2d (840)	39.5	13.4	−1.2	1.4
PsAAP 3b (051)	17.3	10.4	−1.2	−2.1
PsAAP 6a (931) (Cluster 4B)	−13.0	−26.1	−1.0	−1.3
PsAAP 6b (328)	9.1	11.2	1.1	1.0
PsAAP 1 (180)	−17.2	−56.4	−1.9	−1.3
PsCWINV1 (240)	−14.3	−32.8	1.9	1.6
PsCWINV2 (448)	9.1	2.8	1.7	1.9
PsCWINV3 (415)	−222.0	−73.1	−1.6	−1.3
PsCWINV6 (320)	93.0	75.4	1.6	2.8
PsSW 1 (1) (Clade I)	−1.7	−1.2	−3.8	−8.1
PsSW 2a (2a)	1.4	−1.1	−1.3	1.0
PsSW 2b (2b)	8.3	7.9	−3.1	−1.4
PsSW 4 (7) (Clade II)	29.6	8.8	1.5	1.3
PsSW 5a (6a)	−1.0	−31.0	1.6	1.1
PsSW 5b (6b)	24.7	9.7	1.0	1.1
PsSW 7 (4)	1.0	−2.9	−1.7	−2.7
PsSW 12 (13b) (Clade III)	3.3	10.5	−1.8	−2.4
PsSW 13 (13a)	48.5	46.4	−1.3	−1.1
PsSW 17 (17) (Clade IV)	−31.3	−20.2	−2.5	−4.9

(b) Seedline 382, 562 vs Bolero (POD WALLS)

Target genes	Developmental stages												
	382 12d	562 12d	382 14d	562 14d	382 16d	562 16d	382 18d	562 18d	382 20d	382 23d	562 23d	382 30d	562 30d
PsIPT 1 (604)	2.2	2.2	2.1	3.5	1.3	−1.6	−2.4	−2.2	−2.6	−2.8	1.2	1.1	1.4
PsIPT 2 (605)	−1.1	1.1	−1.2	1.5	1.7	1.1	1.0	−1.2	−2.1	1.0	1.6	1.2	1.6
PsIPT 4 (421)	6.5	6.0	1.5	1.5	2.2	−1.5	−1.4	1.1	−1.2	−1.2	1.3	1.2	2.0
PsCKX 1 (930)	1.7	1.9	−1.0	1.6	−1.0	−1.8	−2.3	−2.0	−2.0	−1.3	1.3	1.1	1.4
PsCKX 2 (627)	2.0	2.3	−1.0	1.9	1.1	−1.8	1.2	−1.8	2.0	1.8	3.0	1.1	1.5
PsCKX 5 (942)	1.2	1.2	1.0	1.8	1.8	−1.2	1.8	−1.4	3.6	1.4	1.4	1.4	1.5
PsCKX 7 (910)	3.5	1.7	−1.8	−1.1	1.7	−2.8	−2.0	−1.3	1.2	1.5	1.2	−1.1	1.4
PsSUT 1 (366 Transgene)	1.8	3.7	1.5	2.4	28.4	22.1	7.8	4.2	16.4	30.4	41.3	23.0	13.1
PsSUT 2 (948)	3.4	2.6	1.9	4.3	2.3	1.7	−3.1	−1.1	−1.8	1.9	2.3	2.1	1.6
PsSUT 3 (674)	1.4	1.1	1.5	2.4	4.0	1.5	7.8	5.5	−1.5	3.1	3.5	3.7	2.7
PsSUT 5 (666)	1.1	−1.8	−6.5	3.7	1.2	4.6	−9.1	2.9	6.1	6.2	5.6		
PsAAP 1(3a) (532 Transgene)	92.6	31.8	42.2	13.0	233.1	116.0	44.3	10.7	30.2	26.6	8.5	81.5	23.6
PsAAP 7a (498)(Cluster 1)	1.7	−1.2	−2.0	−2.7	14.6	4.6	−4.3	−6.4	−3.4	2.2	2.2	2.4	3.8
PsAAP 7b (9261)	1.7	1.3	−2.7	−2.1	1.2	−1.6	−1.8	−4.1	−2.1	1.3	1.2	1.3	1.4
PsAAP 2a (675)(Cluster 3A)	1.2	1.5	−2.8	−1.5	3.2	2.1	−1.3	−2.8	−1.9	1.1	1.7	−2.0	−1.8
PsAAP 2c (4401)	2.4	2.5	1.2	−1.0	1.1	−1.2	−1.7	−2.8	1.0	1.9	2.2	2.8	2.5
PsAAP 2d (840)	1.6	2.5	−2.0	−1.8	4.9	1.7	−1.2	−1.2	−1.2	1.2	1.9	4.7	4.8
PsAAP 3b (051)	1.0	1.0	−1.8	−1.9	14.6	11.3	−1.1	−2.3	−1.4	−1.2	−1.6	2.4	2.0
PsAAP 6a (931) (Cluster 4B)	3.5	1.9	−3.0	−1.4	−1.2	−3.5	−2.0	−1.7	−3.7	−1.2	−1.0	1.7	1.7
PsAAP 6b (328)	−2.7	−1.6	−1.4	−1.3	7.4	4.3	1.4	1.3	2.4	1.9	1.6	2.3	2.1
PsAAP 1 (180)	−2.0	−7.1	−2.1	−2.8	1.9	−1.2	−6.7	−8.0	−11.5	3.8	−3.7	−1.7	−3.3
PsCWINV1 (240)	6.8	7.8	−1.1	3.3	3.9	−1.7	−1.4	−1.6	−1.2	1.0	1.8		
PsCWINV2 (448)	2.8	4.0	1.9	5.5	3.3	1.1	−1.2	−1.5	−1.5	−1.3	2.7	−1.9	−3.1
PsCWINV3 (415)	4.1	2.1	1.3	1.5	14.1	2.5	−1.6	1.2	−2.8	−2.9	−1.5		
PsCWINV6 (320)	3.6	6.3	−1.3	3.3	24.9	18.3	1.2	−1.1	−2.0	−1.0	4.3	5.3	6.6
PsSW 1 (1) (Clade I)	−2.5	−2.0	−3.9	−3.2	3.6	6.5	−2.4	−6.7	−1.0	−1.1	−4.2	1.5	2.4
PsSW 2a (2a)	−2.3	1.0	−1.4	1.7	4.2	2.8	−2.8	−2.9	−2.2	1.5	1.3	2.9	5.1
PsSW 2b (2b)	−2.7	1.4	−2.9	−1.1	18.1	6.8	1.0	1.5	1.0	−15.1	−8.3	−1.2	−28.4
PsSW 4 (7) (Clade II)	−13.0	2.5	−6.2	36.9	−4.0	−61.8	−15.1	−143.0	−64.3	1.2	2.5	−2.4	−2.9
PsSW 7 (4)	−2.6	−1.3	−1.4	−1.1	6.1	3.2	−2.6	−4.4	−2.4	−1.3	1.5	5.9	10.3
PsSW 9 (9) (Clade III)	5.2	3.0	−4.0	1.4	18.3	2.0	−1.1	−1.5	−12.8	1.1	1.1	−29.1	−21.8
PsSW 12 (13b)	1.3	2.1	−1.8	1.8	3.3	2.3	1.0	−7.1	1.1	−1.1	−1.7	2.4	1.3
PsSW 13 (13a)	5.3	2.2	−5.4	−2.0	8.4	1.9	−1.8	−15.0	−2.9	−5.4	−5.5	3.5	−1.8
PsSW 15a (534)	4.5	4.1	−2.7	−1.5	2.9	1.1	4.5	−1.5	−6.4	−1.6	−4.4	1.3	1.3
PsSW 15b (445)	12.4	2.0	−4.7	−1.5	3.5	−1.7	−5.6	−4.4	−5.2	1.0	1.1	1.6	2.3
PsSW 15c (624)	3.0	3.5	−1.5	2.3	15.6	4.9	−4.1	−7.1	−2.7	2.4	5.1	1.2	1.7

