RESEARCH ARTICLE

Cellular Plasticity in Response to Suppression of Storage Proteins in the *Brassica napus* Embryo

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Short title: Cellular plasticity in oilseed rapeseed

One sentence summary: Suppressing the genes encoding napin and cruciferin leads to an increase in oleosin accumulation and a reorganization of the intracellular architecture in the developing embryo of oilseed rape.

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Abstract

The trade-off between protein and oil storage in oilseed crops has been tested here in oilseed rape (*Brassica napus*) by analyzing the effect of suppressing key genes encoding protein storage products (napin, cruciferin). The phenotypic outcomes were assessed using nuclear magnetic resonance and mass spectrometry imaging, microscopy, transcriptomics, proteomics, metabolomics, lipidomics, immunological assays, and flux balance analysis. Surprisingly, the profile of storage products was only moderately changed in RNAi transgenics. However, embryonic cells had undergone remarkable architectural rearrangements. The suppression of storage proteins led to the elaboration of membrane stacks enriched with oleosin (6-fold higher protein abundance) and novel ER morphology. Protein rebalancing and amino acid metabolism were focal points of the metabolic adjustments to maintain embryonic carbon/nitrogen homeostasis. Flux balance analysis indicated a rather minor additional demand for cofactors (ATP, NADPH). Thus, cellular plasticity in seeds protects against perturbations to its storage capabilities, and hence contributes materially to homeostasis. This study provides mechanistic insights into the intriguing link between lipid and protein storage, which have implications for biotechnological strategies directed at improving oilseed crops.
Introduction

Cellular architecture depends *inter alia* on the size, number and disposition of the various subcellular organelles (Rafelski and Marshall, 2008). In the seed of oilseed rape/canola (*Brassica napus*), storage parenchyma cells are responsible for the synthesis of triacylglycerols (TAG), deposited in the form of lipid droplets (Chapman et al., 2012), while the major seed storage proteins cruciferin and napin are deposited in large storage vacuoles (Borisjuk et al., 2013). Cruciferin and napin account for 60% and 20%, respectively, of the seed's overall protein content (Crouch and Sussex, 1981), representing around a quarter of a seed’s dry mass. Lipid content represents about one half of the seed’s dry mass and is largely in the form of TAGs. Therefore, the metabolism of developing seeds is dominated by a repertoire of enzymes and transporters involved in the synthesis of both TAGs and storage proteins (Troncoso-Ponce et al., 2011; Schwender et al., 2015; Chao et al., 2017).

Amino acids are the building blocks for the elaboration of seed storage proteins, while sucrose is converted into both fatty acids and TAGs. Both glycerolipids and storage proteins are assembled in the endoplasmic reticulum (ER) simultaneously during seed development and may thus compete for limited resources, including precursors and cofactors (Schwender 2008; Schwender and Hay, 2012). Additionally, the site of accumulation within the embryo overlaps extensively (Borisjuk et al., 2013), suggesting perturbations in the synthesis of proteins may affect lipid synthesis, or *vice versa*. The existence of cross-talk between lipid and protein metabolism has also been established by showing that certain gene loci influence both the protein and lipid content of the *B. napus* seed, but in the opposite direction (Chao et al., 2017). The outcome, as has been noted by breeders, is that the lipid content of mature seed tends to be negatively associated with that of protein (Chao et al., 2017; Patil et al., 2018). It has been suggested that this competition might be transgenically manipulated by redirecting substrate from protein to lipid synthesis (or *vice versa*), but little success has been achieved in implementing this strategy. Indeed in *B. napus*, the repression of genes encoding certain storage proteins was found to favor the compensatory synthesis of other storage proteins (a process named protein re-balancing), rather than to boost the accumulation of lipids (Kohno-Murase et al., 1994, 1995). A similar effect has been noted in soybean (*Glycine max*) (Schmidt et al., 2011), in barley (*Hordeum vulgare*) (Hansen et al., 2007), in maize (*Zea mays*) (Wu and Messing, 2014), and in wheat (*Triticum aestivum*) (Baro et al., 2016). Shifts in carbon partitioning and central metabolism remain extremely difficult to engineer (Sweetlove et al., 2017), and the mechanistic link between oil and protein storage in seeds is far from being understood.
*B. napus* is one of the most important and widely cultivated temperate zone oilseed crops. Increasing its seed lipid content remains a major aim of a number of breeding programs (Abbadi and Leckband, 2011). Here, the dependency between protein and lipid accumulation was analyzed by transgenic suppression of genes encoding the two dominant storage protein classes (napin and cruciferin) using RNAi, along with phosphoenolpyruvate carboxylase, an enzyme that channels glycolytic intermediates towards organic and amino acid synthesis (Rolletschek et al., 2004). The developing seeds set by these transgenic plants were characterized at the transcriptomic, proteomic, metabolomic and lipidomic levels, and the visible consequences of RNAi suppression were explored using microscopy, nuclear magnetic resonance and mass spectrometry imaging. Metabolic fluxes and energy cofactor demands were predicted by flux balance analysis. The identification of the numerous changes at the ultrastructural, molecular and metabolic levels has led to a number of mechanistic insights, highlighting cellular and metabolic plasticity to ensure a sufficient level of homeostasis to support the maturation and viability of the seed.

**Results**

*The effectiveness of RNAi in suppressing napin and cruciferin synthesis*

The pool of storage proteins in *B. napus* is encoded by numerous genes (Gacek et al., 2018); thus, BAC library screening results and *in silico* analysis were applied to identify all genes of interest. For cruciferin, we identified 8 genes encoding the P1, P2 and P3 sub-families of cruciferin, which were all targeted by the hairpin (Supplemental Dataset 1). For napin, 15 (out of 17 identified) genes were targeted, and for phosphoenolpyruvate carboxylase (PEPC), 8 (out of 11 identified) were targeted. The effectiveness with which the targeted genes were down-regulated in the developing embryo by RNAi was evaluated by RT-qPCR of three independent transgenic lines (#576, #578 and #580) and compared with those of their respective wild type segregant (WTS) lines (#577, #579 and #581) (Fig. 1A-C). The abundance of transcripts generated by different genes encoding various cruciferins was reduced by up to 32-fold (-5 at a log2-scale, Fig. 1A). The extent of the suppression was even higher for napins, shown for three napin encoding genes in Fig. 1B. The suppression of other targeted napin and cruciferin-encoding genes became evident in the RNA-sequencing datasets (see further below). A time series analysis showed that the extent of down-regulation was most pronounced in the later stages of seed filling (Fig. 1D). The level of suppression of PEPC was marginal at the level of both transcript abundance and enzyme activity (Fig. 1C,E). Thus, the PEPC RNAi construct was ineffective.
The RNAi approach alters the composition but not the carbon/nitrogen ratio of mature seeds

The visible phenotype of the transgenic plants was essentially identical to those displayed by WTS plants. Measurements of seed content showed mature transgenic seeds contained 10-15% less protein and lipid, and 30-35% more fiber than WTS seeds (Fig. 1F-H). Further compositional analysis revealed that the total nitrogen content and the carbon-to-nitrogen ratio was unchanged, while the water content was statistically lower by ~9% in the transgenic seeds (Supplemental Fig. 1). Starch content (only transiently accumulated in B. napus seeds) was below 1% of dry weight without any consistent changes in the transgenic vs WT lines (Supplemental Fig. 1).

Suppressing the production of napin and cruciferin lowers the embryo’s portion of mature seeds

At maturity the transgenic seeds had a lower weight than WTS seeds despite little difference in seed size (Fig. 2B). To determine what changes may have resulted internally due to RNAi suppression of cruciferin and napin proteins, magnetic resonance imaging (MRI) was used to visualize the seed’s internal structure non-invasively (Supplemental Movie 1). MRI revealed that the cotyledons of mature transgenic seeds were irregularly shaped and left a number of air-filled cavities (void spaces), as exemplified in Fig. 2A. The volume of void spaces was significantly greater by 62-73% than WTS seeds (n = 8-12 seeds, Fig. 2E). In WTS seeds, the radicle is usually covered by cotyledons, and only rarely protrudes beyond the cotyledons (8%), whereas the radicle tip protruded in 87% of the transgenic seed models (white arrows in Fig. 2A; see also Supplemental Movie 2). When assayed using MRI, the transgenic embryos were 7-9% smaller than those of WTS (Fig. 2C), and the volume of the seed coat was 5-8% greater (Fig. 2D). This led to a lower ratio of embryo (em, rich in oils and protein) to seed coat (sc, rich in fiber) in transgenic seeds, and thus to an altered seed composition (Fig. 2F). Despite these changes, there was no loss in the ultimate viability of transgenic seeds, except a delay in the timing of germination (Supplemental Fig. 2).

