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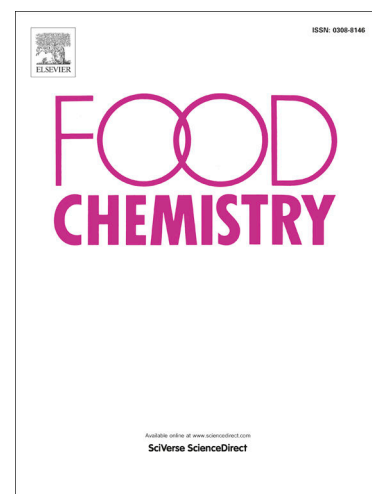
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## Guanosine tetraphosphate (ppGpp) is a new player in *Brassica napus* L. seed development

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### Abstract

Rapeseed oil, constituting 12% of global vegetable oil production, is susceptible to quality degradation due to stress-induced incomplete seed degreening, fatty acid oxidation, or poor nutrient accumulation. We hypothesise that the hyperphosphorylated nucleotide alarmone ppGpp (guanosine tetraphosphate), acts as a pivotal regulator of these processes, given its established roles in nutrient management, degreening, and ROS regulation in leaves. Using qPCR, UHPLC-MS/MS, and biochemical methods, our study delves into the impact of ppGpp on seed nutritional value. We observed a positive correlation between ppGpp levels and desiccation, and a negative correlation with photosynthetic pigment levels. Trends in antioxidant activity suggest that ppGpp may negatively influence peroxidases, thereby safeguarding against chlorophyll decomposition. Notably, despite increasing ppGpp levels, sugars, proteins and oils appear unaffected. This newfound role of ppGpp in seed development suggests it regulates the endogenous antioxidant

system during degreening and desiccation, preserving nutritional quality. Further validation through mutant-based research is needed.

## Keywords:

oilseed rape seeds; fatty acids, *RSH* genes; ppGpp; stringent response; antioxidant system

## Highlights

- Alarmone ppGpp was detected in the seed of *B. napus* L., non-leaf organs.
- ppGpp accumulation peaked at the last stage of seed development.
- Alarmone accumulation did not prevent the build-up of storage compounds.
- ppGpp concentration negatively correlates with chlorophyll content in seeds.

## 1. Introduction

According to the Food and Agriculture Organisation of the United Nations, oil extracted from rape seeds (*Brassica napus* L.) accounts for 12% of global vegetable oil production (FAO, 2022). This oil is a valuable component of a healthy diet, due to its high content of mono- and polyunsaturated fatty acids (MUFA= 62.6% and PUFA = 25.3% of oil, respectively), vitamins E and K, and low level of saturated fatty acids (SFA = 6.6%) (USDA, 2022). Fatty acids (FAs) are synthesised in chloroplasts from acetyl-CoA, and then transported to the endoplasmic reticulum, where they are assembled into triacylglycerols (TAGs). During seed desiccation, TAGs are loaded into vessels to form oil bodies (Frey-Wyssling et al. 1963; Chrispeels and Herman 2000). Rapeseed meal, the byproduct of oil extraction, contains a significant amount of proteins, sugars, and amino acids in favourable quantitative ratios for animal feed (Newkirk 2011). These compounds are mainly deposited in the seed during the grain-filling stage.

The adverse impacts of global climate change have the potential to disrupt proper seed development, leading to a reduction of seed yield, weight, and alterations in its chemical composition. For example, heat stress in *B. napus* results in increased protein content alongside reduced FA concentration. Moreover, such conditions lead to changes in oil quality, with a decrease in omega-3 FAs and an increase in omega-6 FAs (Elferjani et al. 2018). Recent studies have demonstrated that salt stress affects the seed development of *B. napus* by altering the nitrogen (N) and carbon (C) balance within seeds. This effect is achieved through the inhibition of N assimilation and a decrease in the efficiency of N translocation from leaves to seeds (Wang et al. 2022). A full understanding of the complex network of factors that orchestrate nutrient management during grain filling and how it is influenced by environmental cues is especially important for ensuring food security.

The central switch that controls plant and algal metabolism as well as nutrient management during growth and adaptation is the stringent response, initially found to downregulate bacterial metabolism in response to amino acid starvation (Li et al. 2022a; Maekawa et al. 2015).

Interestingly, a similar mechanism known as stringent-like response has been observed in metazoans as well (Sun et al. 2010; Ito et al. 2020; Young et al. 2021). This process is characterised by the production of nucleotides guanosine 5'-monophosphate 3'-diphosphate (pGpp), guanosine tetra- (ppGpp) and guanosine pentaphosphate (pppGpp), referred as (pp)pGpp or alarmone. To date, pGpp and pppGpp were found exclusively in bacteria (Yang et al. 2020; Malik et al. 2023). In plants, ppGpp levels are adjusted by nucleus-encoded, chloroplast localised RelA/SpoT homologue (RSH) proteins. Among these, RSH1 acts as the main alarmone hydrolase. RSH2 and RSH3 are bifunctional enzymes with ppGpp synthase/hydrolase activities, and Ca<sup>2+</sup>- dependent RSH (CRSH) catalyses (p)ppGpp synthesis (Boniecka et al. 2017; Dąbrowska et al. 2006; Masuda et al. 2008a).