<−54.0	18.0 to 53.9	17.9 to 6.0	5.9 to 2.0	−1.9 to 1.9	2.0 to 5.9	6.0 to 17.9	18.0 to 53.9	>54.0

Fig. 3.   Relative expression of gene family members of cytokinin biosynthesis (*PsIPT*), cytokinin degradation (*PsCKX*), sucrose transporters (*PsSUT*), amino acid transporters (*PsAAP*), cell wall invertase (*PsCWINV*) and sucrose efflux carriers (*PsSWEET* = (*PsSW*)) in *Pisum sativum* (*a*) leaves subtending pods 5 and 20 days after flowering (5d and 20d); (*b*) separated pod walls from 12 to 30 DAP (12d to 30d) in the double transgenic lines 382 and 562. Values are fold-changes relative to the expression in the wild-type 'Bolero' line. The colour scale indicates upregulated expression (red), similar expression (white), and downregulated expression (blue) relative to that in the wild-type line.

Seedline 382, 562 vs Bolero (EARLY STAGES)

Target genes	Whole pods (including seed)								Pod wall only						Whole seed					
	−1D		0d		1d		3d		5d PW		7d PW		10d PW		5d SEED		7d SEED		10d SEED	
	382	562	382	562	382	562	382	562	382	562	382	562	382	562	382	562	382	562	382	562
PsIPT 1 (604)	1.7	2.5	−1.2	−1.1	6.0	5.2	−5.4	−2.9	−3.7	5.9	3.3	1.4	4.0	3.5	1.2	−1.7	−2.1	−3.3	−2.5	−5.8
PsIPT 2 (605)	2.5	−1.4	−1.2	−1.1	8.1	5.3	−5.4	−2.9	−3.7	5.9	5.9	1.3	4.1	7.3	1.2	−1.7	−2.1	−3.3	−2.5	−5.8
PsIPT 4 (421)	2.3	1.2	1.5	1.5	6.5	6.0	−1.4	1.1	−1.2	5.3	4.5	1.3	3.0	5.8	1.4	−1.2	−1.1	−1.2	1.2	−7.1
PsCKX 1 (930)	4.9	1.1	1.7	2.2	2.3	2.3	−1.0	1.6	−1.9	7.2	2.8	1.4	6.0	10.0	1.6	1.1	−2.7	1.3	1.2	−7.1
PsCKX 2 (627)	3.0	3.6	3.5	3.8	9.5	14.0	40.4	33.5	33.7	38.0	20.6	14.6	26.9	33.1	35.0	48.0	52.4	55.5	56.4	76.1
PsCKX 5 (942)	1.7	−1.1	3.5	5.4	4.0	−1.0	−1.0	1.4	−1.6	5.2	3.2	1.8	2.8	2.5	3.5	3.4	−1.5	−1.0	3.1	−1.4
PsCKX 7 (910)	3.9	−1.1	1.6	5.4	7.9	6.2	1.3	2.2	3.7	1.5	4.8	2.3	3.4	2.6	3.6	1.7	1.7	1.2	2.3	1.1
PsSUT 1 (366 Transgene)	3.3	2.8	4.0	6.1	1.3	1.5	2.1	3.1	1.2	3.7	2.0	1.5	2.1	1.4	3.0	2.1	1.1	2.2	4.9	3.0
PsSUT 2 (948)	4.2	1.3	1.4	2.6	13.3	10.8	−2.7	−1.9	−3.3	2.3	1.0	−2.3	1.2	1.4	1.1	−1.3	−1.7	−1.5	−1.8	−2.9
PsSUT 3 (674)	1.9	1.8	−1.6	1.1	2.5	3.1	1.1	1.6	1.1	3.6	1.8	1.9	2.2	2.2	2.6	1.7	1.3	1.3	3.1	2.1
PsSUT 5 (666)	7.0	1.7	3.2	1.3	1.5	1.3	1.4	2.5	2.0	−1.9	−1.4	9.2	4.2	5.9	3.8	1.1	1.9	1.6	3.3	1.9
PsAAP 1(3a) (532 Transgene)	183.1	60.2	41.7	66.8	35.2	35.8	99.7	87.5	285.0	233.1	219.3	90.9	319.8	121.7	242.6	37.6	534.5	154.7	1654.8	111.7
PsAAP 7a (498)(Cluster 1)	2.9	1.7	2.9	4.4	3.9	5.9	3.3	4.7	−1.0	2.5	1.8	2.3	4.9	4.7	3.2	1.7	−1.5	1.2	3.5	2.0
PsAAP 7b (9261)	2.7	2.8	2.7	6.4	13.9	6.1	−1.1	1.6	−1.3	3.0	2.1	−1.6	4.1	10.8	1.3	1.3	−2.2	1.4	3.3	1.7
PsAAP 2a (675)(Cluster 3A)	−1.2	−1.0	−1.1	1.0	1.1	1.2	−3.2	1.0	−2.0	1.4	2.1	1.2	1.7	1.4	2.4	1.5	−3.9	−2.0	−2.7	−5.2
PsAAP 2c (4401)	1.3	−1.4	2.5	3.2	12.9	5.7	−1.6	1.2	−3.0	2.9	3.6	1.4	3.0	2.9	1.3	1.0	−1.9	−1.1	−1.7	−3.0
PsAAP 3b (051)	1.1	1.1	1.3	1.4	1.3	1.3	1.8	2.7	1.4	4.6	1.6	−1.5	1.5	1.7	−1.1	−1.5	−2.3	−8.7	−4.8	−4.1
PsAAP 6a (931) (Cluster 4B)	2.0	−1.7	−1.2	−1.1	6.8	5.2	−1.2	−1.9	2.6	1.5	3.0	1.4	6.3	5.2	2.3	−1.0	2.5	1.2	2.1	1.2
PsAAP 6b (328)	4.6	7.6	2.1	3.3	9.1	7.6	1.2	1.9	1.3	5.5	2.1	1.9	6.9	6.2	2.5	2.0	−1.7	−2.6	2.8	2.7
PsAAP 1 (180)	4.2	−1.4	3.7	2.7	7.2	1.9	1.2	−1.0	4.2	−1.6	4.8	10.1	3.4	8.9	3.3	1.3	1.3	1.5	3.3	2.3
PsCWINV 1 (240)	2.8	7.6	−1.3	−1.3	−1.0	1.1	5.4	8.7	−6.2	−26.7	2.3	1.4	−1.2	−4.5	4.1	4.0	2.6	2.0	1.4	1.1
PsCWINV 2 (448)	−1.3	1.9	−2.5	−2.6	−1.1	2.6	7.1	7.0	−3.2	−1.2	−1.2	2.3	−1.4	−1.7	4.6	10.0	15.7	4.0	−2.8	−2.7
PsCWINV 3 (415)	3.0	6.1	6.1	8.1	1.0	−2.1	13.5	12.4	−4.4	−1.9	−2.3	4.1	3.1	−1.3	4.2	8.3	8.0	9.1	1.8	−2.1
PsCWINV 6 (320)	38.2	45.1	−1.4	1.7	−2.9	−2.2	9.8	13.7	−4.4	−1.7	1.1	1.5	1.9	−1.9	−2.1	1.6	2.7	−1.2	−6.8	−10.7
PsSW 1 (1) (Clade I)	7.3	12.7	3.0	5.1	−1.4	−1.7	6.1	6.4	−1.0	−1.1	−1.5	2.2	1.6	−1.4	1.4	1.8	−2.8	−2.5	3.9	3.6
PsSW 2a (2a)	−2.0	1.7	−3.0	−2.0	1.6	4.3	4.6	3.3	−7.4	−2.6	1.5	4.4	−1.4	−1.7	4.1	3.8	3.8	4.0	−3.0	−2.5
PsSW 2b (2b)	−1.1	2.6	−2.9	−1.0	1.0	−1.1	5.8	4.8	−5.4	−2.6	1.7	3.6	−1.9	−5.5	1.2	1.4	−1.8	−1.3	2.5	−1.2
PsSW 4 (7) (Clade II)	4.5	5.0	2.6	2.7	1.5	−5.0	4.1	4.6	−3.3	−1.8	9.6	11.9	38.7	5.2	2.7	3.8	2.8	4.5	2.8	3.2
PsSW 5a (6a)	2.8	6.5	5.0	4.7	−1.3	−1.5	8.1	9.6	−13.6	−33.5	42.2	288.5	93.1	9.6	1.4	2.2	1.9	2.6	2.3	−1.4
PsSW 5b (6b)	6.9	10.0	5.1	3.7	−2.3	−1.2	3.2	3.3	−6.5	−2.7	−3.4	1.1	111.5	16.7	−1.0	−1.3	1.5	1.8	1.1	−3.1
PsSW 7 (4)	6.0	13.0	32.8	72.5	−5.7	−3.4	4.4	3.3	1.0	2.1	2.0	2.9	1.6	−1.6	3.2	3.9	1.2	−1.0	2.4	2.9
PsSW9 (9) (Clade III)	8.2	13.5	239.2	303.2	1.0	−2.6	3.9	4.4	−1.6	2.7	−1.0	1.6	9.6	−2.5	−1.4	2.2	3.4	20.2	−1.2	1.1
PsSW 13 (13a)	−3.3	1.2	−8.0	0.2	−1.9	1.3	13.9	25.3	−8.2	1.4	−8.9	−1.5	1.6	−1.1	3.2	7.1	5.1	3.3	−4.1	−4.3
PsSW 12 (13b)	−1.1	2.8	−5.3	−4.1	−1.5	−1.0	15.5	20.0	−4.9	−3.5	1.3	1.5	9.5	−12.1	3.4	4.3	5.5	3.1	−2.5	−2.2
PsSW 15a (534)	9.0	13.5	1.7	2.9	−13.8	11.3	1.1	1.1	1.5	−13.4	1.1	39.4	9.5	61.0	6.6	4.4	1.8	1.4	1.6	1.7
PsSW 15b (445)	8.1	5.1	−36.5	−12.5	−13.8	11.3	−1.7	−1.3	−2.4	−25.7	−7.2	−17.0	−14.2	1064.8	3680.5	2118.6	4659.0	29157	436.0593	308.0493
PsSW 15c (624)	14.1	10.4	2.9	1.4	1.3	3.7	−2.0	−1.1	−6.8	−1.1	8.3	9.8	12.8	1.7	−1.8	−1.7	6.5	3.7	36.1	12.1
PsSW 17 (17) (Clade IV)	−1.3	1.4	−4.5	−3.0	1.3	2.9	2.1	3.8	−9.7	−3.3	4.2	13.0	1.7	−2.3	3.2	3.2	16.4	4.9	−1.6	−3.1