The intracellular architecture is markedly changed by the loss of napin and cruciferin

Substantial differences in the subcellular organization were observed in the embryos of transgenic and WTS plants. In the early stages of development, parenchyma cells in WTS embryos already featured numerous protein bodies, which expanded over the course of seed development (Fig. 3A,C,E and Supplemental Fig. 3). By contrast, in the transgenic embryos, protein bodies were detected only later in seed development, remained substantially smaller in size and exhibited a crystalline structure (Figs. 3B,D,F and Supplemental Fig. 3). Perhaps the most striking subcellular change was the accumulation of large membrane stacks evident throughout storage parenchyma...
cells of the transgenic embryos (Fig. 3G). The protein bodies formed in WTS embryos were surrounded by densely packed lipid droplets (Fig. 4A). Immunolabeling confirmed that both napin and cruciferin were associated with the WTS protein bodies (Fig. 4C,D), while oleosins (structural proteins of oil body membranes; Huang 2018) were associated with lipid droplets (Fig. 4B). In the cells of transgenic embryos, the abundance of cruciferin was greatly reduced, and what was present was scattered throughout the protein bodies (Fig. 4H); napin was barely detectable (Fig. 4G). On the other hand, oleosin was well represented in the cells of transgenic embryos, both within lipid droplets and throughout the membrane stacks (Fig. 4F). All principal changes in intracellular architecture described above for transgenic line #580 versus its WTS #581 were also evident for other transgenic lines as exemplified in Supplemental Fig. 4.

Both the storage and membrane lipids are altered in transgenic seeds
Analysis of lipids from intact, mature seeds using MRI showed a reduction in total lipid content by ~20% in both transgenic lines analyzed despite similar seed sizes to WTS (Supplemental Movie 3). Regarding lipid distribution, the cotyledons accumulated a higher proportion of lipids relative to the radicle (Supplemental Movie 2), confirming earlier analyses (Borisjuk et al., 2013). This was apparent in all genotypes. Mass spectrometry analysis of developing seeds (~42 DAF) revealed that both the quantity and composition of TAGs and the common membrane lipid, phosphatidylcholine (PC), were affected by suppression of napin and cruciferin production (Fig. 5A). In plants, PC serves as both a structural component of membranes as well as an important metabolic precursor for the synthesis of TAGs. The quantity of PC was nearly four-fold greater in the seeds from transgenic line #580 than in those from WTS line #581, whereas the quantity of TAGs was about 20% lower in the transgenic seeds. For both classes of lipids there was a marked shift in their saturation levels. Polyunsaturated fatty acids were more abundant in both PC and TAG from lipid extracts of transgenic seeds compared to WTS seeds (Fig. 5B).

Mass spectrometry (MS)-based imaging of seed sections confirmed the marked reduction in saturation in the transgenic seeds with respect to TAGs and PC, and also emphasized the heterogeneous distribution of lipid molecular species in different tissues (Fig. 5C). The extent of the reduced saturation was more variable seed to seed in transgenic seeds than in WTS seeds (Supplemental Fig. 5).

Analysis of lipid extracts of dissected seed tissues confirmed MS imaging results and demonstrated that the quantitative shift in saturation for both PC and TAG to more unsaturated species occurred in
all parts of the transgenic embryos (Supplemental Fig. 6). In addition, membrane lipids not involved in TAG accumulation also showed a similar shift to more unsaturated molecular species in the different tissues of the embryo as shown for phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (Supplemental Fig. 7). This may suggest the membrane stacks observed in cells of the embryo (Figs. 3, 4) were associated with alterations in lipid composition, favoring membranes (and storage lipids) with a greater degree of unsaturation. Particularly, marked differences were observed in the region of the embryonic axis where the composition of PCs shifted from 34:1 to 34:2 and 34:3 (Fig. 5B), that of PEs from 34:1 to 34:2 and from 36:3 to 36:4 (Supplemental Fig. 7), and that of PI from 34:1 to 34:2 and 34:3 (Supplemental Fig. 7). However, all parts of the transgenic embryos exhibited shifts in unsaturation in both membrane and storage lipids (Fig. 5C; Supplemental Fig. 6).

Comparative transcriptome analysis evidences a major shift in global gene expression

RNA-seq profiling detected transcripts from 67,885 protein-encoding genes (Supplemental Dataset 2A-J). A comparison of transcript abundances between the transgenic (line #580) and its WTS (line #581) showed 94 genes were down-regulated in the transgenic embryos, while 97 were up-regulated (Supplemental Dataset 2C). Not surprisingly, the most strikingly affected genes were those encoding storage proteins (Supplemental Dataset 2D). The transcript abundance from 12 genes predicted to encode napin and seven to encode cruciferin were significantly lower in line #580 (transgenic) than in line #581 (WTS) embryos (Fig. 6A). In most cases down-regulation was >10 fold, but as high as 111-fold in at least one case. In the WTS embryos, about 50% of the reads were generated from a set of 25 napin- or cruciferin-encoding genes, but this was the case for just 4% of the reads in the transgenic embryos. This represents a large quantitative shift in gene expression. A number of genes encoding proteins associated with sulfur assimilation were down-regulated in the transgenic embryos (see also Supplemental Dataset 2F): the A. thaliana orthologs (At1g04770, At5g48850, At1g36370) of nine of these genes are known to be induced by sulfur deficiency (Maruyama-Nakashita et al., 2005). Transcript abundance of several genes involved in the reduction of sulfate to H₂S also were reduced; their A. thaliana putative orthologs encode a sulfate transporter of the chloroplast envelope (AT5G13550), a chloroplastidic isoform of ATP sulfurylase (AT3G22890), 5'-adenylylsulfate reductase (AT1G62180) and a sulfite reductase (AT5G04590). The set of down-regulated genes also included two putative orthologs of the A. thaliana genes encoding adenylyl sulfate kinase (AT2G14750), an enzyme that provides activated sulfate used in the synthesis of secondary metabolites, as well as a gene encoding serine acetyltransferase (AT2G17640), an enzyme active in cysteine synthesis.

Among the up-regulated genes, those encoding oleosin tended to be expressed at higher levels in the
transgenic than WTS line (Fig. 6B), although none of the individual 19 oleosin encoding genes were statistically significantly different (Supplemental Dataset 2D). Several members of a gene family encoding non-specific lipid transfer proteins (LTPs) were up-regulated in the transgenic line #580 (Supplemental Dataset 2D), the most abundant being BnaC04g30640D (A. thaliana orthologs At5g38180, At5g38195). The B. napus putative orthologs of At3g59970, an A. thaliana gene encoding methylenetetrahydrofolate reductase (C1-metabolism), were up-regulated in the transgenic embryos, and their products were noted as being more abundant at the proteomic level.

GO-term enrichment analysis indicated that among the down-regulated genes in the transgenic line, the biological processes “Sulfate Assimilation”, “Serine Family Amino Acid Metabolic Process” and “Sulphur Compound Biosynthetic Process” were the most significantly over-represented terms, while among the upregulated genes, “Lipid Transfer” and “Lipid Localization” were the most over-represented (Supplemental Dataset 2I).

The rebalancing of the proteome induced by the loss of napin and cruciferin results in the hyperaccumulation of oleosin

Two-dimensional gel electrophoresis-based proteome profiling enabled the identification of 1,124 distinct proteins in B. napus embryos (Supplemental Fig. 8). According to an MS-based analysis, 49 proteins were shown to have increased in abundance and 165 to have decreased in abundance in transgenic embryos relative to WTS (Table 1 and full list of proteins in Supplemental Dataset 3). As expected, the list of proteins with lower abundance was dominated by the vacuolar storage proteins napin2, cruciferin1 and cruciferin4, each of which was reduced in abundance by between five- and ten-fold. The set of reduced-abundance proteins also included several enzymes involved in the tricarboxylic acid cycle, glycolysis, the synthesis of amino acids and polyamines, and sulfur and cofactor (C1) metabolism. Cruciferin1 was recovered from several spots, pointing to multiple isoforms (which confirms earlier observations by Nietzel et al., 2013). There was an indication that the α P1 and β P2 cruciferin chains were reduced in abundance, while the quantity of the α P2, α P3 and β P1 + P3 chains were largely unaffected in the transgenic seeds. Among the up-regulated proteins, the most prominent were oleosin2 (6.7-fold increase in abundance), biotin carboxyl carrier protein2 (a subunit of the acetyl coenzyme A carboxylase complex) and plastidic pyruvate kinase, all of which are involved in the synthesis of fatty acids and/or storage of lipids (Supplemental Dataset 3). Notable among the other up-regulated proteins were two related to translation (Elongation factor 1-α 1 and the 60S ribosomal protein L10), the endoplasmic reticulum stress-related protein disulfide isomerase-
like 1-2, and other proteins involved in either cell wall synthesis or amino acid metabolism. Among the latter group was nitrogen regulatory protein PII, coordinating C/N metabolism (Chellamuthu et al., 2014), and part of the acetyl-CoA carboxylase complex (Feria Bourrellier et al., 2010). We did not observe an increase of other vacuolar storage proteins.