While ppGpp has not been previously detected in non-leaf tissues, a collection of evidence suggests a link between alarmone and grain filling. Firstly, *Arabidopsis RSH2* and *RSH3* knock-out plants, lacking the major (p)ppGpp synthases, produced seeds significantly lighter in weight compared to the wild-type plants (Sugliani et al. 2016).

Secondly, the Target of Rapamycin (TOR), a serine/threonine protein kinase that serves as a master regulator managing trade-off responses between growth and stress adaptation (Flavell 2022), has been associated with the stringent response in algae (Imamura et al. 2018) and in *A. thaliana*. In the latter, TOR controls the translocation of RSH3 into chloroplasts (D'Alessandro et al. 2023). Importantly, TOR establishes a nutrient sink, thereby orchestrating nutrient relocation to energy-demanding organs and tissues like growing seeds (Liang et al. 2013).

Thirdly, ppGpp is known to bind a wide range of proteins that potentially can alter gene expression, and thus the cell capacity to synthesize storage proteins or enzymes involved in nutrient biosynthesis and modification. Our understanding of this ppGpp-interactome is still expanding. In plants, ppGpp regulates processes that alter the chloroplast proteome and energy level: nuclear and chloroplast transcription, chloroplast translation, GTP biosynthesis, cell growth, lipid metabolism, protein and sugar biosynthesis, and the remodelling of photosynthetic apparatus (Tozawa and Nomura 2011; Field 2018; Avilan et al. 2021; Romand et al. 2022; Mehrez et al. 2023). However, the complete network of molecular-level interactions is still under investigation. To date, alarmone interaction with plant proteins has been suggested only for the plastid-encoded RNA polymerase (Sato et al. 2009; Sugliani et al. 2016) and guanylate kinase (Nomura et al. 2014).

Fourthly, the stringent response contributes to plant growth and development (Masuda 2012; Ihara and Masuda 2016; Ono et al. 2021; Maekawa et al. 2015; Sugliani et al. 2016). Importantly, ppGpp was also proposed to regulate nutrient remobilisation and relocation from vegetative tissues into seeds during senescence (Boniecka et al. 2017). This alarmone also plays a vital role in stress response and acclimation when energy resources must be remobilised (Boniecka et al. 2017; Abdelkefi et al. 2018; Romand et al. 2022).

Fifthly, our previous work demonstrated dynamic expression of *BnCRSH* throughout *B. napus* seed development, peaking at the 70<sup>th</sup> day after flowering (DAF). Interestingly, given the Ca<sup>2+</sup>-dependency of CRSH enzymes, this pattern correlated with the amount of calcium in the seeds (Turkan et al. 2023a).

Lastly, RSH gene promoters contain putative *cis*-regulatory elements associated with plant growth/development, light response, hormonal response, and abiotic and biotic stress response regulatory elements (Dąbrowska et al. 2021).

Together these lines of evidence led us to formulate a hypothesis that ppGpp may serve as an important gatekeeper of nutrient reserves in seeds, and thereby in oil and meal. Alterations in ppGpp levels could potentially affect the biochemical makeup of seeds, which, in turn, may impact the quality of the extracted oil. One of the key factors contributing to reduced oil quality is chlorophyll contamination resulting from incomplete breakdown and the oxidation of FAs. In *Arabidopsis thaliana*, ppGpp accumulation prevents the overproduction of reactive oxygen species (ROS), whereas reduced levels of alarmone have been linked to increased oxidation of linolenic acid (Romand et al. 2022). This protective mechanism may be crucial during chlorophyll breakdown (degreening) and seed desiccation, helping to maintain minimal oxidation of storage materials.

To verify our hypothesis, we collected seeds at five developmental stages (35, 56, 63, 70, and 80 days after flowering). Initially, we examined the dynamics of stringent response in developing seeds, by analyzing the expression of representative members of each of the *RSH1-3* families, and measuring ppGpp levels. Importantly, this study represents the first report showing the presence of alarmone in non-leaf tissues. Next, to gain insight into the possible involvement of ppGpp in desiccation, photosynthetic pigment breakdown, ROS management, and nutrient quantity and quality, we investigated the correlation between ppGpp/RSH and water content, chlorophyll and carotenoid levels, activities of antioxidant enzymes (peroxidases, catalases, and superoxide dismutases), as well as lipid, carbohydrate, and protein content.

## **2. Material & Methods**

### **2.1. Plant material and growth conditions**

*B. napus* L. spring cultivar Karo seeds were sown on a growth medium consisting of a mixture of soil (Garden soil AthenA, pH (H<sub>2</sub>O) 5.5-6.5) and vermiculite at a ratio of 3:1 in 5 L (22.5 cm) pots. Plants were regularly watered at intervals of three to four days or as needed depending on the plant developmental stage. Rapeseed plants were grown in a growth chamber, with temperature regulated to 24 °C, at 16 h light and 8 h dark cycles, and light intensity of 250  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Flowers were tagged at the time of opening for determination of days after flowering (DAF). The range of flowering time was 127–136 days after sowing. Seeds were harvested at different developmental stages (35, 56, 63, 70, and 80 DAF), transferred to liquid nitrogen and stored at -80 °C until further procedures.