Colour scale: <−54.0 | −18.0 to 53.9 | −17.9 to 6.0 | −5.9 to 2.0 | −1.9 to 1.9 | 2.0 to 5.9 | 6.0 to 17.9 | 18.0 to 53.9 | >54.0
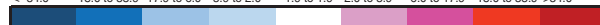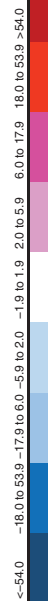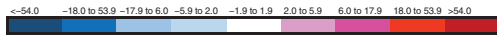
Fig. 4. Relative expression of gene family members of cytokinin biosynthesis (*PsIPT*), cytokinin degradation (*PsCKX*), sucrose transporters (*PsSUT*), amino acid transporters (*PsAAP*), cell wall invertase (*PsCWINV*) and sucrose efflux carriers (*PsSWEET = PsSW*) in double transgenic pea lines 382 and 562. Samples from the earliest developmental stages (−1 to 3 days after pollination (DAP) (−1d to 3d)) include the whole pod; samples from 5 DAP to 10 DAP are dissected and include either the pod wall only (5d PW to 10d PW) or the whole seed including seed coat (5d SEED to 10d SEED). Values are fold-changes relative to the expression in the wild-type 'Bolero' line. The colour scale indicates upregulated expression (red), similar expression (white), and downregulated expression (blue) relative to that in the parental line.

(a) Seedline 382, 562 vs Bolero (SEED COATS)