Amino acid metabolism is a focal point of the metabolic adjustment to the loss of napin and cruciferin

A set of 79 free metabolites was quantified using a liquid chromatography/mass spectrometry platform. Soluble sugars (esp. sucrose) were by far the most abundant metabolite category followed by amino acids (esp. Arg, Asp, Gln, Glu) and organic acids (esp. citrate, malate) (Fig. 7A; Supplemental Dataset 4). Notably, the amount of amino acids and polyamines was almost doubled in the transgenics versus WTS. There was a statistically significant increase in the level of Gln (+163%), Arg (+118%), Val (+38%), His (+136%), Trp (+34%) and, though not statistically significant, a strong increase for some other amino acids (Asn +192%, Asp +55%, ornithine +184%, citrulline +75%). Oxaloacetate (product of PEPC reaction) was significantly increased by 24% in the transgenics vs WTS. Apart from these variations, the loss of napin and cruciferin induced minor changes in the level of other intermediates of cellular metabolism (Fig. 7B).

Neither the respiration nor the photosynthetic activity of the embryo is affected by the loss of napin and cruciferin

The operational efficiency of photosystem II in embryos was assessed by PAM fluorescence, while photosynthetic energy transfer occurring within the seed was measured as a linear electron transport rate (ETR). Both transgenic and WTS embryos exhibited a similar ETR without the formation of any distinct gradient across the embryo (Supplemental Fig. 9A). A comparison of rapid light response curves (Supplemental Fig. 9B) showed both genotypes exhibited a comparable level of photosynthetic activity when illuminated by normal light levels, equivalent to a maximum of 100 photosynthetic active radiation (PAR) (Borisjuk et al., 2013), but at higher light levels, transgenic embryos had lower activity than WTS. There were no apparent differences in the respiratory activity (measured as the rate of total oxygen consumption) between the transgenic and WTS embryos (Supplemental Fig. 9C).

Modeling the metabolic flux and the energy cofactor demands for biosynthesis of lipids and proteins

Since the transgenic interventions were found to change seed composition and metabolic profiles (Figs. 1, 5, 7), there should be associated changes in metabolic flux. To compare central metabolism
flux between the genotypes, we first determined the growth rate and the biomass composition of the developing embryo at about 30 DAF for the WTS and transgenic lines #580 and #581 (Supplemental Dataset 5A,B). In transgenic embryos, there was a statistically significant 0.75-fold reduction in protein level relative to WTS (p-value 1 x 10⁻⁴), while free metabolites were 1.8-fold higher (p-value 5 x 10⁻⁵) (Supplemental Dataset 5B). The decrease in protein in the growing embryos parallels the measurements in dry mature seeds of transgenics (Fig. 1H). The theoretical amino acid requirements for the expressed proteins as determined from the RNAseq data of expressed genes suggests transgenic embryos would use 52% less glutamine and 31% less cysteine (Supplemental Dataset 5C), and the measured sulfur content was ~15% lower in transgenic embryos than in the WTS (4.3 vs 5.1 mg per g), which together is consistent with the proposed shift in cysteine demand and sulfur metabolism.

The embryo dry weight (DW) composition data were used to parametrize a metabolic model of the B. napus developing embryo (Hay et al., 2014). Accumulation of free sugars, amino acids and other polar metabolites were defined in the model according to the free metabolites DW fraction (Supplemental Dataset 5B) and compositional data (Fig. 7). Metabolic fluxes were predicted for the two genotypes by flux balance analysis (Supplemental Dataset 5D). Based on the biosynthetic pathway fluxes, energy cofactor demands for the biosyntheses of lipids and proteins were derived (Fig. 8A; Supplemental Dataset 5E). In the transgenic line, the model revealed an overall reduction in biosynthetic ATP demands (Fig. 8A, ATP demand). ATP demands for protein synthesis were diminished while ATP demands for lipid synthesis increased by a lesser extent (Fig. 8A). With regards to the balance of reducing cofactors, the total redox demand for the transgenic line increased but the redox changes were overall less pronounced than the changes in ATP demands (Fig. 8A). Since it was observed that lipids of transgenic embryos were overall higher in unsaturation than WTS embryos, this may suggest a higher demand for reducing equivalents for fatty acid desaturation and an overall impact on total redox balance. The balance of redox for desaturation of fatty acids, shown in Fig. 8A, is increased by 1.2-fold in transgenic embryos. However, this increase, amounting to 0.5 mmol H₂ equivalents gDW⁻¹, is only 2% of the total redox demand for lipid biosynthesis in the WTS embryos (26.1 mmol gDW⁻¹). Therefore, the overall requirements for reducing equivalents were only slightly impacted by the change in lipid saturation.

In addition to the balances of energy cofactors, we examined the total nitrogen balance of the embryo (Fig. 8B). In particular, since in the transgenic embryo PC was found to be increased almost 5-fold (Fig. 5A), it was of interest to know if the associated increase in PC-bound N had a substantial impact on the total nitrogen balance. Overall, the total nitrogen content per g DW in transgenic embryos was
reduced relative to the WTS (Fig. 8B). The reduction in the protein N level was in part compensated by an increase in N in the free metabolite fraction (Fig. 8B). Although PC-bound N was increased in the transgenic embryos by almost 5-fold, this increase accounted only for about 16% of the decrease found in protein N. This would suggest changes in PC had only minor impacts on overall nitrogen balance.

**Discussion**

The RNAi-mediated suppression of the synthesis of cruciferin and napin was balanced by a remarkable level of plasticity at the ultrastructural, molecular and metabolic levels. The formation of large, oleosin-containing membrane stacks replaced the normal protein-filled vacuoles. Although the precise mechanistic basis for these subcellular changes remain to be determined, several factors revealed here suggest insights into a homeostatic organellar mechanism in seeds that maintains the appropriate storage and compartmentalization of carbon and nitrogen reserves for seed viability.

*Reprogramming of protein storage is accompanied by intracellular re-arrangement*

Despite the transgene-induced reprogramming of the storage process, the seeds appeared to develop normally, were viable, and hardly showed any outwardly visible aberration (although its germination was somewhat slower than that of WTS seed). The most prominent effect of suppressing the synthesis of cruciferin and napin was to alter the intracellular architecture of the embryo’s storage tissues. Along with the expected reduction in the size of protein bodies, the cytoplasm developed numerous, small oil bodies and large, oleosin-rich membrane stacks. This latter phenomenon may well be a consequence of the somewhat unusual features of oleosin (Hsieh and Huang, 2004), which possess a central, uninterrupted hydrophobic domain required for its association with membranes and its deposition into oil bodies (Hope et al., 2002). Oleosins are synthesized on the ER membrane and targeted to intracellular lipid droplets. As neutral lipids are generated in the same compartment, the formation of lipid droplets parallels the incorporation of oleosins into membranes (Wahlroos et al., 2003) together with other lipid droplet-related proteins (Chapman et al., 2012; Huang, 2018). Immuno-labelling showed that oleosin was attached to the lipid droplets in storage cells (Fig. 4), while electron microscopy imaging confirmed a marked increase in the number of small lipid droplets (Figs 3 and S3). An *in vitro* study carried out by Vance and Huang (1987) established that many, small lipid droplets would contain greater amounts of oleosin than fewer, larger lipid droplets due to increased surface area. The formation of small, oleosin-enriched lipid droplets and membrane stacks is thus in accord with the several-fold increase in oleosin content found in the transgenic seed. During the
process of lipid droplet formation and oleosin synthesis, a gradient of enrichment with respect to oleosins and TAG is generated, and, as a result, the quantity of TAG produced has an impact on the final shape of the lipid droplet. As TAG content of transgenic embryos was lower than WTS embryos (Fig. 5), the lipid droplets may have been less efficiently filled, resulting in the creation of smaller and irregularly shaped lipid droplets. A similar phenotype was observed in maize kernel (Ting et al., 1996), where a reduction in the proportion of oleosin to lipid was shown to result in the formation of large lipid droplets (Shimada et al., 2008; Siloto et al., 2006). The mechanistic basis of lipid droplet formation remains obscure (Herman, 2008; Gao and Goodman, 2015), but it seems reasonable to conclude that both a reduced size and aberrant/ellipsoid shape (Fig. 3, Supplemental Fig. 3) in the transgenic embryos were influenced—possibly even determined—by the surfeit of oleosin. The loading of membranes with oleosin and the formation of many small lipid droplets provide the means for the embryo to accommodate an excess of oleosin protein. The geometric shift from spherical to ellipsoid lipid droplets (and in its extreme to membrane stacks) also involves a preponderance of phospholipid (Fig. 5). Thereby, the transgenics can maximize the surface area of lipid storage while maintaining their volume. The mathematical description (formula in Fig. 8D; Michon, 2019) reveals that a five-fold increase of one of the axes of a sphere (while keeping its volume constant) will cause the surface area to increase by about 75%. In other words, the intracellular plasticity allows the plant to easily store excessively abundant membrane proteins (oleosin) without large compromises on overall oil storage capacity (lipid droplet volumes).