### **2.2. Water content**

Forty seeds of each developmental stage (35, 56, 63, 70, 80 DAF) were weighed in 3 replicates. Then the seeds were dried in an oven at 60 °C for 48 h and weighed. Seed water content was calculated as the percentage of water content in the fresh seed weight.

### **2.3. Pigment content**



The pigment content was determined by the modified spectrophotometric method of Lichtenthaler and Wellburn (1983). Seed samples were homogenised in 80% ethanol. Then the homogenate was centrifuged at 1000 g for 5 minutes at 4 °C. The supernatant was collected and stored at 4 °C in the darkness until the absorbance was measured. Absorbance values  $A_{470}$ ,  $A_{648.6}$ ,  $A_{664.2}$  were measured using microplate reader (Synergy 2, Bio-Tek, Winooski, VT, USA). The content of chlorophyll *a* and *b* and the sum of carotenoids were calculated from the following equations:

$$\text{Chlorophyll } a \text{ } [\mu\text{g mL}^{-1}]: \quad C_{\text{chl.a}} = 13.36 A_{664.2} - 5.19 A_{648.6}$$

$$\text{Chlorophyll } b \text{ } [\mu\text{g mL}^{-1}]: \quad C_{\text{chl.b}} = 27.43 A_{648.6} - 8.12 A_{664.2}$$

$$\text{Total chlorophyll } [\mu\text{g mL}^{-1}]: \quad C_{a+b} = 5.24 A_{664.2} + 22.24 A_{648.6}$$

$$\text{Total carotenoids } [\mu\text{g mL}^{-1}]: \quad C_{x+c} = (1000 A_{470} - 2.13 C_{\text{chl.a}} - 97.64 C_{\text{chl.b}}) / 209$$

Values were normalised to dry weight (DW) and expressed as  $\mu\text{g mg}^{-1}$  DW.

#### 2.4. *BnRSH* genes expression

Total RNAs isolation, reverse transcription and qPCR procedures were performed as described before (Turkan et al. 2023a, b). For expression analyses, we chose one gene of each *RSH1-3* type: *BnRSH1\_b* (XM\_013839498.2), *BnRSH2\_b* (XM\_022703426.1), and *BnRSH3\_a* (XM\_013785013.2). Ubiquitin-conjugating enzyme 9 (*BnUBC9*, XM\_013816206.2) was used as a reference gene. Primers for *BnRSH1-3* and *BnUBC9* are the same as in our previous work (Turkan et al. 2023b).

#### 2.5. Alarmone content

Nucleotide pools were isolated from developing rape seeds, according to Ihara *et al.* (2015) and Bartoli *et al.* (2020), with some modifications. We added 1.5 ml of 2 M formic acid to the frozen crushed tissue (~100 mg) in liquid nitrogen, and the samples were incubated for 30 min on ice with gentle shaking. After incubation, 1.5 ml of 50 mM ammonium acetate (pH 4.5) was added. Cell debris was removed by centrifugation at 5000 g for 5 min at 4 °C, and the supernatants were collected. 3.75  $\mu\text{l}$  of 10  $\mu\text{M}$  13C ppGpp internal standard was added to each sample. Then, the extracts were purified with OASIS® WAX SPE cartridges (Waters, Milford, MA, USA) using the following procedures. First, we pre-treated the SPE cartridge with 1 ml of MeOH and then washed it with 1 ml of 50 mM  $\text{NH}_4\text{OAc}$  (pH 4.5). Subsequently, we loaded the sample extracts onto the SPE cartridge and then washed them with 1 ml of 50 mM  $\text{NH}_4\text{OAc}$  (pH 4.5), followed by 1 ml of MeOH. We have eluted the nucleotide pool from the SPE cartridge with 1 ml MeOH/ $\text{H}_2\text{O}$ / $\text{NH}_4\text{OH}$  (20:70:10) solution. The effluent was lyophilised using the CentriVap Cold Trap system (Labconco, Kansas City, MO, USA), dissolved in 100  $\mu\text{l}$  of water, and filtered through DNA Purification Columns (EURx, Gdańsk, Poland). The nucleotides were isolated from three biological replicates and analysed in three technical replicates.

Alarmone ppGpp content in extracts was assessed using liquid chromatography–tandem mass spectrometry (LC-MS/MS) with the Nexera UHPLC and LCMS-8045 integrated system

(Shimadzu Corporation, Canby, OR, USA). We employed Ascentis® Express C18, 2.7  $\mu\text{m}$  HPLC Column (Supelco, Bellefonte, Pennsylvania, U.S.A.). Nucleotides were separated at a flow rate of 0.4  $\text{ml min}^{-1}$  in a linear gradient of solvent A (aqueous solution of 8 mM hexylamine, supplemented with 80  $\mu\text{L}$  acetic acid) and solvent B (acetonitrile) set according to the following profile: 0 min, 96% A; 8 min, gradual change to 30% A, 8.50 min, decrease to 96% A; 20 min, 96% A. The ESI ion source was set to the positive ion mode, and the measurement parameters were automatically optimised. Endogenous 12C ppGpp and 13C ppGpp internal standards were detected in the multiple reaction monitoring (MRM) mode. The ion transition at  $m/z$  603.8  $\rightarrow$  152.1 was used for 12C ppGpp and at  $m/z$  613.9  $\rightarrow$  157.05 for 13C ppGpp detection.