Target genes	Developmental stages													
	12d 382	12d 562	14d 382	14d 562	16d 382	16d 562	18d 382	18d 562	20d 382	20d 562	23d 382	23d 562	30d 382	30d 562
PsIPT 1 (604)	−1.1	1.3	1.2	1.1	1.5	1.3	3.4	−2.1	−1.6	−1.6	−1.3	1.0	1.3	1.5
PsIPT 2 (605)	3.1	4.1	7.4	6.7	2.2	2.8	5.6	9.1	2.8	2.7	6.3	1.7	10.2	7.0
PsIPT 4 (421)	11.0	12.0	10.9	13.3	7.0	8.6	12.1	12.6	4.1	3.6	4.1	3.5	5.3	5.9
PsCKX 1 (930)	2.3	1.9	2.4	2.7	2.7	2.9	5.2	4.6	1.9	2.2	2.0	2.3	1.9	1.6
PsCKX 2 (627)	33.1	22.2	27.6	20.1	28.7	18.4	13.1	25.7	8.1	8.3	8.2	16.4	4.1	8.9
PsCKX 5 (942)	5.9	8.7	−1.6	−1.2	14.5	12.8	2.0	2.3	9.6	10.8	17.5	14.4	25.8	23.0
PsCKX 7 (910)	3.0	2.8	2.6	2.2	2.9	2.3	4.3	4.0	3.4	2.7	3.6	3.2	16.4	16.1
PsSUT 1 (366 Transgene)	1.8	5.2	2.1	6.7	1.6	1.4	3.7	3.1	5.5	8.0	−1.6	−1.2	−1.6	−1.2
PsSUT 2 (948)	24.6	23.1	19.3	17.0	15.7	15.3	16.8	22.6	9.9	10.7	4.5	4.2	1.5	2.1
PsSUT 3 (674)	1.1	1.2	1.7	1.3	2.0	2.0	6.9	8.7	2.7	3.0	1.7	2.7	−1.0	−1.0
PsSUT 5 (666)	1.2	1.5	13.0	10.6	6.0	10.2	28.6	23.4	1.8	2.7	4.2	4.0	3.6	1.1
PsAAP 1(3a) (532 Transgene)	37.5	22.6	120.9	90.4	188.2	113.0	480.8	715.5	510.7	326.5	479.4	222.0	55.1	96.0
PsAAP 7a (498)(Cluster 1)	1.5	1.3	9.5	10.0	10.3	9.4	137.4	49.4	37.8	27.7	57.1	4.7	47.7	34.0
PsAAP 7b (9261)	−1.3	3.3	9.2	5.1	1.3	1.1	3.2	4.4	1.3	1.3	1.3	1.3	−1.3	1.3
PsAAP 2a (675)(Cluster 3A)	5.4	5.0	30.5	21.5	40.1	45.7	249.8	213.1	108.9	89.8	83.0	56.9	12.5	18.5
PsAAP 2c (4401)	3.0	2.8	1.3	1.1	2.9	2.3	4.3	4.0	3.4	2.7	3.6	3.2	16.4	16.1
PsAAP 2d (840)	2.8	1.9	3.8	4.6	2.6	3.2	14.7	17.2	3.6	5.4	−1.4	−1.6	1.8	1.0
PsAAP 3b (051)	20.9	26.4	4.4	9.0	60.0	69.2	23.4	20.5	41.0	37.6	3.5	2.7	1.7	1.4
PsAAP 6a (931) (Cluster 4B)	1.9	2.0	1.4	3.0	1.6	1.3	8.4	6.5	1.9	2.2	1.1	−1.0	17.1	42.6
PsAAP 6b (328)	1.5	1.3	4.5	3.1	3.1	2.1	4.1	3.4	4.3	3.7	2.3	2.5	9.3	1.3
PsAAP 1 (180)	2.4	2.1	1.1	1.2	2.1	1.8	3.5	3.8	2.4	2.9	2.3	2.2	3.0	3.4
PsCWINV 1 (240)	1.6	1.3	19.0	19.2	6.8	5.2	17.4	23.1	−1.0	−1.1	1.1	−1.1	173.4	221.9
PsCWINV 2 (448)	1.2	1.1	1.8	1.4	42.6	47.2	36.3	45.7	10.3	14.1	35.4	32.0	1.4	−1.1
PsCWINV 3 (415)	2.3	1.5	3.2	1.7	1.3	1.6	8.9	3.1	1.6	1.2	3.6	9.3	2.1	7.1
PsCWINV 6 (320)	2.1	1.7	2.8	2.2	1.0	1.1	3.0	2.5	1.3	1.1	2.6	2.3	3.4	3.3
PsSW 1 (1) (Clade I)	1.2	1.4	1.3	2.0	2.6	4.1	−2.1	−1.8	−1.6	1.0	1.0	1.4	10.2	22.6
PsSW 2a (2a)	1.7	1.6	4.5	2.2	−1.4	−1.2	1.6	−2.3	−1.0	−1.2	1.5	−1.4	−1.9	−1.7
PsSW 2b (2b)	1.2	1.7	1.4	2.3	−1.2	2.1	−1.2	−1.2	2.9	5.5	1.8	3.4	1.7	4.7
PsSW 4 (7) (Clade II)	1.2	2.8	1.7	3.1	4.3	11.8	1.7	−1.2	−1.9	−1.1	2.0	2.9	2.9	6.8
PsSW 5a (6a)	2.2	3.4	1.2	2.4	−4.1	2.8	1.4	1.5	−5.7	−2.9	−4.5	−3.1	1.8	17.7
PsSW 5b (6b)	1.1	3.0	−1.2	1.4	−1.9	2.7	−2.6	−1.7	1.6	2.8	−1.1	−2.9	2.1	8.7
PsSW 7 (4)	2.3	4.5	1.6	2.8	1.3	7.1	−2.4	−4.7	−4.8	−5.2	1.0	14.4	−1.2	2.2
PsSW 9 (9) (Clade III)	2.5	10.2	21.8	12.6	21.9	46.1	7.9	42.4	−1.5	−4.9	121.3	63.4	7.6	2.5
PsSW 13a	−1.3	3.3	9.2	5.1	−9.7	−33.6	3.2	−7.5	−3.0	−3.0	1.5	−1.3	−2.2	−2.4
PsSW 13b	−2.0	2.7	3.8	6.8	−3.0	−1.7	3.4	11.7	−1.7	1.4	1.4	−2.9	1.8	1.5
PsSW 15a (534)	1.4	2.5	1.6	1.7	3.2	7.7	1.6	1.6	1.9	3.3	−1.2	1.5	2.8	8.5
PsSW 15b (445)	2.9	3.4	2.1	1.8	−1.7	−1.0	−1.3	−1.5	1.7	3.1	19.8	39.4	8.4	36.0
PsSW 15c (624)	1.7	2.5	1.4	2.4	85.4	58.4	−1.1	1.4	3.7	9.1	−2.0	−1.3	−8.3	−2.6
PsSW 17 (17) (Clade IV)	2.2	3.5	1.7	1.6	−1.1	4.7	−6.5	−3.5	−2.1	−2.0	−7.3	5.0	3.4	34.0

Colour scale: | <−54.0 | −18.0 to 53.9 | −17.9 to 6.0 | −5.9 to 2.0 | −1.9 to 1.9 | 2.0 to 5.9 | 6.0 to 17.9 | 18.0 to 53.9 | >54.0 |

(b) Line 104–SUT1, 382, 562 vs Bolero (COTYLEDONS)