The consequences of the intracellular re-arrangements could be widespread. Lipid droplets are involved in intracellular communication due to their contact with most of the subcellular organelles (Valm et al., 2017), and they are associated with several functions relating to intracellular homeostasis (Huang, 2018; Gao and Goodman, 2015; Shimada et al., 2018). The observed up-regulation of the gene encoding protein disulfide isomerase-like 1-2 (At1g77510) in the present transgenic plants is possibly a response to ER stress (Lu and Christopher, 2008). A further suggested effect on ER functionality relates to the increased presence of both EF1-α and a 60S ribosomal protein, implying that translation may be compromised in the transgenic embryo. Finally, there were numerous changes in the contribution of PC, PE and PI to the membrane lipid content (Fig. 5 and Supplemental Figs. 5-7), all of which are assembled in the ER. Note that the composition of membrane lipids (and membrane proteins) can affect both the fluidity and curvature of the membrane (van Meer et al., 2008), so part of the homeostatic control here may be to synthesize additional membrane lipids with increased numbers of double bonds to accompany changes in membrane architecture required to
store adequate amounts of protein and lipid for seed viability.

The metabolic adjustments associated with the process of intracellular re-arrangement

Although the introduction of the RNAi construct delivered a major reduction in the abundance of transcript encoding cruciferin and napin (Fig. 6A), the overall reduction in total seed protein was only moderate (Fig. 1H). The shift in protein synthesis had the effect of lowering the embryos’ demand for amino acids, most notably for Gln, which most likely accounted for a marked increase in the steady state levels of both Gln and other amino acids (Fig. 7). In the transgenic lines, processes controlling supply and demand of nitrogenous precursors are likely perturbed. Part of the decrease of nitrogen in storage proteins is offset by an increase of nitrogen in the fraction of free metabolites and, to a lesser extent, in the nitrogen-containing membrane lipid PC (Fig. 8B). In soybean, proteome rebalancing involves only minor transcriptional changes, with post-transcriptional/translational regulation playing a greater role (Herman et al., 2014).

An unexpected (but nevertheless clear) result was that the transgenic seeds accumulated less storage lipid than WTS. The following scenarios can be invoked to explain this observation: first, the portion of seed coat versus embryo slightly increases in the transgenic plants (Fig. 2); second, the increased abundance of PII protein (Table 1) may imply a strengthened inhibition over the activity of ACCase, responsible for initiating the synthesis of fatty acids in the plastids (Feria Bourrellier et al., 2010); third, the reduced abundance of several enzymes involved in glycolysis (Supplemental Dataset 3) may lower the flux into fatty acids/TAGs; fourth, the increased level of PC (Fig. 5A) may either indicate a failure to incorporate fatty acids into TAGs and/or reflect an increased demand for PC to synthesize the observed membrane stacks; and fifth, the size and/or spatial distribution of lipid droplets (Supplemental Fig. 3, Fig. 4) may affect the dynamics of lipid accumulation (Miquel et al., 2014).

Another unexpected finding was that the degree of fatty acid unsaturation was elevated in transgenic embryos (Fig. 5). While the increased unsaturation observed was too small stoichiometrically to make a significant contribution to rebalancing redox demand (outcome of FBA-modeling; Fig. 8A), the question remains why a reduction in the seed storage proteins cruciferin and napin would increase the degree of unsaturation in membrane and storage lipids. Plausible explanations are (i) greater resident time of PC in the ER prolonging the availability for fatty acid desaturase enzymes to act, thereby supporting the increase in polyunsaturated fatty acids; (ii) a concerted ER-subdomain
configuration change that favors membrane lipid assembly vs TAG; and (iii) inefficient packaging of newly synthesized TAG that leads to a buildup of improperly assembled cytoplasmic lipid droplets.

Implications for seed improvement
The proteome rebalance in seeds of Brassica seems to be ineffective as compared to soybean and maize (Schmidt et al., 2011; Herman, 2014; Wu and Messing, 2014). Knowing that the rules can be different in different crops is critical for breeding and genetic intervention to improve agriculture.

A stand-out conclusion from the present research is that suppressing the production of one class of storage compound (in this case napin/cruciferin) does not necessarily lead to the redirection of assimilate to an alternative class (TAGs/seed oil). Rather, the growth of the embryo is slowed as a result of a presumed feedback between protein and lipid storage. The conclusion is that attempting to raise lipid or seed oil yields by repressing the accumulation of storage proteins is unlikely to succeed. Instead, a more promising approach would be to focus on increasing the size of the seed, for example by stimulating the supply of assimilate and its uptake into the seed. It has been suggested that over-expressing the genes encoding oleosin could boost the production of storage lipids (Huang, 2018), a strategy which appears to be effective in tissues characterized by a rather minor lipid content, such as the rice (Oryza sativa) grain (Liu et al., 2013) or the potato (Solanum tuberosum) tuber (Liu et al., 2017); however, as yet, there is no support in the literature for this approach working in an oilseed crop. The present data imply that raising the production of oleosin alone will possibly fail to have a positive effect on the seed lipid content. This conclusion would need to be tested more stringently by engineering the up-regulation of oleosin-encoding gene(s) in a background where the production of cruciferin and napin was normal. Up-regulation of oleosin-encoding genes combined with an increase in the transcriptional activator WRINKLED1 (Li et al., 2015), diacylglycerol acyltransferase (Weselake et al., 2008) and other lipid assembly genes offers better chances of actually driving higher oil production.

Overall, the outcome of the present experiments support the quantal synthesis model proposed for animal embryos (Rafelski and Marshall, 2008). This model predicts that the determination of an organelle’s size depends on the amount of precursor synthesized. In the present B. napus transgenic plants, a surplus of oleosins and PC had the effect of inducing the formation of large, oleosin-containing membrane stacks, which replaced the normal protein-filled vacuoles but were not filled with storage lipids. The remarkable plasticity of the seed’s cellular architecture prevents aberrations on the overall seed phenotype, and thereby ensures homeostasis required for sustaining seed
viability and growth.

