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Research

Evidence that auxin is required for normal seed size and starch synthesis in pea

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Summary

- In recent years the biosynthesis of auxin has been clarified with the aid of mutations in auxin biosynthesis genes. However, we know little about the effects of these mutations on the seed-filling stage of seed development.
- Here we investigate a key auxin biosynthesis mutation of the garden pea, which results in auxin deficiency in developing seeds. We exploit the large seed size of this model species, which facilitates the measurement of compounds in individual seeds.
- The mutation results in small seeds with reduced starch content and a wrinkled phenotype at the dry stage. The phenotypic effects of the mutation were fully reversed by introduction of the wild-type gene as a transgene, and partially reversed by auxin application.
- The results indicate that auxin is required for normal seed size and starch accumulation in pea, an important grain legume crop.

Introduction

Auxin is a key plant hormone that is implicated in many aspects of plant growth and development. In recent years our understanding of auxin biosynthesis has improved immeasurably, and it now appears that the main auxin in plants, indole-3-acetic acid (IAA) is synthesized mainly from tryptophan, via one intermediate, indole-3-pyruvic acid (IPyA, Zhao, 2012; Brumos *et al.*, 2014; Tivendale *et al.*, 2014). In the seeds of pea and its relatives, a parallel, chlorinated pathway produces large amounts of chlorinated auxin (4-Cl-IAA; Tivendale *et al.*, 2012).

The importance of the IPyA pathway has been established with the aid of mutations in genes encoding enzymes for the conversion of tryptophan to IPyA and of IPyA to IAA. Mutations affecting these steps have been used to demonstrate the importance of auxin for the first phase of seed development (embryogenesis; Cheng *et al.*, 2007), but to a much lesser extent to investigate the second phase, during which embryos expand and mature.

The garden pea (*Pisum sativum*) has many advantages for research on seed development. The seeds are large, and compounds can be readily analysed in individual seeds. Furthermore, there already exists a range of pea mutations that affect the dry seed phenotype. Because starch is a major component of dry seeds, it is not surprising that several of these affect starch biosynthesis. The most famous is the *rugosus* (*r*) mutation, which causes 'wrinkling' of the dry seeds (Wang *et al.*, 1998). This wrinkling is a striking and distinct phenotype, which enabled Mendel to use the round/wrinkled difference in his pivotal experiments on inheritance (Ellis *et al.*, 2011; Reid & Ross, 2011). Because starch is a key component of human diets, seed-wrinkled mutants, affected in starch biosynthesis, were the focus of a previous mutagenesis program (Wang *et al.*, 1998).

Here we present evidence that in pea seeds, auxin is important for the second, seed-filling, phase of seed development, including starch accumulation. In this species, the gene *PsTAR2* encodes an aminotransferase that catalyses the conversion of tryptophan to IPyA acid, and of chlorinated tryptophan to chlorinated IPyA (Tivendale *et al.*, 2012). A loss-of-function mutation in *TAR2*, *tar2-1*, dramatically reduces the content of 4-Cl-IAA and IAA in pea seeds, analysed whole, with this reduction occurring mainly during the second phase (Tivendale *et al.*, 2012; Cook *et al.*, 2016; Supporting Information Table S1). We show that *tar2-1*

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causes seed wrinkling, and attribute this to reduced starch content in the mutant seeds. The wrinkling, reduced starch content and small size of mutant seeds were reversed by transgenic introduction of the wild-type *TAR2* allele or, at least in part, by auxin application. The activity of certain starch synthesis enzymes is reduced in mutant seeds, as is the expression of genes encoding those enzymes or sub-units of those enzymes.

Materials and Methods

Plant material

The *tar2-1* (referred to as *tar2* in Tivendale *et al.*, 2012) and *tar2-2* mutations were obtained by Targeting Induced Local Lesions in Genomes (TILLING), using the cultivar Caméor as the starting line. The *TAR2* and *tar2-1* plants used resulted from six to eight generations of backcrossing with Caméor.

The *tar2-2* mutation (previously uncharacterized) results in a mis-sense protein (G to A substitution at position 759 in the wild-type (WT) genomic sequence JQ002584 and a G to R amino acid substitution in the corresponding protein, at position 107). For *tar2-2*, there were 5 generations of back-crossing with Caméor. The *tar2-1* mutation was isolated from the TILLING line 918, and *tar2-2* from line 3930. The *rr* line used was Hobart line 53 (Murfet, 1971). Plants from the cross *tar2-1* × *rugosus* (*r*) were genotyped for both genes. The *RB* and *rb* lines were JI3318 and JI3319, respectively.

Growth conditions

Plants (apart from those relating to Figs 3, 5, 6, S6 and Table S2) were grown under an 18 h photoperiod consisting of natural daylight extended morning and evening with high pressure sodium 400 W lamps (Vialox NAV-E, SON-E, Osram, Munich, Germany) in pendant mount fittings (HL400HPSCG, Pierlite, Padstow, NSW, Australia) with acrylic diffusing covers delivering, *c.* 100 μmol m^{-2} s^{-1} at the pot surface. The temperature was regulated such that the maximum usually ranged from 20 to 28°C during the day and the minimum from 12 to 16°C at night. The nutrient solution was Peters Professional Peat-Lite Special (Scotts Australia, Baulkham Hills, NSW, Australia).

The plants used for Figs 3, 5, 6, S6 and Table S2 were grown in 2-l pots in growth chambers under a light : dark regime of 16 h : 8 h, light : dark at 19°C : 16°C. Light intensities were 330 μmol m^{-2} s^{-1} at the pot surface and up to 550 μmol m^{-2} s^{-1} at the tip of the plant. Nutrient solution (0.4% Hakaphos Blau; Compo Expert, Munster, Germany) was applied weekly, starting from 4 wk after sowing. Harvested material was frozen in liquid N_2 and stored at −80°C until preparation.

Determination of genotypes

Mature seed DNA was extracted as described in Tivendale *et al.* (2012), or using the Isolate II Plant DNA kit (Bioline, Alexandria, NSW, Australia). DNA was amplified by PCR as described by Tivendale *et al.* (2012). Where leaf material was genotyped, the Terra PCR direct polymerase mix (Clontech, Mountain View, CA, USA) was used. The genotype was ascertained by RFLP (Tivendale *et al.*, 2012; primers noted in Table S3) or by sequence analysis (Macrogen). As no restriction site was present at the *tar2-2* mutation site, a dCAP primer was used to artificially insert a cut site for the enzyme pstI. To genotype *R* (*Starch Branching Enzyme I*; accession number X80009.1), PCR fragments were visualized by gel electrophoresis (*rr* plants carry an 800-bp insertion; Bhattacharyya *et al.*, 1990).

Electron microscopy and light microscopy

Sections (30 μm) of dry pea seeds were prepared using a Reichert Wein Nr 12054 slide microtome (Vienna, Austria) with a Physitemp BFS-3MP freezing stage (SDR Scientific, Sydney, Australia). For dry 2,4-dichlorophenoxyacetic acid (2,4-D)-treated seeds, cotyledonary material was scraped using a razor blade into 100 μl dH_2O (after removal of the testa) in a consistent area in treated and untreated cases from the plane 90 degrees to the hilum. The sections were gently agitated to release starch granules from cells. Unstained material was examined under a Zeiss Axioscop 2 plus microscope (Carl Zeiss, Göttingen, Germany), equipped with differential interference illumination, with images taken using an Axiocam HRC digital camera. Axiovision 3.1 was used for scaling. For cell size estimation, the sections (unstained) were viewed with a Leica DM 1000 light microscope (Nussloch, Germany) with a ×10 objective, and images taken with a Nikon Digital Sight DS-Fi2 camera (Melville, NY, USA). Cell area was measured using ImageJ v.1.48 (National Institutes of Health, Bethesda, MD, USA), on the basis of sections from five replicate seeds, with 10 images of different sections per replicate. To view starch granules using scanning electron microscopy, droplets of water containing starch granules were placed onto stubs, dried for 1 h at 40°C, coated in platinum using a BalTec SCD 050 sputter coater (Leica Microsystems, Wetzlar, Germany) and imaged with a Hitachi Su-70 field emission scanning electron microscope (Tokyo, Japan).