## 2.6. Protein extraction

Protein extraction was conducted as described before (Konieczna et al. 2023). Approximately 100 mg of the fresh plant tissue was homogenised at 4  $^{\circ}\text{C}$  with a 0.05 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenate was centrifuged at 10,000 g for 15 min, and the supernatant was collected.

## 2.7. The activity of antioxidant enzymes

The activity levels of peroxidase (POD) catalase (CAT) and superoxide dismutase (SOD) were determined using methods adapted from (McCord and Fridovich 1969; Aebi 1984; Lück 1962) as described before (Konieczna et al. 2023). Statistical analyses were performed based on three biological replicates analysed in three technical replicates. The activity of each enzyme was normalised to the total protein content.

## 2.8. Protein content

Protein content was measured as described before (Konieczna et al. 2023). The analysis was performed in three biological replicates and three technical replicates.

## 2.9. Sugar content

The total amount of sugars was determined spectrophotometrically using the phenol-sulfuric acid method (DuBois et al. 1956). Soluble sugars were extracted from 100 mg of plant tissue in 1 ml of 80% ethanol and centrifuged at 2800 g for 5 min. The reaction mixture contained 200  $\mu\text{L}$  of distilled water, 10  $\mu\text{L}$  of the ethanolic plant extract, 200  $\mu\text{L}$  of 5% phenol and 1 mL of 95% sulphuric acid. After the samples had cooled down, the absorbance was measured at 490 nm using a microplate reader (Synergy 2, Bio-Tek, Winooski, VT, USA). Glucose was used as a standard. The analysis was performed in three biological replicates and three technical replicates.

## 2.10. Oil content

The oil content in rape seeds was determined according to a procedure described by Quéro *et al.* (2016) with minor modifications. Approximately 100-150 mg of plant tissue ground in liquid



nitrogen was transferred to tubes, and 1 mL of n-hexane-isopropanol (2:1) was added to each sample, and mixed vigorously for 5 min. The homogenates were centrifuged at 9000 g for 5 min at 4 °C and the supernatants containing oil were recovered. The oil extraction from each seed sample was repeated two times. The supernatants were collected, and the solvent evaporated until reaching a constant weight of the recovered oil.

### 2.11. Fatty acids composition

Total lipids were extracted and analysed according to Marmon *et al.* (2017) and Miklaszewska *et al.* (2023). Seeds were collected at different developmental stages (35, 56, 63, 70, and 80 DAF). One ml of a methanolic solution containing 2.75% (v/v) H<sub>2</sub>SO<sub>4</sub> (95%–97%), 2% (v/v) dimethoxypropan, and 2% (v/v) toluene was added to the samples. Then, samples were shaken at 80°C for 1 h and washed with 1 mL *n*-hexane and 1.2 mL of saturated NaCl solution. After centrifugation at 1000 g for 10 min at RT, the upper phase was transferred to a new glass tube and dried under nitrogen streaming. FAs were separated and quantified by gas chromatography (Agilent Technologies, Santa Clara, USA) using tripentadecanoate (tri-15:0) (Merck KGaA, Darmstadt, Germany) as an internal standard and added to each sample before the methylation. The analysis was performed in three biological replicates.

### 2.12 . Scanning electron microscopy

Analysis of oil content was performed using scanning electron microscopy (SEM). For SEM analysis, seeds from each stage were dried at 90 °C for 1 hour. Before analysis, seeds were sputtered with SC7620 palladium gold using a nano-plating sprayer (Quorum Technologies, Lewes, UK). Imaging was performed using high-resolution scanning electron microscopy (scanning electron microscope /focused ion beam) Quanta 3D FEG (FEI company, Hillsboro, Oregon, United States). HV was 10.0 kV, the mode was SE, the pressure was 2.27e-3 Pa. The analysis was performed in three biological replicates.

### 2.13. Confocal microscopy

As described before in Miklaszewska *et al.* (2023), samples (embryo and endosperm) were incubated with 1 mM BODIPY™ 505/515 for 30 min and washed with phosphate-buffered saline (PBS, pH 7.2). Samples were observed under a confocal laser scanning microscope (CLSM) Olympus F3000 (Olympus, Tokyo, Japan) with excitation at 505 nm and emission at 515 nm for BODIPY. All images were analysed and processed using Image J software. The analysis was performed in three biological replicates.