Methods

Plant transformation

To simultaneously down-regulate the genes encoding PEPC1, PEPC2, napin and cruciferin, protoplasts recovered from hypocotyls of two-day-old seedlings of *B. napus* var. PPS02-144B were isolated following Glimelius et al. (1984), then co-cultivated for three days with *Agrobacterium tumefaciens* strain C58C1Rif (pGV4000) harboring the binary vector pTJCL032. The pTJCL032 T-DNA contained a chimeric hairpin fragment consisting of a 120-nt segment of PEPC2 (*BnaA05g34260D-1*), a 103-nt segment of PEPC1 (*BnaA08g01200D-1*) driven by the *A. thaliana* oleosin promoter (sequence description of promoter in Thomas and Li, 1998), as well as a 179-nt segment of napin (*BnaA01g16230D-1*) and a chimeric 200-nt segment comprising portions of the genes encoding the P1, P2 and P3 sub-families of cruciferin, both driven by the *B. napus* napinA promoter (Stålberg et al., 1993). The relevant sequences are given in Supplemental Table 1. The fragment was isolated from *B. napus* accession PPS02-144B. In addition, the vector harbored the gene *bar* to allow for the phosphinothricin-based selection of transformed cells. Following 14–20 days in culture, colonies were plated onto K3 medium (Nagy and Maliga, 1976) containing 0.6% w/v Seaplaque agarose, 0.1 M sucrose, 5 mg/L AgNO₃, 250 mg/L carbenicillin, 250 mg/L ticarcillin, 0.5 g/L MES, 20 mg/L PPT, 0.25 mg/L 2,4-D, 0.025 mg/L NAA and 0.025 mg/L BAP. To induce shoots, colonies of diameter 2–3 mm were transferred onto Murashige and Skoog (1962) medium solidified with 0.5% w/v agarose (Invitrogen, Belgium), and supplemented with 2% (w/v) sucrose, 5 mg/L AgNO₃, 250 mg/L carbenicillin, 250 mg/L ticarcillin, 0.5 g/L MES, 10 mg/L PPT, 1 mg/L BAP and 0.5 mg/L NAA. Regenerants were potted into soil and raised in a greenhouse. Based on Southern blot analysis, only transgenic plants with single inserts were selected for further analysis (Supplemental Fig. 10). Three independent transgenic lines were isolated from each primary transformant and were paired with a WTS. All WTS were checked for the absence of hairpin sequences using TaqMan assays (Supplemental Fig. 11) according to the procedure described by Martin et al. (2005).

Southern blot analysis

Genomic DNA was isolated from 4 leaf discs according to the CTAB protocol (Doyle and Doyle, 1987). Ten milligrams of genomic DNA samples was digested overnight with either EcoRI or EcoRV restriction enzymes (New England Biolabs) and two hybridization membranes were prepared for the transformation events, one for hybridization with the marker gene-specific probe (bar), the other for
hybridization with the target DNA-specific probe (pdk-intron). The digested DNA was separated on a 1% TAE agarose gel (12 h, 25 V) and transferred to a Hybond-XL membrane (Amersham). For DNA probes, template DNA was PCR amplified by Taq polymerase (Promega), purified using the Qiagen Gel purification kit and radioactively labelled with either [α-32P]dCTP (bar probe) or [α-32P]dTTP (pdk-intron probe) using the High Prime DNA Labelling Kit (Roche). Hybridizations were performed using a standard hybridization protocol (Sambrook and Russell, 2001). After washing, the hybridization membranes were exposed to a Kodak Hyperfilm for 5 days, after which the films were developed.

Plant growth and sampling
Plants were grown in a chamber set to deliver a constant temperature of 18°C, a 16 h photoperiod (400 µmol quanta m⁻² s⁻¹) and a relative humidity of 60%. The same embryo materials were used for the transcriptomic, proteomic and metabolomic analyses: developing embryos, sampled 30-45 days after flowering (DAF) were harvested, snap-frozen in liquid nitrogen and stored at -80°C until required.

Assays for PEPC activity and respiration
The maximum catalytic activity of PEPC was measured in embryo extracts using an indirect spectrophotometric assay (Rolletschek et al., 2004). Embryonic respiration was deduced from the rate of oxygen consumption: intact embryos harvested at ~35 DAF were incubated in air-tight flasks (five embryos per flask, ten replicates per genotype), following Munz et al. (2017).

Chlorophyll fluorescence imaging
The chlorophyll fluorescence generated by freshly sectioned embryos harvested at ~35 DAF was quantified using an Imaging-PAM chlorophyll fluorometer (Borisjuk et al., 2013). The resulting data relate to the effective quantum yield of photosystem II (measured at 37 µmol quanta per m² s), used to represent the ETR across the embryo section.

Metabolite, starch and sulfur analysis of the immature embryo
Metabolic intermediates were extracted from frozen immature embryo samples in a chloroform/methanol/water mixture and analyzed using liquid chromatography coupled to MS, as detailed by Schwender et al. (2015). Each sample was measured three times (technical replicates), and each genotype was replicated three times (biological replicates with ~10 embryos each). The data were quantified using authentic standards and external calibration. Starch contents in the immature embryo were assessed by subjecting the insoluble residue to spectrophotometry, following
Borisjuk et al. (2013). Total sulfur was measured using an iCAP 6500 inductively coupled plasma optical emission spectroscope (ICP-OES) (Thermo Fisher Scientific, Dreieich, Germany) combined with the CETAC ASXPRESS™ PLUS rapid sample introduction system and a CETAC autosampler (CETAC Technologies, Omaha, NE, USA). A 50 mg sample of freeze-dried, ground embryos was digested in 2 mL 69% HNO₃ (Bernd Kraft GmbH, Duisburg, Germany) using an UltraClave IV microwave device (MLS GmbH, Leutkirch im Allgäu Germany). Digested samples were made up to 15 mL with deionized water. Element standards (Bernd Kraft) were used as external standards, and Yttrium (ICP Standard Certipur, Merck, Darmstadt, Germany) as an internal standard for matrix correction. For each genotype three replicate samples were measured pooled from ~ 50 seeds from three different plants each.

Proteomics
For proteomics, three replicate samples with each ~50 embryos (35 DAF) were used. Embryo proteomes were profiled by 2-dimensional isoelectric focusing/SDS gel electrophoresis following methods provided by Schwender et al. (2015). Visible spots were detected and numbered using Delta2D software. Spots classed as differentially abundant were excised from the gel and their protein constitution was analyzed using MS. The threshold applied for differential expression was a 1.5-fold difference in the relative spot volume.

Mature seed phenotyping
Seeds were harvested at ~65 DAF and stored at 4°C until required. The lipid content in ~50 mg seed samples was measured using an MQ60 time domain nuclear magnetic resonance device (Bruker GmbH, Ettlingen, Germany) following Borisjuk et al. (2013). The seeds’ content of fiber, starch and moisture was measured in the same samples using a near infrared spectroscope (MPA, Bruker GmbH), calibrated according to the supplier’s protocol. Total nitrogen and total carbon content were measured in pulverized samples of mature seeds using an elemental analyzer (vario EL cube; Elementaranalysensysteme, Hanau, Germany) according to manufacturer’s instructions and acetanilide as elemental standard.

Histological procedures
Sections of embryos were stained with Ponceau. For staining, 0.1% Ponceau S (w/v) was dissolved in 5% acetic acid (w/v). The microscope slides were placed on a heating plate (60°C) and covered with the staining solution. After 2 minutes, the slides were carefully rinsed with distilled water and
dried. Immunostaining based on affinity-purified anti-cru- ciferin, anti-oleosin and anti-napin polyclonal antibodies used in 1:100 - 1:500 dilution (Tiedemann et al., 2008) and Alexa488-antiRabbit antibodies (1:500-1000) for secondary labelling (Cat # A-11008; Invitrogen/ThermoFisher Scientific, Karlsruhe, Germany) was performed following Borisjuk et al. (2013). Transmission electron microscopy including tissue preparation was performed exactly as described previously (Verboven et al., 2013).

Germination assay
Mature seeds were spread on filter paper moistened with 1 mL sterile water in a sealed Petri dish and held at 23°C under a 14 h photoperiod (100 µE m⁻² s⁻¹). Each dish contained 25 seeds, and each genotype was represented by three dishes. The formation of a visible radicle protrusion was taken as a successful germination.

MRI
MRI was used to generate three dimensional models of seeds and to map the distribution of lipids. The analysis relied on an Avance III HD 400 MHz NMR-spectrometer (Bruker GmbH, Rheinstetten, Germany) based on the procedures detailed by Borisjuk et al. (2013). NMR data processing was performed using MATLAB (MathWorks, Natick, MA, USA). Segmentation and seed modeling was performed using AMIRA software (FEI Visualization Sciences Group, Merignac, France). For details, see Munz et al. (2017).

Electrospray ionization-mass spectrometry (ESI-MS)
Lipids were extracted from both whole seeds (20-30 mg) and dissected seed tissues, and then subjected to direct infusion ESI-MS analysis as described by Sturtevant et al. (2017, 2019). Hand-dissected tissue samples were prepared from 20-30 mg seed imbibed under vacuum. The internal standards PC-14:0/14:0 (1,2-dimyristoyl-sn-glycero-3-phosphocholine) and TAG-17:0/17:0/17:0 (glyceryl triheptadecanoate) were added to the samples to quantify PC and TAGs.