Analysis of starch content and amylose/amylopectin ratio

In order to obtain the data presented in Figs 2 and 4, whole dry seeds (including the testa) were harvested from *TAR2 tar2-1* plants (in order to ensure that the growth environment was identical), or in subsequent experiments, *R tar2-1*, *r tar2-1*, *R TAR2* and *r TAR2* plants, with individual genotyped seeds comprising a replicate. The seeds were ground to a fine powder with a mortar and pestle in liquid nitrogen. The total starch content and amylose/amylopectin ratio of the resulting powder was assessed using the Amylose Amylopecin Assay Kit (Megazyme, Wicklow, Ireland). Absorbance at 510 nm was measured using a Lightwave S2000 UV/Vis spectrophotometer (Biochrom WPA, Cambridge, UK). Alternatively (Figs 3, 6), powdered samples were extracted twice with 80% ethanol at 60°C and supernatants were pooled in separate tubes, vacuum-evaporated and dissolved in sterile water. The sucrose content of the supernatant fraction was determined

enzymatically, according to Heim *et al.* (1993). The starch-containing insoluble residuals were solubilized in 1 N KOH for 1 h at 95°C and neutralized 5 N HCl. Starch was hydrolysed with amyloglucosidase and glucose formation was determined enzymatically, as described by Rolletschek *et al.* (2002).

Application of 2,4-D

The synthetic auxin, 2,4-D, was introduced into seeds by application to the leaf subtending the pod concerned, which was the first-formed pod or the pod immediately above that pod. 2,4-D was dissolved in 10% ethanol in water, containing 0.1% Tween 20. The aliquot applied was 20 µl, containing 50 µg ml^{-1} of 2,4-D, and the solvent was spread over both leaflets of the subtending leaf. The application began when the pod was 6–8 mm wide, *c.* 14 d after pollination (DAP), and continued weekly for 4 more weeks.

Extraction, purification and quantification of hormones and sucrose by UPLC-MS

Whole seeds (including the testa) were harvested, from homozygous mutant or WT plants, with individual seeds (each from a different plant) comprising a replicate. The FW of harvested seeds was between 200 and 400 mg. The seeds were weighed, placed into Eppendorf tubes, frozen in liquid nitrogen and stored at −70°C. Indole-3-acetic acid (IAA) and chlorinated auxin (4-Cl-IAA) were extracted and quantified (Tivendale *et al.*, 2012; Lam *et al.*, 2015); the internal standards were: [$^{13}C_6$] IAA (from Cambridge Isotope Laboratories, Andover, MA, USA), [$^{2}H_4$] 4-Cl-IAA and $^{13}C_{12}$ sucrose (Sigma-Aldrich).

Samples were analysed using a Waters Acquity H-series UPLC coupled to a Waters Xevo triple quadrupole mass spectrometer, with a Waters Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm × 1.7 µM particles). For auxins, the UPLC-MS conditions were as described by Tivendale *et al.* (2012), except for IAA, where the flow rate was 0.25 ml min^{-1}. The mass spectrometer was operated in positive ion electrospray mode with needle voltage of 2.5 KV. The ion source temperature was 130°C. The desolvation gas was nitrogen at 950 l h^{-1}. Cone gas flow was nitrogen at 50 l h^{-1}, and the desolvation temperature was 450°C. The conditions, and ions monitored, for sucrose were as described in Mason *et al.* (2014). The transitions monitored for auxins were: for IAA, *m/z* 176.07 → 130.06; for [$^{13}C_6$] IAA, *m/z* 182.09 → 136.08; for 4-Cl-IAA, *m/z* 210.05 → 164.05; and for [$^{2}H_4$] 4-Cl-IAA, *m/z* 213.05 → 167.05; Data were analysed using MassLynx software (2005). For all samples, the ratio of endogenous ion intensity to internal standard ion intensity was calculated and used to determine the concentration of hormone per gram FW.

Assay of ADP–glucose (ADPG), UDP–glucose (UDPG) and hexose phosphates

For the measurement of ADPG, UDPG and hexose phosphates, frozen embryo material was ground in liquid nitrogen. Aliquots (30 mg) of this powder were extracted in 250 µl of ice-cold chloroform/methanol (3 : 7, v/v). The mixture was held at −20°C for 2 h with occasional mixing and water soluble compounds were extracted from the organic phase by the addition of 200 µl water. After centrifugation at 10 621 ***g*** for 10 min, the upper, aqueous methanol phase was transferred to a new 2-ml collection tube and kept at 4°C. The lower phase was re-extracted with 200 µl as already described and the second aqueous phase was combined with the first. The combined extracts were evaporated to dryness using a vacuum dryer and were dissolved in 100 µl of water.

A slightly modified procedure to that described by Schwender *et al.* (2015) was used to separate metabolites, by hydrophilic interaction chromatography. A liquid chromatograph (Ultimate 3000; Dionex, Sunnyvale, CA, USA) was coupled to a triple quadruple mass spectrometer (API4000; ABSciex, Singapore) in negative ion mode. Aliquots (1 µl) of the extract were passed through an aminopropyl column (Luna NH2; 250 × 2 mm, particle size of 5 µm; Phenomenex). The LC solvents were: solvent A, 20 mM ammonium acetate plus 20 mM ammonium hydroxide in 95:5 water:acetonitrile, pH 9.45; and solvent B, acetonitrile. The gradient was: 0 min, 75% B; 2 min, 75% B; 6 min, 10% B; 20 min, 0% B; 24 min, 0% B; 25 min, 75% B; and 30 min, 75% B. Nitrogen was used as a curtain gas, nebulizer gas, heater gas, and collision gas. The ion spray voltage was set to −4000 V, the capillary temperature was 450°C, and the dwell time for all compounds was 100 ms. Compound identities were verified by mass and retention time matches to authenticated standards. External calibration was applied using authenticated standards.

Enzyme activity assays

Quick-frozen and pulverized embryo material was extracted in 5 volumes of 100 mM MOPS (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 2 mM DTT. Homogenates were centrifuged for 5 min at 10 000 ***g*** and 4°C. Supernatants were immediately assayed for enzyme activities of sucrose synthase (Weber *et al.*, 1996), UDP-glucose pyrophosphorylase (Kleczkowski *et al.*, 2005), phosphoglucomutase (Manjunath *et al.*, 1998) and ADP-glucose pyrophosphorylase (AGPase, Weber *et al.*, 1995). Each enzyme assay was confirmed to be dependent on substrate and linear in respect to time and amount of extract.