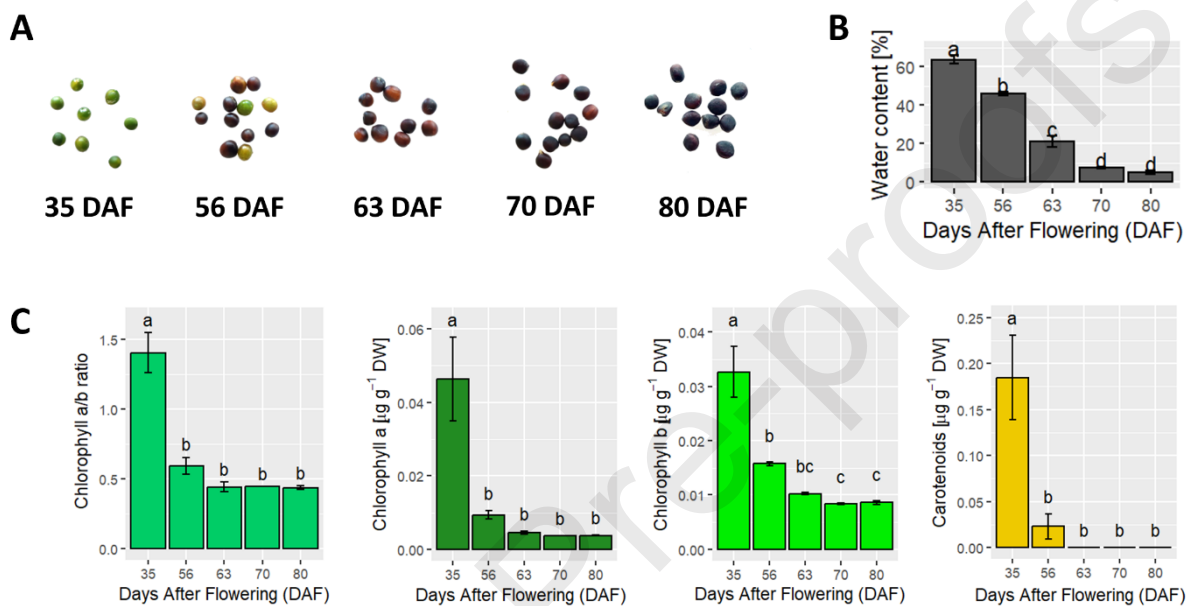
### 2.14. Statistical analysis

Data analysis was performed using one-way ANOVA and post hoc Tukey's multiple range test and visualised in R using 'ggplot2', 'multcomp' and 'multcompView' packages. The results are presented as mean values of tree replicates and standard deviation (SD). Significant differences between treatments were marked at  $p \leq 0.05$ .

## 3. Results

### 3.1. Desiccation and degreening of seeds during maturation

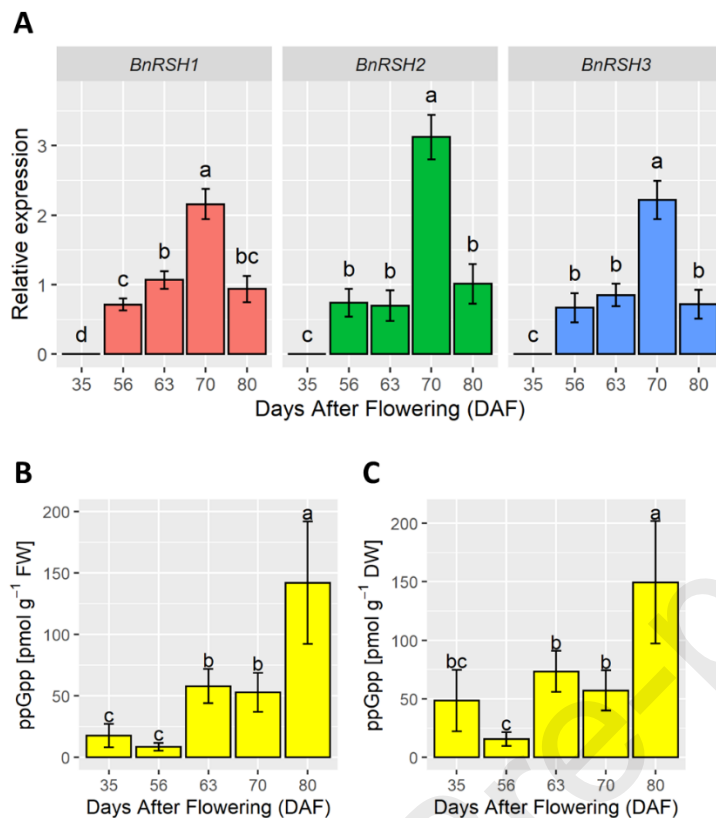
At the examined developmental points *B. napus* L. seeds successively desiccated and lost their green colouration due to the loss of chlorophylls and carotenoids (Fig. 1). Seed colour change progressed through the whole studied time period (Fig. 1A) with the highest magnitude of pigment breakdown between 35<sup>th</sup> and 56<sup>th</sup> DAF (Fig. 1C). Water content of 60% at 35<sup>th</sup> DAF gradually dropped until it reached 7% at 70 DAF and stabilized (Fig. 1B).