Target genes	Developmental stages																				
	12d 104	12d 382	12d 562	14d 104	14d 382	14d 562	16d 104	16d 382	16d 562	18d 104	18d 382	18d 562	20d 104	20d 382	20d 562	23d 104	23d 382	23d 562	30d 104	30d 382	30d 562
PsIPT 1 (604)	3.8	1.4	3.0	2.8	3.2	2.9	−1.1	1.2	2.3	2.9	6.3	2.6	−1.1	1.9	1.8	1.3	1.2	1.1	–	–	–
PsIPT 2 (605)	2.4	−2.2	3.4	−1.3	6.1	8.0	5.6	−2.2	2.0	1.6	7.4	3.1	−1.2	1.7	1.7	1.5	1.2	−3.1	–	–	–
PsIPT 4 (421)	1.5	11.0	12.0	1.8	10.9	13.3	2.4	7.0	8.6	6.8	12.1	12.6	1.4	4.1	3.6	1.0	4.1	3.5	1.0	5.3	5.9
PsCKX 1 (930)	4.9	−2.5	1.3	−1.4	2.3	2.2	−1.2	−4.3	2.8	4.8	5.7	1.7	−1.3	1.1	1.2	1.4	1.9	−1.7	3.7	48.3	14.2
PsCKX 2 (627)	2.1	−2.2	2.7	−2.4	4.9	4.7	2.2	−4.3	−1.1	−1.1	4.7	1.7	−1.7	−1.4	−1.4	−1.1	−1.5	−4.5	−1.2	2.6	1.4
PsCKX 5 (942)	–	1.5	1.2	–	1.2	1.3	–	1.1	1.0	–	1.3	1.1	–	1.4	1.5	–	1.0	1.0	–	1.2	1.1
PsCKX 7 (910)	1.3	2.4	4.4	−1.1	2.4	1.4	−5.6	−1.9	−1.2	−1.1	−4.0	−4.0	−4.9	1.0	−1.0	1.4	9.9	1.7	21.7	34.9	4.1
PsSUT 1 (366 Transgene)	−1.7	−3.6	−1.2	1.1	7.2	2.1	1.7	−3.6	3.9	1.6	2.7	−1.3	−1.2	−1.0	3.3	−1.8	−1.2	1.1	−3.7	2.8	8.7
PsSUT 2 (948)	2.7	2.3	3.5	1.1	7.2	2.1	1.4	2.6	3.4	1.6	2.7	−1.3	1.2	1.2	4.0	1.8	1.5	2.1	3.7	10.4	32.3
PsSUT 3 (674)	−8.3	1.1	1.7	−1.9	3.0	4.4	−1.5	−3.1	1.2	−1.2	3.3	1.1	−2.4	−1.1	−1.3	−2.3	2.1	−3.3	−1.4	3.6	3.4
PsSUT 5 (666)	1.9	−1.1	3.5	−1.1	10.5	19.1	1.6	−2.3	12.4	−1.4	3.5	5.3	1.9	1.3	12.1	2.1	1.9	1.5	1.0	16.2	5.2
PsAAP 1(3a) (532 Transgene)	−2.8	13.0	1.9	−1.2	13.7	2.1	−6.3	1.5	1.7	−3.1	3.6	−6.3	−9.7	1.7	−3.4	−3.2	4.3	−2.4	−17.9	5.1	2.6
PsAAP 7a (498)(Cluster 1)	−2.6	−1.9	2.2	2.3	2.0	2.1	−1.7	−16.2	−4.2	−1.7	−2.1	−4.1	−3.0	1.2	−1.9	−2.6	2.1	−5.6	6.1	24.5	17.4
PsAAP 7b (9261)	−1.5	1.4	−4.1	2.6	4.1	8.1	−2.5	−30.1	−1.7	540.2	87.1	70.7	1.5	−1.1	1.3	−1.3	1.1	−2.4	2.9	3.9	3.1
PsAAP 2a (675)(Cluster 3A)	−7.3	−1.8	−1.6	2.2	7.8	11.8	−4.9	−6.8	−1.0	10.4	5.6	1.1	1.7	1.8	1.0	3.7	3.6	1.4	5.1	5.8	1.5
PsAAP 2c (4401)	3.2	−1.6	5.1	3.0	7.0	7.9	2.4	−3.0	1.4	1.0	−1.1	2.0	−1.0	1.2	1.4	1.6	2.5	−1.1	3.6	4.8	5.2
PsAAP 2d (840)	1.5	−1.6	2.0	−2.1	3.7	3.9	−1.8	−9.3	−3.7	1.4	7.7	1.2	−1.6	1.4	−1.4	1.3	1.8	−3.7	3.3	4.5	2.6
PsAAP 3b (051)	4.7	−2.0	−1.2	2.2	−1.6	−1.6	−1.5	−10.1	−1.8	122.9	21.1	11.1	1.3	3.6	−1.9	−1.5	1.4	−2.3	5.3	9.3	8.6
PsAAP 6a (931) (Cluster 4B)	1.2	2.0	2.6	−1.7	−5.4	5.2	2.0	7.3	−3.6	−4.9	7.8	1.6	1.5	1.6	1.0	1.1	1.7	−2.6	2.3	3.7	1.3
PsAAP 6b (328)	−4.3	−2.6	1.0	−1.7	2.6	2.8	−3.0	−5.7	−2.2	−1.1	1.5	−7.2	−1.7	1.5	−1.6	−3.1	−1.1	−8.1	8.4	20.9	3.0
PsAAP 1 (180)	3.9	−1.9	1.9	1.8	1.6	2.0	−1.8	−1.4	−3.1	−1.1	1.4	1.2	−2.3	7.0	2.0	1.9	3.6	1.7	−23.0	−1.1	−1.6
PsCWINV 1 (240)	−1.2	3.1	1.1	−10.5	−2.8	2.4	12.6	−1.4	6.9	1.4	2.9	1.6	−5.1	−1.6	−1.1	7.8	4.1	10.2	2.0	1.6	1.4
PsCWINV 2 (448)	−1.4	−3.2	3.7	5.2	9.0	17.9	31.6	−3.6	6.3	6.3	9.5	3.8	−1.0	3.0	4.0	11.6	13.7	4.5	183.3	240.4	499.4
PsCWINV 3 (415)	−4.7	−2.1	−2.6	−1.6	2.7	4.8	8.1	−1.9	−1.1	−1.2	1.2	−1.7	1.6	1.5	1.2	1.4	2.9	−1.1	−4.8	−16.4	−12.3
PsCWINV 6 (320)	1.4	−1.0	2.5	−1.2	3.4	37.8	5.1	−1.2	7.1	−11.2	−4.2	−7.9	−17.2	2.4	3.1	16.6	3.1	1.4	7.2	2.9	3.1
PsSW 1 (1) (Clade I)	–	1.1	2.8	–	4.8	4.9	–	1.9	2.1	–	1.3	1.1	–	1.4	3.1	–	1.7	−1.3	–	8.3	5.5
PsSW 2a (2a)	−1.6	−1.7	1.6	−1.5	4.5	2.2	1.7	−1.4	−2.7	1.6	−2.3	−2.4	−1.0	−1.2	−1.0	−1.5	−1.4		−11.8	−1.9	−1.7
PsSW 2b (2b)	–	1.2	2.8	–	6.6	5.0	–	3.6	1.6	–	−1.6	−1.1	–	2.1	2.0	–	24.8	8.9	–	20.5	5.7
PsSW 4 (7) (Clade II)	–	1.8	1.6	–	4.5	4.3	–	3.7	2.0	–	1.4	2.0	–	1.4	1.5	–	30.9	3.9	–	1.6	7.3
PsSW 5a (6a)	–	1.7	4.9	–	4.8	11.2	–	2.9	1.4	–	−2.5	−1.9	–	1.6	1.5	–	23.2	4.3	–	8.7	1.7
PsSW 5b (6b)	–	1.2	2.1	–	4.6	4.6	–	1.6	1.1	–	1.1	1.2	–	1.0	1.0	–	2.1	1.4	–	1.3	2.0
PsSW 7 (4)	–	1.1	1.9	–	3.0	4.5	–	1.5	1.1	–	1.0	1.8	–	3.1	1.1	–	1.4	−1.4	–	2.1	5.0
PsSW 13 (13a) (Clade III)	24.8	−1.3	3.3	−1.1	9.2	5.1	−4.6	−9.7	−33.6	3.0	3.2	−7.5	−5.3	−3.0	−3.0	1.3	1.5	−1.3	−5.6	4.7	−2.4
PsSW 12 (13b)	3.7	−2.0	2.7	−1.6	3.8	6.8	2.8	−3.0	−1.4	−1.4	3.4	11.7	−2.6	−1.7	1.4	1.1	1.4	−2.9	−12.2	−4.1	−6.7
PsSW 15a (534)	–	−1.6	2.3	–	14.0	5.4	–	2.8	1.2	–	−1.4	−1.3	–	−1.8	−1.3	–	7.5	−1.3	–	10.6	10.8
PsSW 15b (445)	–	1.1	2.7	–	5.2	4.5	–	−3.3	−1.7	–	−1.2	−1.2	–	−14.1	−1.2	–	−1.2	−2.0	–	11.5	−1.1
PsSW 15c (624)	–	3.8	1.9	–	2.9	5.4	–	−1.1	−1.0	–	2.0	2.0	–	−1.3	2.9	–	−1.1	1.1	–	2.6	3.5
PsSW 15d (623)	–	1.1	1.9	–	6.8	5.1	–	1.5	2.7	–	1.1	1.3	–	1.1	1.1	–	1.7	1.3	–	1.8	4.1