Quantitative real-time PCR

Mature green seeds of *TAR2* and *tar2-1* were harvested, and RNA extracted as described by Tivendale *et al.* (2012). Four replicates were taken for each genotype, each comprising one seed. cDNA was synthesized (Tetro cDNA synthesis kit, Bioline) using random hexamers. First-strand cDNA was diluted 20-fold, with 1 µl used per 10-µl quantitative real-time PCR reaction using SYBR Green chemistry (SensiFAST™ SYBR® No-ROX Kit; Bioline). Samples were set up with a CAS-1200N robotic liquid-handling system (Corbett Research) and run for 50 or 55 cycles in a Rotor-Gene RG3000A Dual-Channel machine (Qiagen). Three technical replicates were performed for each biological

replicate, and the concentrations were calculated relative to a curve containing seven serial 10-fold dilution points of original pooled cDNA taken equally from each replicate. Reaction efficiencies and correlation coefficients were as described by Tivendale *et al.* (2012). Pea 18s ribosomal RNA levels were utilized as described by Ozga *et al.* (2003), in place of a housekeeper gene. Primers used to amplify *PGM1*, *PGM2*, *AGPS1*, *AGPS2* and *AGPL1* (accession numbers AJ250769.1, AJ250770.1, X96764.1, X96765.1, X96766.1, respectively) are listed in Table S3, or, for *TAR1* and *TAR2*, are given in Tivendale *et al.* (2012).

USP::TAR2 complementation

The coding region of *PsTAR2* full-length cDNA was amplified by standard PCR methods using a Phusion DNA polymerase (Thermo Scientific, Karlsruhe, Germany) and the primer pair for *TAR2* (Table S3). The resulting PCR product was restricted and cloned under control of the seed-specific *Vicia faba USP*-promoter (Zakharov *et al.*, 2004) into the binary vector pBar, yielding *USP::TAR2*. Plasmids were sequence-verified and transformed into *Agrobacterium tumefaciens* strain EHA 105. Transgenic pea plants were produced by *Agrobacterium*-mediated transformation of excised WT (*TAR2*) embryo axes of the pea cultivar Erbi (Schroeder *et al.*, 1993). In order to generate crossings between *USP::TAR2* and *tar2-1*, pollen of homozygous *USP::TAR2* plants was transferred onto emasculated flowers of homozygous *tar2-1* plants. Segregation of T-DNA insertion was monitored by Southern Blot using a [^{32}P] dCTP labelled PCR-fragment of the *ocs* terminator (Miranda *et al.*, 2001). PCR-based genotyping of WT *TAR2* and mutant *tar2-1* alleles was performed in accordance with Tivendale *et al.* (2012). To allow separation of native *TAR2* and *tar2-1* alleles from the *USP::TAR2* insertion, an alternative reverse primer for *TAR2* (Table S3), complementary to a *PsTAR2* intron sequence, was used.

Promoter analysis

The promoter regions of the pea starch-related genes, *AGPL1*, *AGPS1*, *AGPS2* and *PGM2*, and of the sucrose synthase-encoding gene *SUS1*, were analysed *in silico* for auxin response elements (AuxREs), *cis*-acting elements that confer auxin responsiveness to genes, using the motif search tool FIMO v4.11.2 (http://meme-suite.org/tools/fimo; Grant *et al.*, 2011). Only previously published AuxREs were considered in the analysis.

Statistical analysis

The significance of differences between means was determined by Student's *t*-test.

Accession numbers

The sequences reported in this paper are in the GenBank database: accession nos. JQ002584 and X80009.1.

Results

The *tar2-1* mutation causes seed wrinkling

The seeds of *tar2-1* plants are strongly wrinkled at the dry stage (Fig. 1). Because seed wrinkling in pea is associated with impaired starch synthesis (Wang *et al.*, 1998), we investigated the starch characteristics of dry *tar2-1* seeds. The starch content of *tar2-1* seeds was markedly reduced, both on a per gram (Fig. 2) and per seed basis (Table 1), but with no change in the relative proportions of amylose and amylopectin (Fig. 2). Mutant seeds had dramatically smaller starch granules than did WT seeds, and mutant granules were abnormally 'pointy', simple in structure and sometimes heart-shaped (Fig. 2a,b). The mutant granules at the dry stage resembled WT granules at a green (immature) stage (Fig. S1), indicating that starch deposition in the mutant granules lags behind that in the WT seeds.

In some respects the phenotype of *tar2-1* seeds resembles that of the other pea wrinkled mutants, including Mendel's *r* mutant (Fig. S2). However, *tar2-1* seeds typically have a large depression in the side of each cotyledon (Fig. 1), with a concomitant flattening in the plane 90 degrees to the hilum, characteristics that are not caused by the other wrinkled-seed mutations. However, in *tar2-1* seeds, the reduction in starch content on a per gram basis (*c.* 40%) was similar in magnitude to that reported previously for Mendel's *r* mutation (Wang *et al.*, 1998). The *r* mutation results in an accumulation of sucrose (Stickland & Wilson, 1983), and we found that in *tar2-1* seeds also, sucrose accumulated (Fig. S3).

In order to further investigate how the *r* and *tar2-1* mutations interact, if at all, we generated the *r tar2-1* double mutant. On the *r* background, *tar2-1* increased the severity of wrinkling, reduced the size of starch granules (Fig. S2), and further reduced the starch content (Fig. 2), indicating that although both *tar2-1* and *r* affect starch accumulation, and consequently seed shape, they do so by affecting different steps in the starch biosynthetic pathway.

The *tar2-1* mutation reduces seed size

Mature *tar2-1* seeds were markedly smaller than WT seeds, as shown by DW data (Table 1). A smaller, but statistically

Fig. 1 Phenotypes of *TAR2 TAR2* and *tar2-1 tar2-1* pea seeds at the dry stage. This illustrates the large depression in the side of the cotyledon in the mutant.