**Figure 1.** Desiccation and degreening in *B. napus* L. developing seeds collected at 35, 56, 63, 70 and 80 days after flowering. Photographs of representative seeds (A). Relative water content (B). Chlorophyll *a/b* ratio and pigment content on a dry biomass basis (C). Bars represent mean values  $\pm$  SD ( $n=3$  biological replicates). Different letters indicate significant differences between means at  $p < 0.05$  according to one-way ANOVA and Tukey post hoc test.

### 3.2. *BnRSH* genes expression

The expression of *BnRSH* genes during *B. napus* L. seed development was investigated. The analysis showed that genes representing each of the *BnRSH1-3* types were expressed in each developmental stage except at 35 DAF (Fig. 2A). *BnRSH1* ((p)ppGpp hydrolase), *BnRSH2* (hydrolase/synthase of (p)ppGpp) and *BnRSH3* (hydrolases of (p)ppGpp) were significantly expressed at 56 DAF, and reached maximal levels at 70 DAF. Interestingly, the highest increase in transcript accumulation was observed between 63 and 70 DAF, representing a two-fold increase for *BnRSH1* and *BnRSH3*, and three-fold for *BnRSH2* (Fig. 2A). These data indicate that (p)ppGpp metabolism is induced during seed maturation.



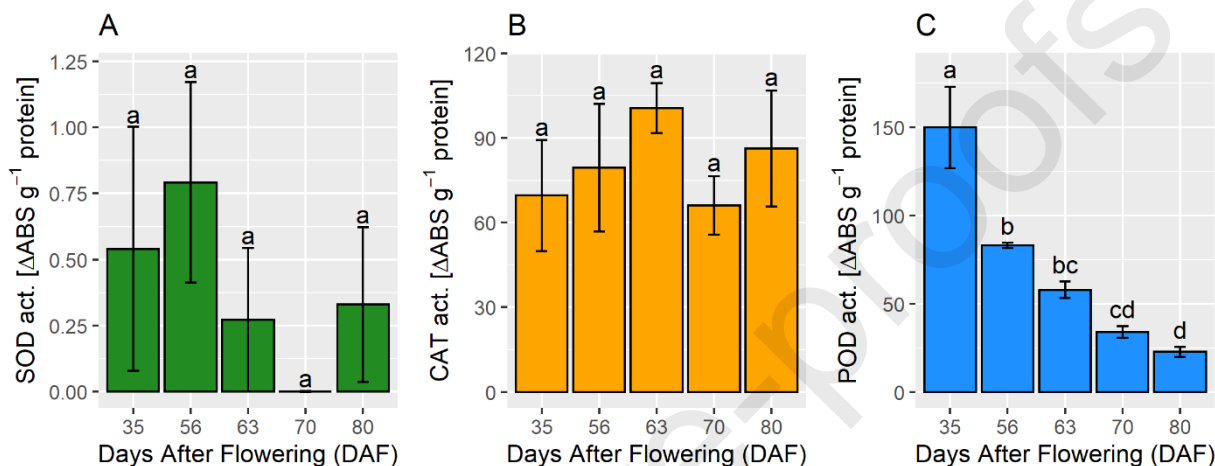
**Figure 2.** Regulation of the stringent response at different stages of *B. napus* L. seed development (35, 56, 63, 70, and 80 DAF). The relative expression level of *BnRSH1-3* genes (A). Content of ppGpp expressed on a fresh (B) or dry (C) biomass basis. Bars represent mean values  $\pm$  SD (n=3 biological replicates). Different letters indicate significant differences between means at  $p < 0.05$  according to one-way ANOVA and Tukey post hoc test.

### 3.3. Accumulation of ppGpp

The content of alarmone ppGpp was determined in developing rape seeds. To verify whether the higher ppGpp level in the last developmental stage results from the lower water content, the results were analysed on both a fresh (Fig. 2B) and an adjusted dry (Fig. 2C) weight basis. The direct analysis of alarmone content in dry biomass was impossible because of the great instability of ppGpp at high temperature, which is necessary during the drying procedure. Thus, the data for the FW was recalculated to DW using means of water content (Fig. 1B). The analysis showed that ppGpp level increased during seed development (Fig. 2B, C). The highest ppGpp content was recorded for seeds collected at 80<sup>th</sup> DAF, 5 or 3-fold higher than at earlier stages (35 and 56 DAF) on a fresh (Fig. 2B) or dry (Fig. 2C) weight basis, respectively. Our results therefore indicate that there may be specific roles for ppGpp during seed maturation, when nutrients are redirected and recycled to the developing seeds, and also during seed desiccation.

### 3.4. The activity of antioxidant enzymes

The activity of antioxidant enzymes (POD, CAT, and SOD) of rape seeds collected from five different developmental stages (35, 56, 63, 70 and 80 DAF) was analysed. POD activity gradually declined over the subsequent DAF (Fig. 3C), while CAT and SOD activities did not change significantly during seed development: CAT activity was constantly high (Fig. 3B), and SOD activity remained very low (Fig. 3A).