Fig. 5.   Relative expression of cytokinin biosynthesis (*PsIPT*), cytokinin degradation (*PsCKX*), sucrose transporters (*PsSUT*), amino acid transporters (*PsAAP*), cell wall invertase (*PsCWINV*) and sucrose efflux carriers (*PsSWEET* (*PsSW*)) gene family members in (*a*) seed coats; (*b*) cotyledons from the double transgenic pea lines 382 and 562 and the single transgenic line 104-SUT1. Seed coats and the embryo axes were separated from cotyledons from 12 days after pollination (DAP) to 30 DAP (12d to 30d). Values are fold-changes relative to the expression in the wild-type 'Bolero' line. The colour scale indicates upregulated expression (red), similar expression (white), and downregulated expression (blue) relative to that in the wild-type line. Expression measurements not made are noted as '-'.
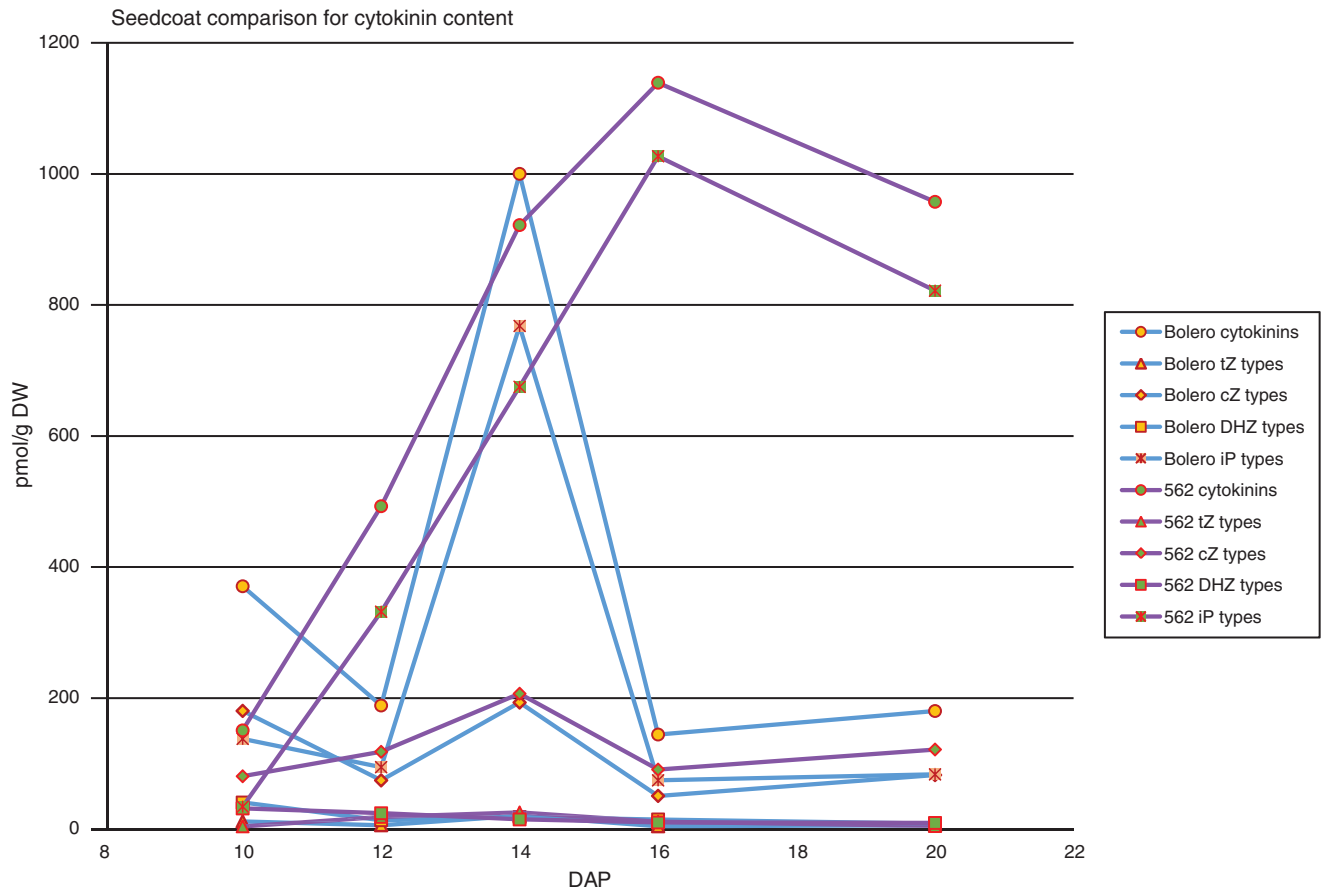
Fig. 6. Cytokinin content of pea seed coats from 12 days after pollination (DAP) to 20 DAP in the wild-type line 'Bolero' and the double transgenic line 562. Total cytokinin, *trans*-zeatin (tZ)-types, *cis*-Z (cZ)-types, dihydro-Z (DHZ)-types and isopentenyladenine (iP)-types are shown.

Clade III *SWEET* GFMs were upregulated relative to the 'Bolero' control. *PsIPT4* and *2* were consistently upregulated, as was *PsCKX2*. Both *PsIPT4* and *PsCKX2* were also upregulated in transgenic field pea donor parent 1/55-AAP1(3a) (Fig. S4).

Cotyledons

In contrast to the leaves, pod walls and seed coats (Figs 3, 4), neither the *AAP1(3a)* transgene nor the *SUT1* transgene were consistently elevated in the cotyledons of the double transgenics from 12 DAP relative to the 'Bolero' control during seed development (Fig. 5b). In 104-SUT1 cotyledons, expression of the *SUT1* transgene and the *AAP1(3a)* GFM was generally more similar to 'Bolero' than to the double transgenics. From 12 DAP, the expression of *PsIPT4* was consistently elevated throughout seed development in the double transgenic lines, but not so in the 104-SUT1 line, relative to the 'Bolero' control (Fig. 5b). At 14 DAP, expression of most of the *IPT, CKX, SUT, AAP, CWINV* and *SWEET* GFMs were strongly elevated in the double transgenics, but not in the single transgenic line 104-SUT1.

Endogenous cytokinins

Multiple cytokinin forms were detected in the pea tissues, including the nucleotides, free bases and ribosides of both zeatin (Z)-type and isopentenyl adenine (iP)-type cytokinins in the double

transgenic and wild-type pea lines (Supplementary material Table S1). Both c*is*- and *trans*-Z derivatives were detected along with metabolites of dihydrozeatin. *O*-glucoside forms as well as 7- and 9-glucoside forms of zeatin were detected, but not the 7- or 9-glucoside forms of iP. The *cis*-Z nucleotide (*c*ZRMP) was the most significant form in the pod wall, while isopentenyladenosine-5′-monophosphate (iPRMP) was in the seed coat. The N-glucosides were in slightly greater amounts in seed coats and the *O*-glucosides in pod walls relative to other organs (Table S1).