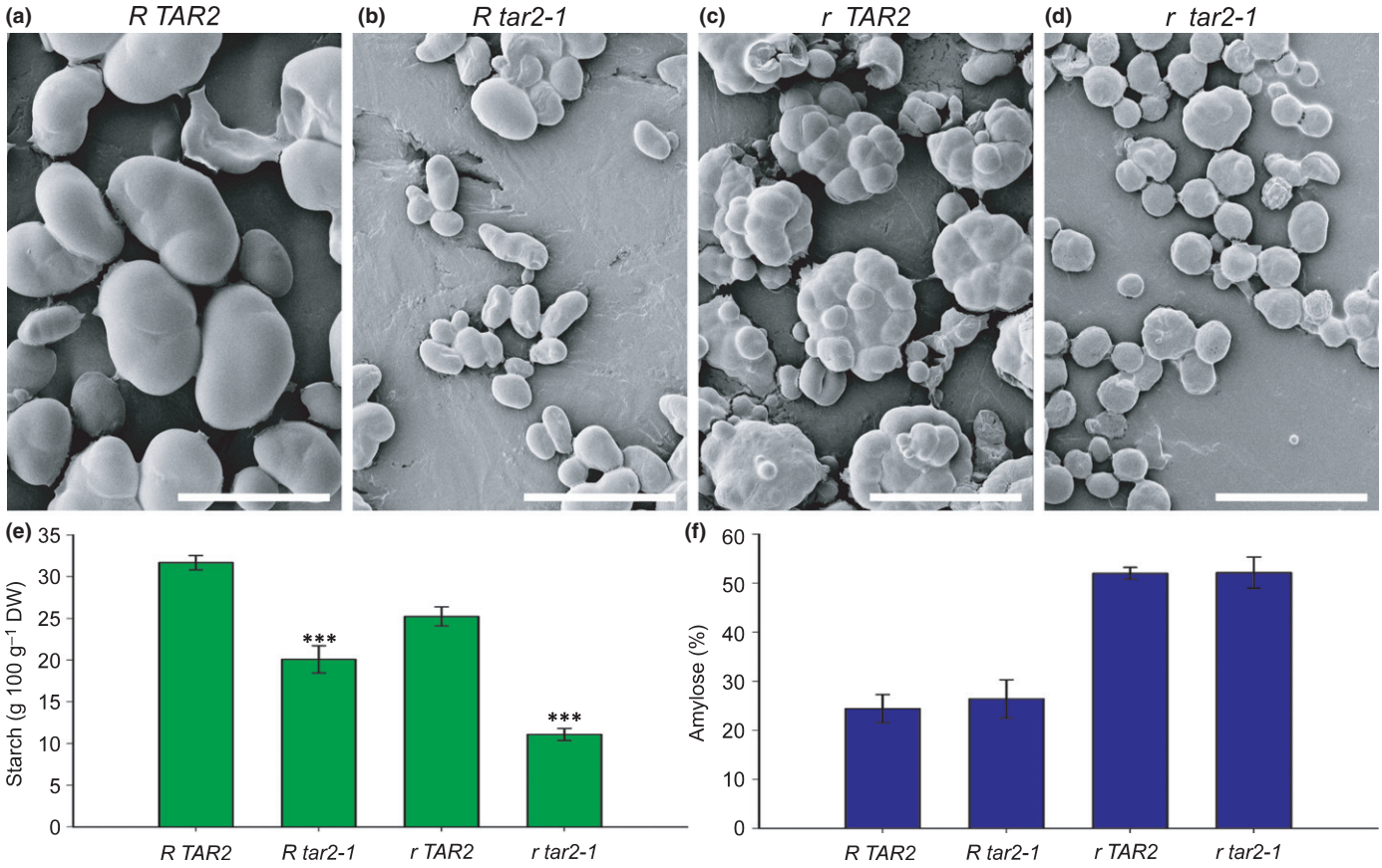

Fig. 2 The effects of *tar2-1* on starch granule morphology. Scanning electron microscopy (SEM) images of starch granules in (a) *R TAR2*, (b) *R tar2-1*, (c) *r TAR2* and (d) *r tar2-1* pea seeds at the dry stage, as well as (e) overall starch content and (f) percentage of amylose, in seeds carrying the *tar2-1* and *r* mutations. Seeds giving rise to (a) and (b) are on a different genetic background to those in (c) and (d); valid comparisons are therefore only possible between (a) and (b) or (c) and (d). Shown are the mean concentrations ± SE ($n = 4$). *** Indicates that the difference between *TAR2* and corresponding *tar2-1* means is significant at the 0.001 level. Bars, 40 μm.

Table 1 Effects of *tar2* mutations on pea seed characteristics

Genotype	Weight per seed (mg) ± SE	Cell area (μm^2) ± SE
TAR2	265 ± 4.6 (a)	10 343 ± 883 (a)
tar2-1	108 ± 5.9 (b)	4071 ± 320 (b)
tar2-2	220 ± 7.8 (c)	8301 ± 556 (a)

Shown are the average weights (mg) of dry seeds from the pod one node above the node of first flower for *TAR2* (Caméor), *tar2-1* and *tar2-2* plants, as well as 2D cell areas (μm^2). Data are means ± SE, from four replicate plants of each genotype. Means within a column with different letters indicate a significant difference at the 0.01 level.

significant, reduction was observed for *tar2-2* seeds (Table 1), which, furthermore, were not wrinkled. The reduced seed size in *tar2-1* seeds cannot be fully accounted for by the decrease in starch, which was typically *c.* 60 mg per seed whereas the total reduction in dry seed weight was at least 150 mg in (Tables 1, S1). Much of the reduction in seed size can be accounted for by the smaller cells in mutant seeds at the dry stage (Table 1). On the basis of seed size, *tar2-1* seeds were distinguishable from those of the other starch synthesis mutants, the seeds of which are typically similar in size to WT seeds (e.g. Stickland & Wilson, 1983).

The phenotype of *tar2-1* seeds is attributable to the *tar2-1* mutation itself

The *tar2-1* mutation was obtained by a TILLING approach, as described by Tivendale *et al.* (2012). After eight generations of back-crossing to the parental cultivar, Caméor, followed by six generations of single-plant selection, the wrinkled character co-segregated with the *tar2-1* mutation (Fig. S4). Among *c.* 300 individuals, no homozygous *TAR2* plant produced wrinkled seeds resembling those of *tar2-1* plants. Mutant *tar2-1* plants occasionally produced seeds (less than 5%) that were not wrinkled, but not fully WT in appearance either (described here as 'dimpled'); in other words, there is slightly variable expressivity of the wrinkled trait in this mutant.

In order to definitively test whether seed wrinkling results from the *tar2-1* mutation, rather than from a mutation in a closely linked gene, we crossed a line carrying the transgene *USP::TAR2* (as well as the native *TAR2* gene) with the *tar2-1* mutant. From the progenies of this cross, we identified plants that were homozygous for *tar2-1* and homozygous for the *TAR2* transgene, all of which produced 100% round seeds (13 plants, 654 seeds). Plants that were homozygous for *tar2-1* and lacking the transgene produced 100% wrinkled seeds (8 plants, 354 seeds). Nine plants

that were homozygous for *tar2-1* and hemizygous for the transgene produced 307 round seeds and 76 wrinkled seeds; a small deviation from the expected 3 : 1 ratio. By growing empty vector control plants in parallel, it was shown that transformation by itself did not affect the seed phenotype.

In summary, therefore, introduction of the *TAR2* transgene counteracted the wrinkled phenotype of the *tar2-1* mutation (Fig. 3), indicating that it is the *TAR2* gene itself that is responsible for the round seed phenotype. Introduction of this gene also restored the starch content and seed size to those of WT seeds (Fig. 3).

Application of the synthetic auxin, 2,4-D, partially rescued the seed phenotype

The *tar2-1* mutation appears to specifically affect IAA and 4-Cl-IAA (Cook *et al.*, 2016), and the preceding results therefore implicate auxins in the control of starch accumulation and seed shape. Therefore, we tested the effects of applying auxin. For this purpose we used the synthetic, mobile auxin, 2,4-D, because previous experimentation showed that when this auxin is applied to a mature pea leaf, it is exported and can be detected in seeds from the pod subtended by the application leaf (S. D. Cook & J. J. Ross, unpublished). The phenotype of *tar2-1* seeds was partially rescued by 2,4-D applied in this way, with treated seeds showing a reduced degree of wrinkling and increased starch concentrations (Fig. 4). However, 2,4-D did not restore starch content of mutant seeds to that of the WT (Fig. 4). Application of 2,4-D restored seed DW to *c.* 70% of the WT weight and starch granules from treated mutant seeds resembled those of the WT (Figs 4, S5). Application of 2,4-D at the same dose did not affect the appearance of WT seeds, nor their starch content (Fig. 4).