**Figure 3.** The activity of SOD (A), CAT (B), and POD (C) in rape seeds at 35, 56, 63, 70, and 80 DAF. Bars represent mean values  $\pm$  SD ( $n=3$  biological replicates). Different letters indicate significant differences between means at  $p < 0.05$  according to one-way ANOVA and Tukey post hoc test.

### 3.5. Sugar and protein accumulation

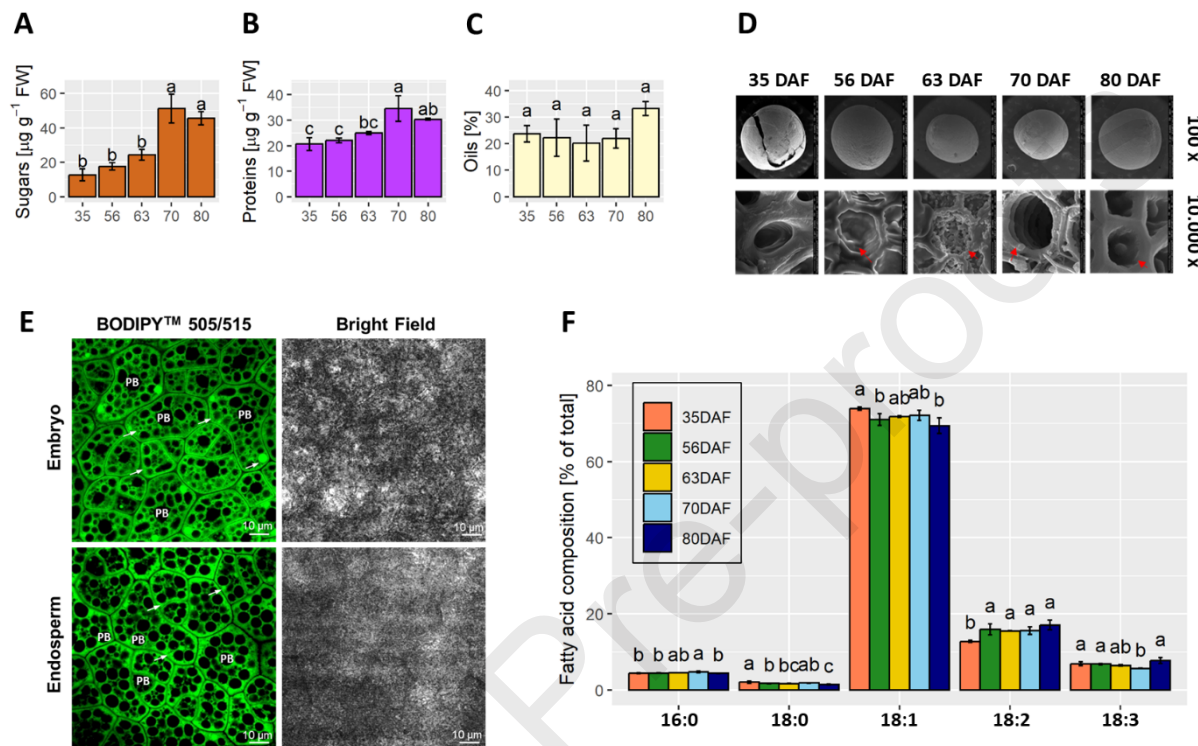
Our results showed that the contents of sugars and proteins increased successively during seed maturation. Sugar and protein levels stabilised at 70<sup>th</sup> DAF with the 2.5, and 1.5 fold higher levels, respectively, as compared to 35 DAF (Fig. 4A,B). The total oil content did not significantly change during seed development (Fig. 4C).

### 3.6. Oil reserves in seeds

We also analysed the distribution of oil bodies in seed coats and embryo or endosperm using SEM and confocal laser scanning microscopy (CLSM), respectively. Oil bodies (OBs) were first visible at the 56<sup>th</sup> DAF, and they successively spread in subsequent stages of seed development (Fig. 4D). At 80 DAF, in both cotyledons and endosperm, small OBs were found to accumulate in the cytoplasm around protein bodies (PBs). Few larger OBs were found in the peripheral parts of the cells of cotyledon as well endosperm (Fig. 4E).

To assess the quality of stored oils, we determined the fatty acid composition (FAC) of the total oil extracted from developing seeds (Fig. 4F). In all analysed stages, the most represented FA was unsaturated oleic acid (C18:1 = 69.0-73.0%), followed by linoleic (C18:2 = 12.0-17.0%) and

linolenic acid (C18:3 = 6.4-7.7%). Much lower amounts of saturated palmitic (C16:0 = 4.4-4.7%) and stearic (C18:0 = 1.4-2.0%) acids were found during rape seed development. The analysed FAs were relatively stable during seed development stages, with a slight but significant shift of SFAs in favour of unsaturated ones (Fig. 4F).