In the wild-type 'Bolero', the total cytokinin content of the pod walls was relatively constant (Table S1), whereas it peaked in the seed coat at 14 DAP (Fig. 6). A decrease in total cytokinin levels was apparent in the cotyledons over time. Relative to pod wall and cotyledons, the seed coat contained the greatest amounts of cytokinin – predominantly as iPRMP.

The most obvious difference between wild type and the double transgenic line was the increased concentration of iPRMP in the seed coats over a longer period of development than in the 'Bolero' control (Fig. 6). This was reflected, but to a lesser extent, in the amounts of iP and iPR in the seed coats.

Discussion

Peas are domesticated temperate legumes and have often been chosen as a model to study regulation of C : N source to sink

transport processes because of their relatively large seed and ease of manipulation (Patrick and Offler 1995, 2001). The seeds are the main harvestable product and used either for sucrose or starch (C storage pool) or protein (N storage pool). Process peas and field peas differ in significant ways. Process peas are generally determinate and harvested immature when sucrose content is high. They have been intensively bred for high sucrose content at ~20–25 DAP. The desiccated seeds from process peas are wrinkled and normally have green cotyledons. Field peas are generally indeterminate and harvested when peas are mature and dry. They are round and can have yellow or green cotyledons. They are used for animal feed and for protein and starch extraction. Through genetic modification followed by a backcrossing and selfing program (Fig. S1), we have produced novel process pea hybrids that overexpress both the *PsSUT1* gene (recurrent process pea parent), and the *PsAAP1* (*3a*) gene (donor field pea parent) so the resulting lines are phenotypically similar to a process pea. After fertilisation, embryo development proceeds through morphogenesis involving rapid cell division followed by a period of cell expansion, then by maturation and accumulation of storage products, and finally by desiccation and dormancy (Weber *et al*. 2005).

Final yield in the double transgenic lines is driven by expression changes in source leaves and seed coats

The most marked changes in gene expression in the double transgenics relative to 'Bolero' control occurred in the leaves subtending the young pods and in the seed coats throughout development of the cotyledons.

The 35S::*PsAAP1*(*3a*) transgene was strongly expressed in the leaves 5 DAP subtending young pods (5 DAP) as were other members of Cluster 3A. As Cluster 3A *AAP* GFMs are involved in the loading of amino acids into the phloem (Tegeder *et al*. 2007; Tan *et al*. 2010; Tegeder and Ward 2012), we suggest this implies an increased 'pushing' of amino acids towards the sink tissues.

Clade III *SWEET*s were upregulated in the younger transgenic leaves. Members of this clade have been identified as being involved in the mobilisation of sucrose out of the leaves (Chen *et al*. 2012). Release of sucrose into the leaf apoplast by the SWEETs was matched by sucrose import through SUTs into companion cells of the phloem in the transgenic leaves as indicated by the strong upregulation of *SUT*s in the transgenic leaves subtending the pods 5 DAP. Clade IV *SWEET17*, known to be involved in import of hexoses to the vacuole for storage (Chardon *et al*. 2013), was strongly downregulated. This again emphasises the effect of the transgenes on these leaves as sources of sucrose for the developing pods and, along with the decreased *PsIPT* expression and the enhanced *PsCKX*7 expression, supports the transitioning of young sink leaves into strong source leaves (Ninan *et al*. 2019).

Cytokinins have been implicated in regulating cell division during early seed development (Jameson and Song 2016) and in enhancing sink strength (Quesnelle and Emery 2007; Jameson and Song 2016). They have been shown to control pod number in legumes (Kambhampati *et al*. 2017) and seed number in

Arabidopsis (Bartrina *et al*. 2011), oil seed rape (Schwarz *et al*. 2020), and rice (Ashikari *et al*. 2005).

In pods and seed, at the early stages of development during mitotic cell division, strong expression of the *AAP* transgene was induced by the 35S promoter. We suggest that the increased expression of the *AAP1*(*3a*) transporter increased amino acid import into seed enabling the development of an increased number of seeds. The resulting increased N levels in the developing pod and seed tissues may have been the stimulus for the increased cytokinin biosynthesis in the seed coat (Gu *et al*. 2018; Kieber and Schaller 2018). This conjecture is supported by the findings of Götz *et al*. (2007) who showed increased cytokinin in *Vicia* seeds overexpressing an *AAP* gene. The cytokinins in turn could have enhanced not only the activity of CWINV, but also the expression of both the *SWEETS* and *SUTs* (Jian *et al*. 2016) leading to seeds with a transiently increased sugar content. In *Vicia faba*, CWINV activity in the seed coat was positively correlated with seed size, leading Weber *et al*. (1996) to suggest that hexose supply to the developing seed was a controlling element of final seed size. Wang and Ruan (2012) suggested invertase-mediated sugar signalling and cross-talk by hormones contribute to regulation of cell cycle control genes. Here, the double transgenics showed increased *PsCWINV* expression after fertilisation and in whole seed relative to the 'Bolero' control creating the high-sugar environment that promotes cell division (Weber *et al*. 2005). Together, the activity of transporters, CWINV and cytokinin is likely to have contributed to the increased seed number and seed size.

Delivery of metabolites to the expanding cotyledons is enhanced in the double transgenic lines

We separated the cotyledons from the seed coat and pod walls from 12 DAP, which is close to the beginning of the cotyledon cell expansion phase, to further understand the delivery of metabolites from the maternal seed coat for release into the seed apoplasm and subsequent uptake by the cotyledons via their transfer cells.

A function of cytokinin in seed is to control sink strength through activity of CWINV, whereas cytokinin degradation is controlled by CKX (Jameson and Song 2016). In the double transgenic cotyledons, expression of the cytokinin biosynthetic gene *IPT* was upregulated from 12 DAP, while *CKX* expression was not, again suggesting enhanced uptake of sugars and amino acids. By 14 DAP there was strong upregulation of gene expression in the cotyledons of the double transgenics compared with 'Bolero' or the single transgenic line 104-SUT1 for most of the genes tested. This suggests that the cotyledons of the double transgenics were in the metabolically highly active cell expansion stage and, through the signalling role of sucrose (Patrick *et al*. 2013; Li and Sheen 2016) along with elevated expression of the *PsIPT* genes, appeared to be enabling enhanced uptake of C and N possibly via the epidermal transfer cells at the cotyledon surface (Patrick and Offler 2001; Offler *et al*. 2003). At a similar developmental stage Lu *et al*. (2020) reported increased endosperm sucrose, supporting increased sucrose flow from the seed coat to the cotyledons.