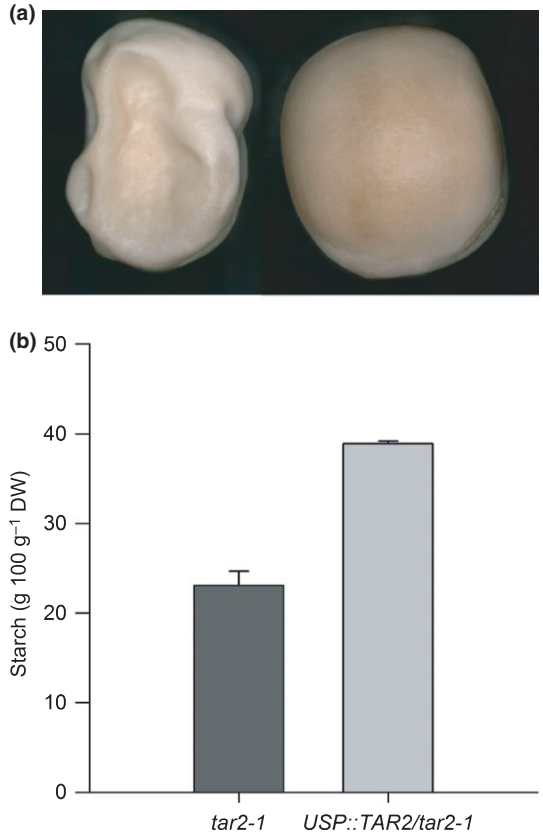

Fig. 3 Rescue of the wrinkled *tar2-1* phenotype by transgenic introduction of *TAR2*. (a) Pea seeds from plants of genotype *tar2-1* with no transgene (and lacking the vector; left) and with the *TAR2* transgene (right). (b) Corresponding starch concentrations. Bars are SEs ($n = 5$); $P < 0.001$. Dry seed weights: *tar2-1* with no transgene, 152 ± 6 mg; *tar2-1* with the *TAR2* transgene, 331 ± 7 mg.

The *tar2-1* mutation reduces the activity of enzymes related to starch synthesis, and the expression of the corresponding genes

In order to further investigate the basis of the reduced starch synthesis in *tar2-1* seeds, we monitored the contents of intermediates involved in the sucrose to starch pathway in 24-d-old *tar2-1* embryos. In plastids, the immediate starch precursor, ADP-glucose, is formed from glucose-1-phosphate by AGPase, whereas glucose-1-phosphate is formed from glucose-6-phosphate by phosphoglucomutase. In the cytosol, UDP-glucose is converted to glucose-1-phosphate by UDP-glucose pyrophosphorylase (Wang *et al.*, 1998). Analysis by LC-MS showed that in *tar2-1* embryos the ADP-glucose content was significantly reduced, by *c.* 40%, whereas the concentration of hexose phosphates was higher than in the WT (Table S2). The concentration of UDP-glucose was unaltered in the mutant. These metabolic changes were consistent with those in *rb* embryos, affected in AGPase activity (Hylton & Smith, 1992), and provide evidence that decreased ADP-glucose availability reduces starch synthesis in *tar2-1* embryos. We next analysed the activities of four enzymes concerned with the conversion of sucrose to starch (Wang *et al.*,

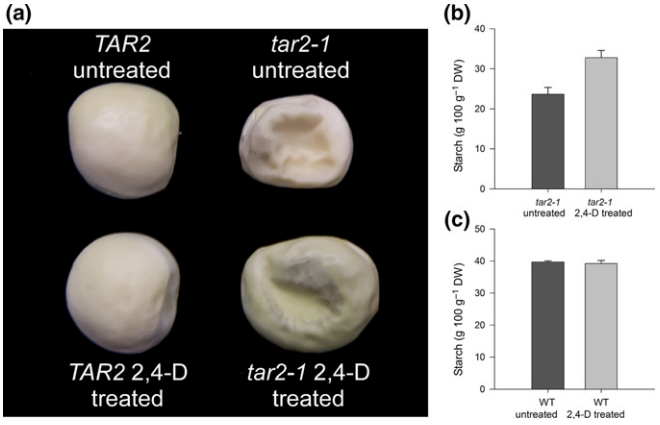

Fig. 4 Partial rescue of the wrinkled *tar2-1* phenotype by application of 2,4-dichlorophenoxyacetic acid (2,4-D). (a) Phenotypes of dry pea seeds. The DWs (mg) of untreated and treated *tar2-1* mutant seeds from adjacent nodes of an individual plant were 127 ± 16 ($n = 4$) and 200 ± 14 ($n = 5$; $P < 0.05$), respectively. DWs of *TAR2* seeds, untreated and treated, were 280 ± 9 ($n = 4$), and 266 ± 12, respectively ($n = 5$). (b, c) Starch levels (per weight basis) in control and 2,4-D-treated *tar2-1* (b) and WT (c) seeds. Bars are SEs ($n = 3$; $P < 0.05$ in b and n.s. in c). In (b), on a per seed basis the concentrations were (mg): control, 28.6 ± 6.8 and 2,4-D-treated, 70.6 ± 3.9 ($P < 0.01$).

1998). Starch synthases and starch branching enzyme were not monitored because *tar2-1*, unlike *r, rug5* and *lam* (Wang *et al.*, 1998), does not affect the ratio of amylose to amylopectin (*RUG5* and *LAM* encode starch synthases).

The activity of AGPase was the most affected, with an almost three-fold reduction in the mutant (Fig. 5: $P < 0.001$ at all time points). Phosphoglucomutase activity was also reduced in mutant seeds ($P < 0.01$ at all time points), whereas that of UDP-glucose pyrophosphorylase was essentially unaltered. The activity of sucrose synthase was slightly elevated in the mutant 24 DAP ($P < 0.01$). To determine the basis of reduced enzyme activity, we monitored the expression of the genes that encode the subunits of AGPase (*AGPS1*, *AGPS2*, and *AGPL1*; Fig. 5; Burgess *et al.*, 1997), as well as the genes that encode phosphoglucomutase *(PGM1* and *PGM2*). In mutant seeds, there was a statistically significant reduction in mRNA level for the large AGPase subunit gene (*AGPL1*, also known as *RB*), whereas the reductions for those encoding the two small subunits (*AGPS1* and *AGPS2*) were significant only at the 0.1 level (Fig. 5). There was also a significant reduction in mRNA level for *PGM2* (Fig. 5), whereas the reduction for the second, *PGM1*, was significant only at the 0.1 level. *PGM2* equates to the pea *RUG3* gene, and encodes the plastidial form of phosphoglucomutase, whereas *PGM1* encodes the cytosolic form (Harrison *et al.*, 2000).

We next analysed the promoter regions of *AGPL1*, *AGPS1*, *AGPS2* and *PGM2,* and of the sucrose synthase-encoding gene *SUS1* (Barratt *et al.*, 2001) for auxin response elements (AuxREs; Table S4). AuxREs are *cis*-acting elements involved in the transcription of auxin-inducible genes such as *PsIAA4/5* (Ballas *et al.*, 1993, 1995; Li *et al.*, 1994; Ulmasov *et al.*, 1995). The *AGPS2* promoter had seven AuxREs, including the canonical TGTCTC (Liu *et al.*, 1994), and in addition, the well-established TGTCAC pea AuxRE and five TGTC variants (Table S4; Ballas *et al.*, 1993; Guilfoyle *et al.*, 1998; Mironova *et al.*, 2014). The *PGM2* promoter contained 10 AuxREs: TGTCTC, the palindromic GAGACA element (Ballas *et al.*, 1993; Ulmasov *et al.*, 1997), two TGTCAC and six TGTC variants. In the *PGM2* promoter, two of the 10 AuxREs were downstream of the putative TATA box; however, it has been suggested that AuxREs in the 5′UTR may also impart auxin responsiveness (Mironova *et al.*, 2014). The *AGPL1* and *AGPS1* promoters had four and six TGTC variants, respectively, but did not contain the canonical AuxRE, TGTCTC. However, although these findings indicate that the genes concerned are auxin-responsive, it should be noted that the promoter of the *SUS1* gene also contained AuxREs (although not TGTCTC), despite the finding that sucrose synthase activity was not reduced in extracts from mutant seeds (Fig. 5).