MALDI-MS imaging of seed sections
Seeds (40-45 DAF) were embedded in 10% w/v gelatin held at 40°C. The solidified gelatin block encapsulating a single seed was trimmed and frozen at -80°C, and then held at -20°C for three days prior to cryosectioning. Seed sections (30 μm thick) were prepared using a CM1950 cryo-microtome (Leica Biosystems, Nussloch, Germany) and thaw-mounted on Superfrost Plus microscope glass slides (Fisherbrand, 12-550-15, Waltham, MA, USA). The sections were lyophilized for 2.5-3 h and then held
under vacuum until required for MS imaging. Sections used to image PC and TAGs were coated with 2,5-dihydroxybenzoic acid (DHB), while those used to image PE and PI were coated with 1,5-diaminonaphthalene (DAN); the sublimation coating procedure followed Hankin et al. (2007). The sections were then subjected to MALDI-MS imaging using a MALDI-LTQ-Orbitrap-XL mass spectrometer (ThermoScientific, San Jose, CA, USA). The DHB-coated sections were analyzed in positive ionization mode and the DAN-coated sections in negative ionization mode. The MALDI ionization conditions were: 12 μJ/pulse, ten laser shots per step, raster step size of 40 μm. The device was set to a resolution of 60,000 and an m/z scan range of 600-1,200. Raw data were processed as described by Horn and Chapman (2014), using Metabolite Imager software and plotted as mol%: low values are shown in green and high values in red. The PC images were derived from the [M+H]+ adduct, those for TAGs from the sum of the [M+Na]+ and [M+K]+ adducts, and those for PE and PI from the [M-H]- adduct.

**RNA extraction and RT-qPCR**

Total RNA was extracted from developing embryos (harvested at different stages from 22 to 45 DAF) following the protocol supplied with a Spectrum™ Plant Total RNA kit (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), and then treated with RNase-free DNaseI (New England Biolabs, Ipswich, MA, USA) to remove any contaminating DNA. A 1 μg aliquot of total RNA was converted to single strand cDNA using a RevertAid First Strand cDNA Synthesis kit (ThermoScientific). A 100 ng template of the single strand cDNA provided the template for RT-qPCRs formulated with SYBR® Green PCR Master Mix (Invitrogen). The necessary primers, designed using Primer3 software (primer3.ut.ee) to generate an amplicon of size 150–200 bp, targeted the 3′-UTR sequence of the targeted genes (Supplemental Table 2). The specificity and amplification efficiency of each primer pair was checked by a melting curve analysis. Each reaction was subjected to a 95°C/60 s denaturation, followed by 40 cycles of 95°C/30 s, 55°C/30 s, 72°C/30 s, and the reactions were completed with a 72°C/10 min final extension. Estimates of transcript abundances, based on three biological replicates, each represented by three technical replicates, were derived by applying the 2^-ΔΔCt method (Livak and Schmittgen, 2001). The reference sequence was UBC9 (ubiquitin-conjugating enzyme 9; accession nr XM_013800933).

**Library construction and RNA-seq**

RNA was extracted and processed from three samples of embryos (30 DAF) harvested from the transgenic line #580 and WTS line #581, using the TruSeq RNA Kit (Illumina, San Diego, CA, USA) kit
following the manufacturer's instructions. The resulting libraries were sequenced using an Illumina HiSeq™ 2500 device.

Reference genomes and data analysis
The version 5 genome assembly of *B. napus* cultivar “Darmor-bzh” (Chalhoub et al., 2014) was retrieved from www.genoscope.cns.fr/brassicanapus/data/. This assembly comprises a set of 101,040 protein-encoding gene models. Gene annotation data for the various gene IDs were obtained from the Universal Protein Resource (www.uniprot.org/). Since only one transcript per locus is predicted to be generated, the analysis did not consider alternative transcripts. As an additional reference, the *A. thaliana* TAIR10 genome release (Lamesch et al., 2012) was accessed to retrieve protein sequences (representative gene models, ftp://ftp.arabidopsis.org/home/tair/Sequences/blast_datasets/TAIR10_blastsets/TAIR10_pep_201103_representative_gene_model_updated.fa), genomic coordinates (ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR10_genome_release/TAIR10_gff3/TAIR10_GFF3_genes.gff) and functional descriptions (ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR10_genome_release/TAIR10_functional_descriptions_20140331.txt). The SynOrths v1.0 package (Cheng et al., 2012; brassicadb.org/brad/tools/SynOrths/) was used to link the protein-encoding genes in the *B. napus* and *A. thaliana* genomes. RNA-seq reads were aligned using bowtie2 v2.2.3 software (Langmead and Salzberg, 2012; www.bioconductor.org) and differential transcription was deduced using the DEseq v1.20.0 package (Anders and Huber, 2010; www.bioconductor.org). The BLAST algorithm v2.2.27+ (Altschul et al., 1997) was used to derive sequence alignments.

Gene annotation and read quantification
Our analysis of RNA-seq data emphasizes higher level aggregation of gene expression over detailed quantification of single gene products. This is meaningful if, for example, the combined transcript abundance originating from different syntelog gene copies is considered to represent the totality of transcript encoding an active enzyme. In addition, as inferring transcript abundances at the gene level is likely a more reliable method than attempting the quantification of individual transcript isoforms (Soneson et al., 2015), it has been assumed that gene expression aggregated onto a higher level might be more robust. These factors have particular relevance to *B. napus* given its tetraploid status (Chalhoub et al., 2014). The strategy adopted was therefore to first establish orthologies between *B. napus* and *A. thaliana* genes, taking advantage of the SynOrths v1.0 package with *B. napus* set as
the query genome and *A. thaliana* as the reference genome; the resulting *B. napus* RNA-seq read counts were thereby transferred to their *A. thaliana* gene orthologs. Inconsistencies arising in the SynOrths output were manually resolved based on Blast alignments. For this integration of orthology relations, it needs to be considered that SynOrths simplifies the complexity resulting from tandem array duplications in the reference genome by reporting only one gene as a representative for each group of genes in a tandem gene array. Accordingly, we applied the tandem array mapping as obtained from the SynOrths output to the Chalhoub et al. (2014) synteny information. Finally, for those *B. napus* genes for which no *A. thaliana* ortholog was found, non-syntenic orthology was deduced based on a Blastp alignment against the *A. thaliana* proteome. Orthology was assumed for the best alignment between a *B. napus* and an *A. thaliana* sequence, provided that the e value was <10, the alignment included >40% identity and the aligned length represented at least 60% of the length of the query sequence. The whole procedure resulted in the association of 95,197 *B. napus* genes with 21,282 *A. thaliana* genes (Supplemental Dataset 2H), with 5,843 *B. napus* genes (5.8%) remaining without any *A. thaliana* ortholog.

Alignment of RNA-seq reads to transcripts
High-throughput paired end sequencing reads were aligned against predicted *B. napus* transcripts using bowtie2 v2.2.3 software, counting only paired end alignments. According to the SAM-format output ("mapping quality"), for 87% of the alignments there was a <1% probability of no correspondence with the read’s true point of origin in the genome. The alignment to 101,040 predicted *B. napus* transcripts resulted in the detection of 67,885 (67%) of the *B. napus* protein-encoding genes. All but 1,591 of *B. napus* reads (equivalent to 99.5%) were associated with 19,062 *A. thaliana* genes.

Differential transcription
Differential transcription between transgenic and WTS samples was detected using the DEseq package (Anders and Huber, 2010), applying default parameter settings. The read counts were step normalized in order to adjust for differences in sequencing depth between samples (Anders and Huber, 2010). To guard against the possibility that the presence of a small number of highly abundant transcripts introduced a bias following the normalization procedure, the impact of omitting the 22 most highly represented genes from the data set was tested. (For the WTS line #581, about 50% of the reads were derived from genes encoding about 20 putative 2S and 12S seed storage proteins, while in the transgenic line #580, this was the case for only about 5% of the reads.) The test resulted in only one change in the call of differential transcription (gene *BnaC06g21090D*). To limit the number
of false positives, the Benjamini-Hochberg multiple testing adjustment was included (adjusted p-values, padj). As recommended by Schurch et al. (2016), the significance threshold was set as 5% for the adjusted p-value and a more than four-fold change in transcript abundance (equivalent to padj<0.01 and |log₂fold change|>2) (Supplemental Dataset 2C).