PsSUT2 and *PsSUT5* were upregulated more highly in the seed coat and in the cotyledons than the *PsSUT1* transgene as
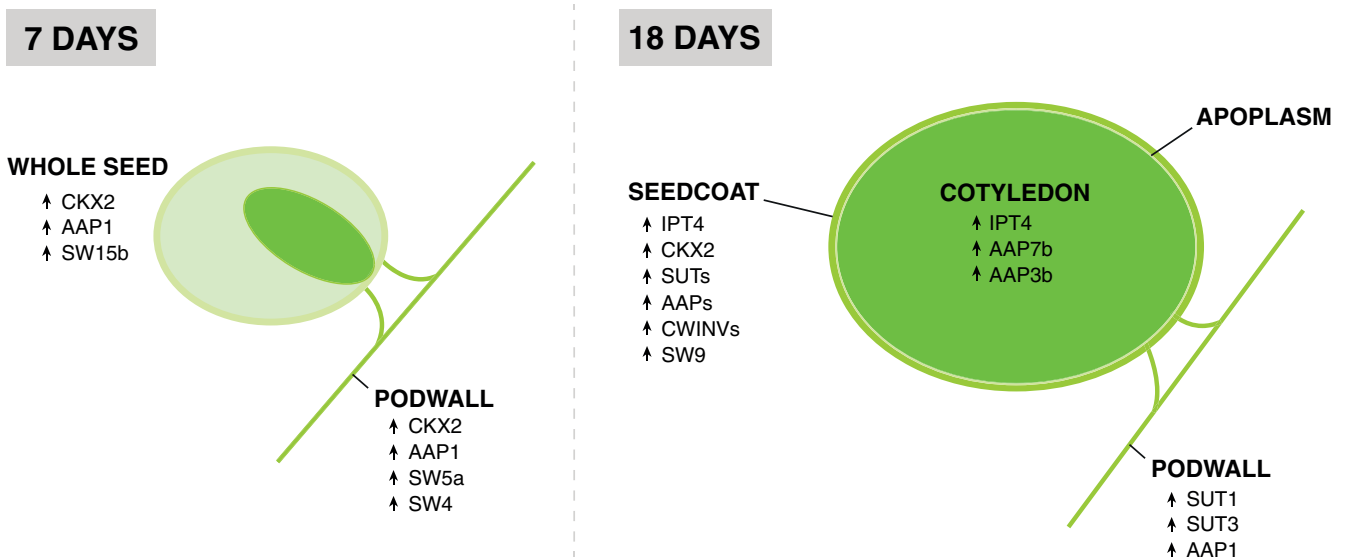
Fig. 7. Summary of gene expression regulation in the seed of the double transgenic lines 382 and 562 compared with Bolero at (*a*) 7 days after pollination (DAP), and (*b*) 18 DAP, showing the upregulation occurring in the podwall, seed coat, and cotyledons.

seen in Lu *et al.* (2020). Sauer and Stolz (1994) suggest that *SUT1* and *SUT2* may have evolved from a common ancestral gene and showed that, in *Arabidopsis*, they may operate differently at different pH or that possibly one transporter may be involved in loading, while the other one may be more involved with unloading.

The transition from the morphogenesis to maturation and storage activities has been associated with a decrease in invertase expression and activity (Wang and Ruan 2013) and, in forage brassica, expression of both *BnIPT* and *BnCWINV* was shown to be restricted to the morphogenesis phase of seed development (Song *et al.* 2015). In the double transgenic lines, we show that the expression of these genes continued into the maturation phase (Figs 5, 7) and appeared to disturb the clear transition from morphogenesis to storage activities (Weber *et al.* 1995, 2005).

Concurrent expression of SUT1 *and* AAP1(3a) *transgenes has the potential to lengthen the optimal harvest opportunity for process peas and increase yield*

As the pea seed progresses towards maturation, starch and protein synthesis increase in the cotyledons while sucrose content decreases. Process peas are generally harvested at a tenderometer (note: a tenderometer is a device that measures tenderness as the force required to effect shearing) reading of 95–110 (D. Goulden, pers. comm.). This is equivalent to a seed stage ~19–23 DAP and a seed dry matter content of 20–25%. During this time starch production has increased to where its quantity is about the same or slightly greater than that of sucrose. Harvesting process peas is a balance between sweetness and yield. The crossover point, at which time the concentration of sucrose and starch are approximately equal, was delayed in the double transgenic lines by 1–2 days. This delay meant a potentially useful increase in the harvest window for the pea crop from being just 2–3 days to being 3–4 days. Essentially the double transgenic cotyledons contain a higher sucrose pool,

which maintains sweetness of the pea for longer. This increase in sucrose is reflected in the small burst in gene expression in the cotyledons at 18 DAP, and also significantly elevated gene expression in the seed coats. In particular, we note *IPT*, *CKX*, *SUT*, *AAP7*, and *CWINV* gene family members were upregulated in the seed coats and *IPT*, *AAP*, and *CWINV* in the cotyledons. As a consequence, upregulation of these and/or other genes, led to increased assimilate transfer from the seed coat via the apoplast and into the cotyledons, finally resulting in enhanced accumulation of storage products.

During maturation in the double transgenics, *AAP* GFMs were upregulated and in particular *AAP7*. While a role for AAP7 has yet to be determined (Tegeder and Ward 2012), its strong upregulation at 18 DAP in both seed coat and cotyledons, indicates a possible role in the switch from cell expansion to maturation and storage compound accumulation.

Cytokinins have been associated with enhancing sink strength and seed loading in legumes (Emery *et al.* 2000; Hwang *et al.* 2012; Kambhampati *et al.* 2017). In our double transgenic line 562, the increase in endogenous cytokinin in the seed coat continued through the cotyledon expansion phase and into the maturation and storage product accumulation stage (Fig. 6). It is unlikely that this cytokinin was imported from the phloem, as developing seeds, in contrast to the pod wall, do not appear to act as a sink for xylem or phloem cytokinins (Jameson *et al.* 1987; Emery *et al.* 2000), although they actively accumulate nutrients. In pea, both the endogenous data (Table S1) and the gene expression data (Fig. 5*a*) indicate that the seed coat is also a site of cytokinin biosynthesis, as has been suggested for soybean and lupins (Singh *et al.* 1988; Emery *et al.* 2000). Enhanced biosynthesis within the seed coat is the most likely source of the elevated cytokinin that led to the extended accumulation of iPRMP in the double transgenic line compared with the wild-type 'Bolero'. Recently, Kambhampati *et al.* (2017) suggested that the iP-type cytokinins may play a significant role during storage product accumulation in soybean – similar to the pattern

seen here in pea. The enhanced cytokinin is, therefore, likely to have affected the overall source-sink dynamics leading to a high sugar environment for metabolism and maturation. High sugar also stimulates N uptake through amino acid synthesis and increased seed/grain protein (Weschke *et al*. 2000; Rosche *et al*. 2002; Rosche *et al*. 2005; Zheng 2009; Lu *et al*. 2020). By 30 DAP, pea C and N are being accumulated into storage pools (protein and starch) in preparation for desiccation and dormancy.

Conclusion

By developing double transgenic homozygous lines for *SUT* and *AAP* we were able to investigate effects of pyramiding these transporters in early pod stages, seeds, seed coats, cotyledons and in leaves subtending pods. By separating the seed coat from the developing cotyledon from the cell expansion stage, we highlighted the effect of the seed coat on seed development. When *SUT* and *AAP* were overexpressed separately in pea lines, seed metabolite levels were enhanced and resulted in increased seed yield and protein content (Miranda *et al*. 2001; Zhang *et al*. 2015; Lu *et al*. 2020). In the double transgenic homozygous lines, gene expression was further upregulated and greater increases in protein content and seed yield were obtained. The C : N dynamics of the double transgenic lines were changed allowing increases in the cotyledon sucrose pool thereby potentially extending the harvest window for the process pea crop. It is evident that cytokinins have a key role in seed development, as interplay of cytokinin signalling along with sucrose signalling ensured that normal seed development proceeded and operated at an increased capacity to enhance yield.

Conflicts of interest

The authors declare no conflicts of interest.

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