Timing of the effects of *tar2-1*: the mutation does not affect embryogenesis

Seed development in pea is considered to occur in two main stages. The first phase (embryogenesis) is characterized by strong mitotic activity and organogenesis in the embryo, whereas in the second phase, embryonic cells expand and storage products are produced (Borisjuk *et al.*, 2002). In pea, the first phase lasts until the overall seed weight is *c.* 50 mg, *c.* 12 DAP (Borisjuk *et al.*, 2002).

During the first phase of seed development *tar2-1* seeds closely resembled those of the WT in overall appearance, and the embryos were also similar in the two genotypes (Fig. S6). The *tar2-1* mutation began to reduce embryo FW, and overall seed FW (compared with the WT) 12–14 DAP (Fig. 6). A reduction in starch content of the embryos was first observed at the 14 d stage, and an increase in sucrose content at 18 DAP (Fig. 6). A reduction in the content of bioactive auxin was observed at 14 DAP in a previous study (Tivendale *et al.*, 2012). In summary, the effects of the *tar2-1* mutation are not manifest until the beginning of the second, seed-filling, phase of seed development (Borisjuk *et al.*, 2002). Consistent with that, the final cell size is dramatically reduced in the *tar2-1* mutant (Table 1).

The *tar2-1* mutation primarily affects the seed phenotype, and indirectly reduces germination and seedling growth

The germination rate of small, strongly wrinkled *tar2-1* seeds was markedly reduced compared with the WT, with fewer than half of the wrinkled seeds germinating (Table 2). However, *tar2-1* seeds from the same mutant plant as the wrinkled seeds, but which were merely 'dimpled' (because of low expressivity), germinated at the WT rate of 100% (Table 2). Wrinkled seeds that did germinate gave rise to small seedlings (40–50% of the WT height), whereas dimpled seeds germinated to produce seedlings that were 70–80% of the WT size at 13 d after sowing, in terms of leaflet size, plant height and petiole length (Table 2; Fig. S7), and usually of WT height by the pod-bearing stage (Fig. S7). With regard to the additional traits of rate of leaf expansion, node and time of flowering, flower shape, pod length and number of seeds per pod, those plants from dimpled *tar2-1* seeds were very similar to WT plants (Table 2; Fig. S7). Indeed, shoots derived from *tar2-1* seeds (wrinkled or dimpled) did not show developmental defects. Plants that germinated from dimpled *tar2-1* seeds produced wrinkled seeds, showing that the occasional low expressivity of *tar2-1* seed traits does not have a genetic basis. Consistent with that, the dimpled seeds were from the uppermost two pod-bearing nodes, and the wrinkled seeds from the remaining lower two nodes, from the same parental plant, which had resulted from eight generations of single plant selection (after a back-crossing program with Caméor).

The wrinkled character is determined by the zygotic genotype

Further evidence indicates that the phenotype of mutant *tar2-1* seeds is determined by the zygotic genotype, rather than by the maternal genotype or phenotype. When *TAR2* pollen was used to pollinate *tar2-1* ovules developing on a *tar2-1* plant the resulting seeds were WT in appearance (Fig. S8). Furthermore, when *TAR2 tar2-1* plants were allowed to self-fertilize, the resulting seeds showed a 3 : 1 segregation for the WT: wrinkled character (apart from examples of variable expressivity), despite the fact that the testas of all these seeds were heterozygous (Fig. S4).

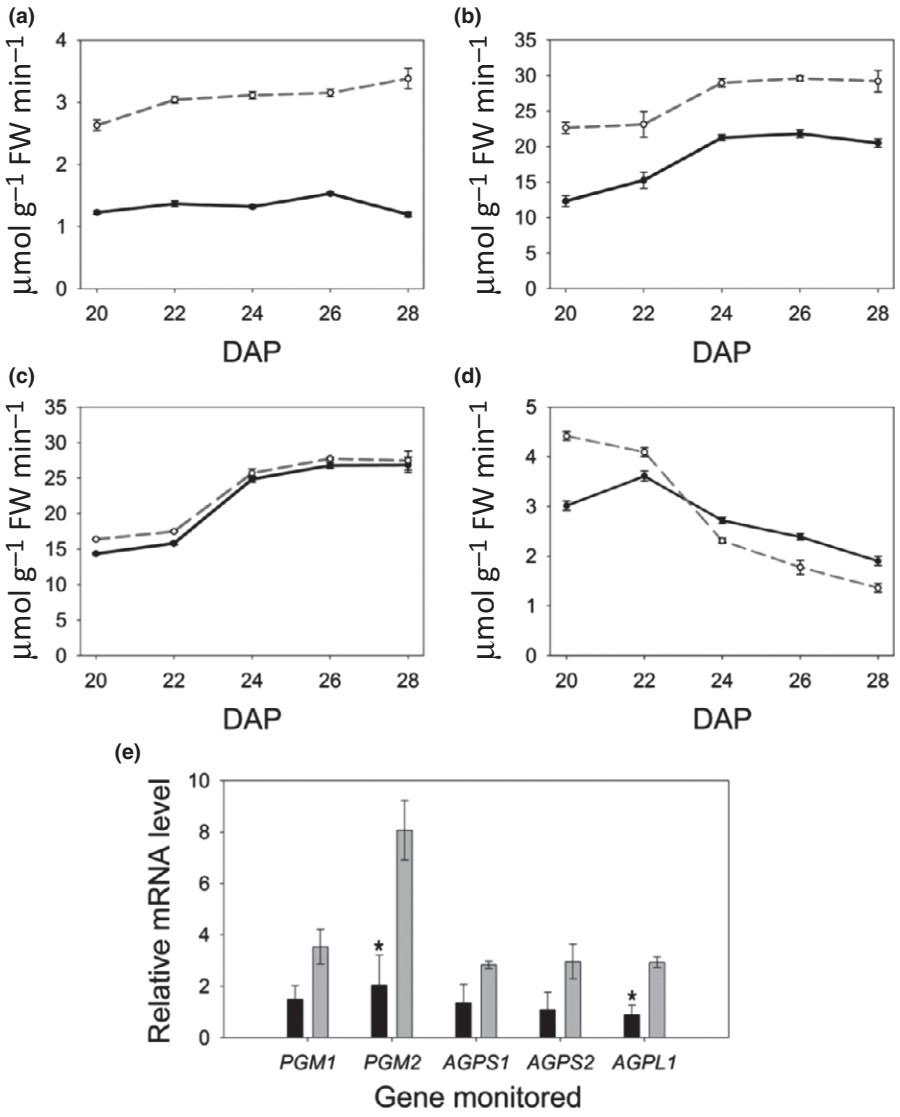

Fig. 5 Effects of *tar2-1* on the activity of enzymes related to starch synthesis and expression of the corresponding genes. Enzyme activity (μmol g^{-1} FW min^{-1}) of (a) ADP glucose pyrophosphorylase; (b) phosphoglucomutase; (c) UDP glucose pyrophosphorylase; and (d) sucrose synthase in *TAR2* (grey dashed line) and *tar2-1* (black solid line) pea seeds, between 20 and 28 d after pollination (DAP). (e) Relative mRNA levels of *PGM1* and *PGM2* (encoding different isoforms of phosphoglucomutase), and two small and one large subunits of ADP glucose pyrophosphorylase, *AGPS1*, *AGPS2* and *AGPL1* in *TAR2* (grey) and *tar2-1* (black) pea seeds; * indicates a significance difference at the $P < 0.02$ level. Shown are mean concentrations ± SE (where SE exceeds the symbol). (a–d), $n = 5$; (e) $n = 4$. The reference gene was pea 18s (Ozga *et al.*, 2003).