**Metabolic seed model**

Simulations of metabolic flux distributions in developing wild-type (line 581) and transgenetic (line 580) embryos by flux balance analysis were performed based on *bna572* (Schwender and Hay, 2012; Hay et al., 2014), a metabolic model describing growth and storage accumulation in *B. napus* developing embryos. In one study (Borisjuk et al., 2013), this model was parametrized for in-planta relevant conditions at 30 DAF, specifically into three tissue-specific embryo sub-models representing the inner cotyledon, outer cotyledon and radicle, respectively. In this study the same in-planta developmental stage (30 DAF) is to be modeled, and therefore some essential model constraints were adapted for the former study by essentially superimposing the three sub-models according to biomass proportions. This way estimates were derived for the intensity of photosynthetic active light reaching the embryo within the developing silique (photon flux) as well as for the ATP-drain flux, a generic reaction summarizing the impact of non-growth associated ATP consumption on metabolism. As further model inputs, embryo-specific growth rates were determined from embryo dry weight determinations between 17 and 37 DAF (Supplemental Dataset 5A). The biomass composition of embryo material was determined at 30 DAF (Supplemental Dataset 5B). Organic solvent extraction and liquid:liquid fractionation into a chloroform soluble (lipid), methanol/water soluble (polar), and insoluble cell polymer fraction as well as elemental analysis was performed as reported earlier (Lonien and Schwender, 2009). To integrate lipidomic data into the model (Supplemental Dataset 5C), the lipid metabolism subnetwork of *bna572* was expanded for explicit simulation of lipid molecule species. Flux simulations were performed using the COBRA toolbox 2.0.5 (Schellenberger et al., 2011) with TOMLAB CPLEX solver package (version 12.3, http://tomopt.com) implemented in MATLAB (version R2013a, http://www.mathworks.com). Simulations were done by minimization of substrate uptakes (sucrose, glutamine) and optimization of the models with function ‘optimizeCBmodel’, and option ‘allowLoops’ set to ‘false’. The SBML standard compliant XML model is given in Supplemental Dataset 6.

**Calculations and Statistics**

Mathematical calculations were performed using Excel 2010 (Microsoft Corp., Redmond WA, USA),
and statistical analyses using software MATLAB (version R2019b, http://www.mathworks.com). The significance of differences between mean abundances was tested using one-way ANOVA with an alpha of 0.05. All test results are given in Supplemental Dataset 7.

**Accession Numbers**

Cru1 - BnaC07g48660D; Cru2 - BnaA02g22500D; Cru3 - BnaA01g08350D; Nap2 - BnaA01g16180D; Nap3 - BnaC01g43250D; Nap4 - BnaC01g19300D; PEPC1 - BnaA08g01200D; PEPC2 - BnaC03g69540D

Primary transcriptome data are available at the EMBL-EBN (https://www.ebi.ac.uk/ena) under accession number PRJEB36839.

Proteome raw data are available via ProteomeXchange with identifier PXD018919.

**Supplemental Data**

**Supplemental Figure 1.** Compositional analysis of mature transgenic seeds (lines 576, 578, 580) versus the respective WTS (lines 577, 579, 581).

**Supplemental Figure 2.** Germination of transgenic seed carrying an RNAi construct designed to repress genes encoding PEPC, napin and cruciferin.

**Supplemental Figure 3.** The intracellular structure of early storage stage cells.

**Supplemental Figure 4.** Intracellular architecture in transgenic line #578 versus its WTS #579.

**Supplemental Figure 5.** Distribution of lipid molecular species.

**Supplemental Figure 6.** The content of phosphatidylcholine (PC) and triacylglycerols (TAGs) of dissected parts of the whole embryo.

**Supplemental Figure 7.** The level and distribution of membrane lipids in the seed.

**Supplemental Figure 8.** Proteomic profiling of the embryo.

**Supplemental Figure 9.** Photosynthetic and respiratory activity in the seed.

**Supplemental Figure 10.** Southern-blot analysis of transgenic lines.

**Supplemental Figure 11.** Outcome of TaqMan assays and glufosinate treatment.

**Supplemental Table 1.** Hairpin sequence information.

**Supplemental Table 2.** Primers used for quantitative real–time PCR analysis of PEPC, napins, cruciferins and the reference gene UBC9.

**Supplemental Movie 1.** Representative, three-dimensional models of mature *B. napus* seeds of transgenic line 580 versus the WTS line 581, based on magnetic resonance imaging.

**Supplemental Movie 2.** Three-dimensional models of lipid distribution in mature *B. napus* seeds...
based on magnetic resonance imaging.

**Supplemental Movie 3.** Comparison of mature *B. napus* seeds of transgenic lines 578/580 versus the respective WTS lines 579/581, based on magnetic resonance imaging.

**Supplemental Movie Legends.**

The following Supplemental Data Sets were submitted to the Data Dryad Repository and are available at [http://datadryad.org/resource/doi:10.5061/dryad.dbrv15dz0](http://datadryad.org/resource/doi:10.5061/dryad.dbrv15dz0).

**Supplemental Dataset 1.** Identification of hairpin targets for the knock-down of cruciferin, napin and PEPC.

**Supplemental Dataset 2.** Transcriptome dataset.

**Supplemental Dataset 3.** Proteome dataset; mass spectrometry-based identification of differentially expressed proteins.

**Supplemental Dataset 4.** Levels of metabolic intermediates measured using mass spectrometry and chromatography.

**Supplemental Dataset 5.** FBA-Modeling results of the biomass composition, metabolic fluxes and bioenergy cofactors for lipid and protein biosynthesis of embryos (30 DAF).

**Supplemental Dataset 6.** Model files (SBML and spreadsheet formats) and code for simulation of metabolic flux distributions.

**Supplemental Dataset 7.** Statistical test results.

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**Author contributions**

L.B. and H.R. designed and carried out the research, analyzed the data with the help of H.-P.B and P.D., and wrote the article. C.L. and C.K. were responsible for the generation of the proteomic, respiration and PAM imaging data and sampling; J.S., S.McC. and H.S. for the transcriptomic data and FBA modeling; A.G. for near infrared spectroscopy, biomass fractionation and statistics; E.M. for the MRI data; Tr.R. and K.C. for the lipidomic and MALDI imaging data; N.H. for the metabolomic data; and S.O., Tw.R. and T.B. for the histological data and structural modeling. K.vanA., S.B. and M.V.A. constructed the transgenics.
Table 1. Proteins classified as differentially abundant between transgenic and WTS embryos, based on two-dimensional gel electrophoresis profiling followed by their identification by MS. Full list is given in Supplemental Dataset 3.

| Spot Volume | Up-regulated proteins in transgenic (line 580) | ATG code |
|-------------|------------------------------------------------|----------|
| Diff.       |                                                 |          |
| 6.75        | Oleosin S2-2                                   | At3g27660|
| 4.71        | Biotin carboxyl carrier protein of acetyl-CoA carboxylase 2, chloroplastic (BCCP2) | At5g15530|
| 3.73        | Elongation factor 1-alpha 1                    | At1g07940|
| 3.32        | 60S ribosomal protein L10                      | At1g26910|
| 3.32        | Osmotin-like protein                            | At4g11650|
| 3.18        | MLP-like protein 31                             | At1g70840|
| 2.74        | Oleosin S2-2 O                                 | At3g27660|
| 2.72        | Oleosin 21.2 kDa                               | At5g40420|
| 2.66        | Glutathione S-transferase U5                    | At2g29450|
| 2.56        | Nitrogen regulatory protein PII homolog         | At4g01900|
| 2.35        | Beta-galactosidase 3                            | At4g36360|
| 2.35        | Probable serine protease EDA2                   | At2g18080|
| 2.32        | Isopentenyl-diphosphate Delta-isomerase II      | At3g02780|
| 2.25        | Alpha-galactosidase                             | At5g08370|
| 2.25        | UDP-sulfoquinovose synthase, chloroplast precursor | At4g33030|
| 2.19        | Importin subunit alpha-1                       | At3g06720|
| 2.19        | Protein disulfide isomerase-like 1-2            | At1g77510|
| 2.19        | Pyruvate kinase isozyme A, chloroplastic        | At3g22960|