Consistent with observations that it is primarily the cotyledons/embryo that are affected in the mutant, we found that in WT seeds at the 13 DAP stage, *TAR2* was more strongly expressed in the embryo than in the liquid endosperm, whereas the opposite was the case for *TAR1* (Table S5). A third *TAR* gene, *TAR3*, was previously shown to be only weakly expressed throughout pea seed development (Tivendale *et al.*, 2012). The germination and growth rate of the *tar2-2* mutant resembled that of the WT, and the seeds of this weaker mutant were not wrinkled.

Discussion

Auxin is required for normal seed growth and starch deposition in pea

The *tar2-1* mutation was the first shown to directly impair auxin biosynthesis in the seeds of a grain legume (Tivendale *et al.*, 2012). The effects of *tar2-1* indicate that auxin deficiency results in small, wrinkled seeds, and our evidence indicates that the wrinkling is due to reduced starch content. It has been shown previously that mutations which affect starch synthesis typically result in wrinkled seeds (Wang *et al.*, 1998). In these mutants, as in *tar2-1*, sucrose accumulates, and it is thought that the resulting increase in osmotic potential causes increased water flow into the seeds. On drying, the seeds display the wrinkled phenotype (Bhattacharyya *et al.*, 1993).

The strongly reduced DW of *tar2-1* seeds distinguishes this mutant from other seed wrinkling mutants of pea. Mendel's *rugosus* (*r*) mutant, for example, produces seeds of wild-type (WT) size (Stickland & Wilson, 1983). The normal size of *r* seeds indicates that reduced starch biosynthesis alone does not necessarily result in reduced DW. Consistent with that, the reduction in starch content per seed in the *tar2-1* mutant accounted for < 50% of the overall decrease in DW per seed (Tables 1, S2). Therefore, there are two discernible effects of auxin deficiency in pea seeds: reduced seed size and reduced starch content. It cannot be discounted that the second effect is a

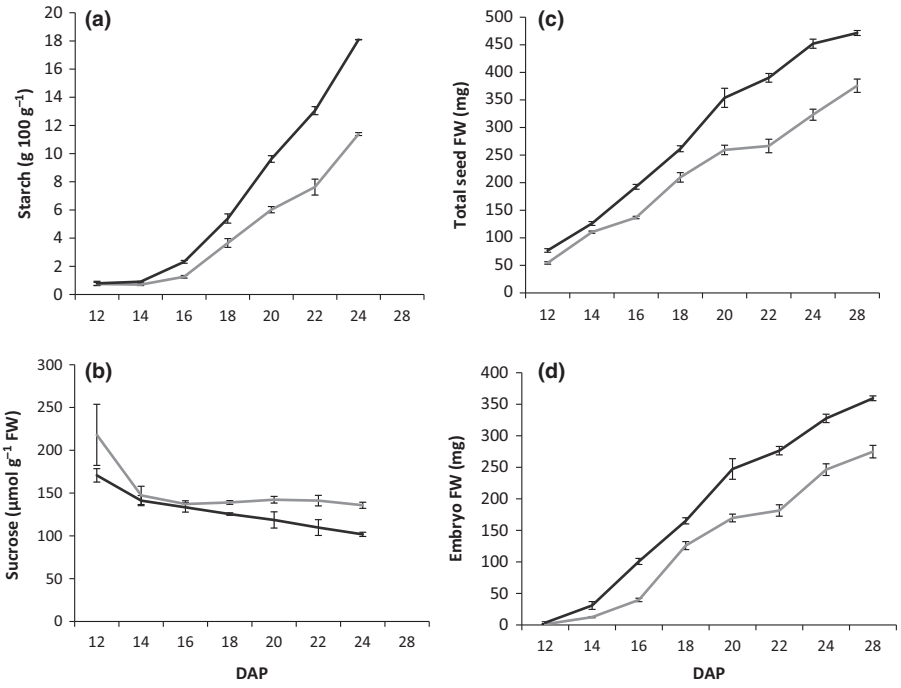

Fig. 6 Time course of (a) starch and (b) sucrose content of embryos, (c) total seed FW, and (d) embryo FW for wild-type (WT; black line) and *tar2-1* (grey line) pea seeds. Data are means ± SE ($n = 3$–12). All differences between *TAR2* and *tar2-1* were significant ($P < 0.05$) at and after 12 d after pollination (DAP) for weights, at and after 14 DAP for starch content, and at and after 18 DAP for sucrose content.

Table 2 Vegetative and reproductive characteristics of *TAR2* and *tar2-1* pea plants

	TAR2 ($n = 10$)	*tar2* (from wrinkled seeds; $n = 3$)	*tar2-1* (from dimpled seeds; $n = 7$)
Germination	100% (10 of 10)	37.5% (3 of 8)	100% (7 of 7)
Leaf at node 4 (mm)			
Leaflet length	29.4 ± 0.6 (a)	18.0 ± 1.5 (b)	24.9 ± 0.7 (c)
Leaflet width	25.6 ± 0.7 (a)	12.8 ± 1.5 (b)	19.2 ± 1.0 (c)
Petiole length	14.9 ± 0.4 (a)	9.3 ± 0.9 (b)	12.4 ± 0.4 (c)
Plant height day 21 (mm)	138.5 ± 3.1 (a)	60.7 ± 7.3 (a)	107.4 ± 6.0 (a)
Leaves expanded day 21	6.0 ± 0.0 (a)	5.0 ± 0.0 (b)	6.0 ± 0.0 (a)
Flowering node	12.0 ± 0.0 (a)	10.7 ± 0.3 (b)	11.9 ± 0.1 (a)
Flowering time (d)	35.7 ± 0.2 (a)	38.5 ± 0.5 (b)	36.4 ± 0.3 (a)
Pod length	73.8 ± 1.1 (a)	55.0 ± 8.9 (a)	70.0 ± 0.72 (b)
Seeds per pod	5.2 ± 0.2 (a)	2.3 ± 0.9 (a)	4.3 ± 0.3 (b)

Plants of genotype *tar2-1* are split into two groups: those produced by wrinkled seeds and those from dimpled seeds (seeds with low expressivity). The pod length and seeds per pod data are from pods at the flowering node (node of first flower). All seeds produced by the 10 *tar2-1* plants were wrinkled. Data are means ± SE; within a row, different letters indicate a significant difference compared with the wild-type (WT), at the $P < 0.05$ level.

consequence of the first, although the presence of AuxREs in the promoters of starch-related genes is consistent with a direct effect of auxin on starch synthesis. AuxREs have been reported also in the promoter of an Arabidopsis starch synthesis gene (*PGM*; Mazarei *et al.*, 2003) and in those of tomato AGPase subunit-encoding genes (Sagar *et al.*, 2013). It cannot be discounted either that the auxin deficiency in *tar2-1* seeds impairs the transport of sucrose within the expanding cotyledon; for example, the formation of sucrose transfer cells at the cotyledonary surface may be affected by auxin status (Andriunas *et al.*, 2013), but this in itself would not explain the accumulation of sucrose in mutant embryos. It should be noted also that in pea there is no vascular connection between the seed coat (maternal tissue) and the developing embryo (Zhou *et al.*, 2009). Therefore, although it is well known that auxin deficiency can disrupt vascular development in shoots, it appears that *tar2-1* does not result in abnormal seeds by affecting vasculature.

The rescue of *tar2-1* seeds by the *TAR2* transgene shows the importance of the *TAR2* gene itself for normal seed growth and starch biosynthesis. Furthermore, our evidence indicates that it is auxin, and not another (putative) product of TAR2 activity, that is responsible for the effects on seed size and starch concentrations, because an auxin, 2,4-D, increased the size and starch content of *tar2-1* seeds (Fig. 4). Furthermore, there is no evidence that TAR2 activity produces biologically active compounds other than indole-3-acetic acid (IAA) and chlorinated auxin (4-Cl-IAA). Indeed, even within the (small) range of auxins in plants, TAR2 action appears quite specific, because *tar2-1* did not reduce the seed content of another auxin, phenylacetic acid (Cook *et al.*, 2016). Across a range of species, there is no evidence

that mutations in *TAR* genes affect phenotypes by a mechanism other than an effect on auxin content (Zhao, 2012).

The *TAR2* gene is not required for initial seed formation and embryogenesis. However, auxin is known to be important at this stage (Cheng *et al.*, 2007), and we suggest that initial seed set and development can proceed normally in the *tar2-1* mutant because other TARs, such as TAR1, compensate for the lack of functional TAR2. We showed previously that *TAR1* is mainly expressed in the days following anthesis (Tivendale *et al.*, 2012), and we show here that *TAR1* is strongly expressed in the liquid endosperm whereas *TAR2* is mainly expressed in the embryo. We suggest that as the embryo grows, and *TAR1* expression tails off (Tivendale *et al.*, 2012), auxin produced by TAR2 in the embryo becomes important for embryo growth and that this explains why *tar2-1* begins to affect seed growth and starch content after 12–14 d, at which stage WT embryos begin to rapidly expand.

The evidence that *tar2-1* does not affect embryogenesis, and is not a seed developmental mutant, is consistent with the observation that *tar2-1* seedlings do not show developmental defects, but rather are simply reduced in size. The reduction in seedling size appears to be a consequence of the wrinkling and small size of the progenitor seed, rather than a direct *tar2-1* effect in the seedling. The main, primary effect of the *tar2-1* mutation is on the seed-filling phase of seed development. In pea, mutations that affect embryogenesis, and which can therefore be classified as developmental mutants, typically affect the morphology of the embryo before the seed-filling stage (Johnson *et al.*, 1994), whereas this does not occur with *tar2-1* (Fig. S7).

Previous evidence from other species indicates that in some circumstances, strong auxin signalling upregulates starch synthesis, whereas in other cases it has the opposite effect. In rice leaf sheaths, auxin reportedly inhibited the expression of starch synthesis genes (Ishimaru *et al.*, 2013) and disruption of an enzyme (TGW6) that purportedly produces IAA from an IAA conjugate reduced seed size. However, TGW6 shares similarities with strictosidine-synthase-encoding genes, suggesting that this gene might not act specifically on auxin status (Ishimaru *et al.*, 2013). A positive regulatory role for auxin in starch synthesis in rice was suggested by Abu-Zaitoon *et al.* (2012), who reported concurrent increases in auxin and starch concentrations during rice grain development; furthermore, IAA application was reported to increase the starch content of rice grains (Javid *et al.*, 2011), and studies on a rice auxin response factor, OSARF18, also indicate a positive role for auxin signalling in starch synthesis (Huang *et al.*, 2016). In cultured tobacco cells, exogenous auxin downregulated starch-related genes (Miyazawa *et al.*, 1999), whereas by contrast, a repressor of auxin action, SlARF4, downregulated AGPase gene expression in tomato fruits (Sagar *et al.*, 2013), consistent with our findings. Again, in Arabidopsis root tips, starch granule production was inhibited by constitutive expression of an auxin action repressor from rice (Luo *et al.*, 2015). These findings indicate that although auxin affects starch accumulation, there may be an optimum auxin concentration for that process.

In maize, an auxin biosynthesis mutation, *de-18*, reduced auxin content and seed size (Bernardi *et al.*, 2012), whereas in *Medicago truncatula*, a mutation that alters auxin distribution within reproductive tissues led to small seeds (Noguero *et al.*, 2015). In rice, the gene *Big Grain 1* is thought to increase seed size by affecting auxin transport (Liu *et al.*, 2015), whereas in *Jatropha curcas*, overexpression of the *Auxin Response Factor 19* gene resulted in larger seeds (Sun *et al.*, 2017). These results are consistent with our findings. Another pea hormone biosynthesis mutation, *lh-2*, which reduces seed gibberellin content, also reduces seed size (Swain *et al.*, 1993, 1995). However, at least on the genetic background used in those studies (cultivar Torsdag), *lh-2* does not cause seed wrinkling. Furthermore, *lh-2* causes extensive seed abortion (Swain *et al.*, 1993, 1995), whereas *tar2-1* does not.

Conclusions

In the grain legumes, seed development and starch accumulation is of crucial importance for human diets, and we need to maximize our understanding of factors regulating these processes. In the present paper we report new insight into signalling connections between auxin, seed size and starch in starch-rich seeds, improving our understanding of how plants regulate their carbohydrate economy. Interestingly, in pea and its relatives, it may be the abundant, seed-specific, chlorinated form of auxin (Lam *et al.*, 2015) that is important for seed growth. This chlorinated form has been shown to exhibit strong auxin activity in several bioassays (Reinecke, 1999).

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Author contributions

E.L.M. and L.J.Q. measured hormone and carbohydrate concentrations and examined starch granules; T.M. performed transgenic work, measured starch concentrations and performed time course studies on seed and embryo growth; S.E.D. genotyped the plants; M.D., A.I.B. and R.T. conducted TILLING; G.A. provided promoter sequences; J.J.S. and S.U. genotyped plants and assisted with analyses; D.S.N. conducted mass spectrometry and A.G-M. analysed AuxREs; and J.J.R. supervised the research, carried out genetic aspects and drafted the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Starch granules from dry and immature *TAR2*, and from dry *tar2-1* seeds.

Fig. S2 Phenotypes of *r TAR2* and *r tar2-1* seeds at the dry stage.

Fig. S3 Concentrations of sucrose in WT and *tar2-1* seeds at the green stage (approx. 300 mg).

Fig. S4 Co-segregation of *tar2-1* with the wrinkled phenotype.

Fig. S5 Starch granules from dry *TAR2* and *tar2-1* seeds with or without 2,4-D.

Fig. S6 The *tar2-1* mutation does not result in abnormal seeds or embryos during the first phase of seed development.

Fig. S7 Shoot phenotypes of WT and *tar2-1* plants.

Fig. S8 The wrinkled character of mutant *tar2-1* seeds is determined by the zygotic genotype.

Table S1 Concentrations of starch and 'nonstarch' dry matter, and of auxins, in WT and mutant seeds

Table S2 Contents of ADP-glucose, UDP-glucose and hexose phosphates in embryos of mutant and WT lines

Table S3 Nucleotide sequences of primers used

Table S4 Auxin response elements identified in pea starch synthesis gene promoters

Table S5 Relative mRNA levels of *TAR1* and *TAR2* in different parts of young WT pea seeds

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