| Spot Volume | Down-regulated proteins in transgenic (line 580) | ATG code |
|-------------|------------------------------------------------|----------|
| Diff.       |                                                 |          |
| 10.17       | Cruciferin CRU1                                 | AT4G28520|
| 10.17       | Malate dehydrogenase, mitochondrial             | AT1G53240|
| 5.94        | 12S seed storage protein CRU1                   | AT5G44120|
| 5.94        | 12S seed storage protein CRU4                   | At1g03890|
| 5.63        | Napin-2                                        | AT4G27150|
| 4.89        | Elongation factor 1-gamma 1                     | AT1G09640|
| 4.89        | Elongation factor Tu, chloroplastic             | AT4G20360|
| 4.89        | Monodehydroascorbate reductase, cytoplasmic isoform 3 | AT3G09940|
| 4.89        | S-adenosylmethionine synthase                   | AT1G02500|
| 4.89        | S-adenosylmethionine synthase 2                 | AT4G01850|
| 4.89        | S-adenosylmethionine synthase 3                 | AT2G36880|
| 4.53        | Methylenetetrahydrofolate reductase 1           | AT3G59970|
| 4.35        | Succinyl-CoA ligase [GDP-forming] subunit alpha-1 | At5g08300|
4.22 Biotin synthase At2g43360
4.22 Fructose-bisphosphate aldolase, cytoplasmic isozyme At4g26520
4.08 Branched-chain-amino-acid aminotransferase-like protein 2 At5g27410
3.96 Eukaryotic translation initiation factor 3 subunit H At1g10840
3.77 Phosphopantothenate--cysteine ligase 2 At5g02080
3.51 Cruciferin AT4G28520
3.51 Napin At4g27140
3.51 Napin-3 At4g27160
3.45 Probable E3 ubiquitin-protein ligase ARI12 At1g05880

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Fig. 1: The effect of suppressing cruciferin and napin achieved by introducing an RNAi construct. (A-C) Transcript abundances in the developing embryo (30 DAF) of genes encoding (A) cruciferin (Cru), (B) napin (Nap), (C) PEPC. Abundances are given in the form of log2-fold changes in the transgenic embryos (lines 576, 578, 580) versus the respective WTS (lines 577, 579, 581). Values shown as individual data points; n = 3 biological repeats, each comprising pooled RNA extracted from 10 embryos. (D) Log2-fold changes in transcript abundances over the course of embryo development of genes encoding napin and cruciferin in transgenic line 580 (versus WTS line 581) (n=3 biological repeats with 9 embryos each). The expression level in A-D was normalized to that of BnUBC9. (E) Maximum catalytic activity of PEPC in embryo extracts (n = 6 replicates with 3 embryos each). (F-H) Content of (F) lipids, (G) fiber, (H) protein in mature seeds. Values in (E-H) are shown in the form mean ± SD (error bars); n = 12 replicates each comprising ~5 seeds in (F); n = 6 replicates each comprising ~20 seeds in (G,H). Asterisks indicate that means differed significantly from WTS values (p<0.05, one-way ANOVA) (ns, not statistically significant). Mature seeds from 3 independent batches were analyzed regarding parameters (F-H) with similar results.
Fig. 2: The effect on seed phenotype of suppressing cruciferin and napin via the introduction of an RNAi construct. (A) Three-dimensional seed models for two transgenic lines (578, 580) and their corresponding WTS (579, 581) acquired by MRI. Red arrows indicate air-filled cavities (void space); white arrow indicates radicle tip. (B) Weight of mature seeds, n=6 with ~20 seeds each. (C-F): Volume of embryo (C), seed coat (D), void space (E) and the percentage of each component to total seed volume (F). Asterisk in (B-E) indicates that means differed significantly from WTS values (p<0.05, one-way ANOVA) (ns, not statistically significant). Data in C-F were derived from the MRI-based analysis of 8 individual seeds per genotype. See also Supplemental Movie 1-3. Abbreviations: em-embryo, ic-inner cotyledon, oc-outer cotyledon, ra-radicle, sc-seed coat, vs-void space.
Fig. 3: Fine structure of the seed embryo’s storage tissues. (A,B) Protein bodies, visualized using Ponceau staining, in early storage stage embryos formed by (A) WTS line #581, (B) transgenic line #580. (C,D) Electron micrographs of protein bodies in early storage stage embryos formed by (C) WTS line #581, (D) transgenic line #580. (E,F) Electron micrographs of protein bodies in mature storage stage embryos formed by (E) WTS line #581, (F) transgenic line #580. (G) The transgenic embryos formed distinct large oleosin-containing membrane stacks (marked with arrows).
Fig. 4: The ultrastructure of the embryo storage tissues. (A,E) Protein bodies (pb) surrounded by lipid bodies (lb) in the embryos of (A) WTS line #581, (E) transgenic line #580. The oleosin-containing membrane stacks present in embryos formed by transgenic line are arrowed. (B-D, F-H) Immunolabelling used to highlight the presence of (B,F) oleosin, (C,G) napin, (D,H) cruciferin in the embryos of (B-D) WTS and (F-H) transgenic lines.
Fig. 5: The level and distribution of storage and membrane lipids in the seed. (A) Mean contents of phosphatidylcholine (PC) and triacylglycerols (TAGs) as measured using ESI-MS; data are given in nmol/mg seed. (B) Mean contents of individual molecular species of PC/TAGs, as measured using ESI-MS; data are given in mole percent (mol%); both sets (A,B) are plotted for each of the three replicates with the standard deviation lines centered around the mean. (C) MS-acquired images showing the spatial distribution of PC and TAGs. The abundance of each compound type is shown as mol% on a colored scale from yellow (low) to green (high). Images captured by light microscopy of the sections used for MS imaging are shown to the left of the MS-acquired ones. See also Supplemental Fig. 4 for biological replicates. Bars: 1 mm.
Fig. 6: The impact of the RNAi transgene on the transcriptome. Read counts of *B. napus* genes ranked according to their expression levels in WTS line #581 (A) or transgenic line #580 (B); only 30 highest ranking genes are shown. The genes associated with the highest read counts in the WTS were mostly identified as encoding storage protein (napin or cruciferin). These represented ~50% of the counts in WTS line #581, but only 4% in transgenic line #580. In the transgenic line, 50% of the read counts are contained in 614 of the highest ranking genes. For gene identifiers and further information, see Supplemental Dataset 2J. *n* = 3 biological repeats, each comprising pooled RNA extracted from 10 embryos. Significance threshold was set as 5% for the adjusted *p*-value and a more than four-fold change in transcript abundance. Asterisks indicate that means differed significantly between WTS and the transgenic line.
Fig. 7: Steady state profile of metabolites in the developing *B. napus* embryo and related changes in gene expression. (A) Metabolite abundance (ordered by categories; number in parenthesis indicate quantified compounds) present in the embryos of transgenic line #580 and WTS line #581. (B) Central metabolism network view. Heat map of metabolites indicates mean values relative to that obtained in WTS; increased (decreased) abundance of compounds in red (blue) color. Three biological replicates with each sample consisting of ~10 embryos were analyzed. Numbers refer to enzymes for which differential gene expression was evident at the level of either the transcriptome or proteome. Bold frame refers to statistical significance (one-way ANOVA; p<0.05).
Fig. 8: Modeling of the impact of the RNAi transgene on the embryonic cells of *B. napus*. (A) Demands in energy cofactors (ATP, reducing equivalents) for the biosynthesis of proteins and lipids. The reducing equivalents for fatty acid desaturation are shown separately of the balance for lipids. Details in Supplemental Dataset 5E. (B) Allocation of nitrogen among different biomass fractions (based on biomass and elemental composition in Supplemental Dataset 5C). (C) Increase of surface area in response to ellipsoid parameters: increasing one of the axes of a sphere while keeping its volume constant will result in an increase of the surface area of the ellipsoid over the sphere. Calculation based on the formula in (D); (D) Gain of surface by reshaping lipid bodies (LB). Left panel shows distinctly shaped LB in electron micrograph. Right panel exemplifies how the transition from sphere to ellipsoid (at constant volume) causes tremendous gain in surface area (SA). Transgenic embryonic cells apply this strategy to incorporate the excess membrane protein oleosin; the stacks are partially a consequence of the excess oleosin and the preponderance of phospholipid at the partial expense of triacylglycerol.
**Cellular Plasticity in Response to Suppression of Storage Proteins in the Brassica napus Embryo**  
Hardy Rolletschek, Jorg Schwender, Christina König, Kent D. Chapman, Trevor Romsdahl, Christin Lorenz, Hans-Peter Braun, Peter Denolf, Katrien van Audenhove, Eberhard Munz, Nicolas Heinzel, Stefan Ortleb, Twan Rutten, Sean McCorkle, Taras Borysyuk, André Gündel, Hai Shi, Michiel Vander Auwermeulen, Stéphane Bourot and Ljudmilla Borisjuk  
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