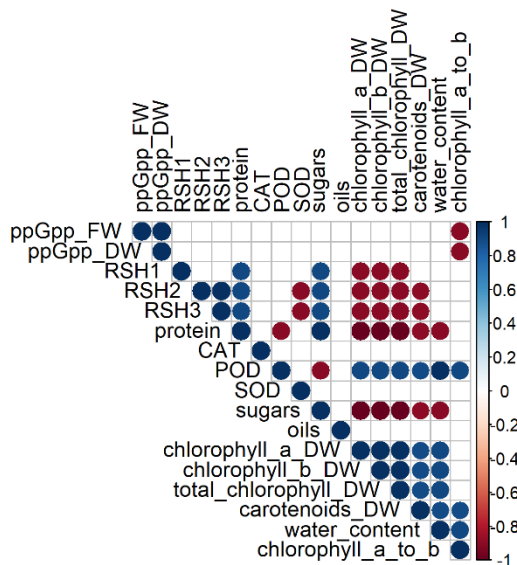


**Figure 4.** Nutritional quality of developing *B. napus* L. seeds at 35, 56, 63, 70, and 80 DAF. Content of total sugar (A) protein (B) and oils (C). D. Scanning electron micrographs. Red arrows indicate oil bodies. E. Representative confocal laser scanning microscopy (CLSM) images of the embryo and endosperm cells of oilseed rape seeds (80 DAF) stained by BODIPY<sup>TM</sup> 505/515; green signal – oil bodies (OBs; arrows indicate exemplary OBs); regular black spots – protein bodies (PBs; exemplary protein bodies are indicated with PB white initials). Bar = 10  $\mu\text{m}$ . F. Fatty acid compositions. C16:0 – palmitic, C18:0 – stearic, C18:1 – oleic, C18:2 – linoleic, C18:3 –  $\alpha$ -linolenic. In panels A-C and F, bars represent mean values  $\pm$  SD (n=3 biological replicates). Different letters indicate significant differences between means at  $p < 0.05$  according to one-way ANOVA and Tukey post hoc test 0.05 according to one-way ANOVA and Tukey post hoc test. The comparisons for fatty acid composition (F) were conducted within each fraction separately.

### 3.7. Correlation of all measured parameters

To gain more insight into possible dependencies between the measured parameters, we carried out a correlation analysis (Fig. 5). Interestingly, ppGpp levels negatively correlated with chlorophyll

*a/b* ratio, *BnRSH* expression negatively correlated with pigment content, and peroxidase activity positively correlated with pigment content, water content and chlorophyll *a/b* ratio (Fig. 5).



**Figure 5.** Correlation analysis of parameters during *B. napus* L. seed maturation. Correlation plot of all parameters created using Spearman method and R ‘Hmisc’ and ‘corrplot’ packages; insignificant correlations ( $p > 0.05$ ) are left blank.

#### 4. Discussion

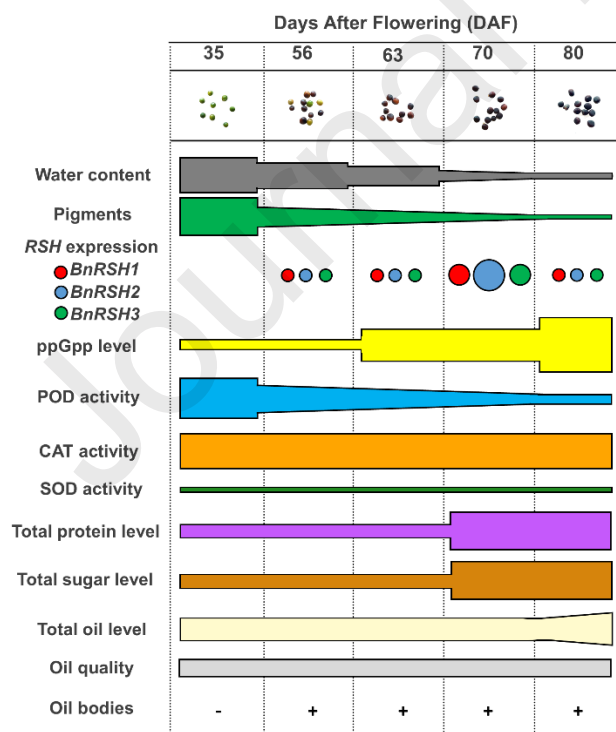
The impact of (p)ppGpp on plant growth and development was previously explored by investigating *A. thaliana* *RSH* mutant lines. Studies using *RSH3*-overexpression lines that overaccumulate alarmone revealed that (p)ppGpp inhibits photosynthesis, can cause dwarf phenotypes in land plants, and results in pale-green leaves, decreased chloroplast gene mRNA levels, and acclimation to nitrogen deprivation (Romand et al. 2022; Sugliani et al. 2016). Our knowledge of this phenomenon at the molecular level is still scarce and fragmentary. ppGpp is known to inhibit chloroplast transcription. Sugliani *et al.* (2016) showed that ppGpp is a potent regulator of the expression of chloroplast genes *in vivo* and influences photosynthesis (Sugliani et al. 2016). They also showed that the (p)ppGpp accumulation inhibits the transcription of nucleus-encoded polymerases (NEP) and the bacterial-like plastid-encoded polymerase (PEP) genes in developing seedlings (Field 2018; Sugliani et al. 2016; Mehrez et al. 2023). However, the (p)ppGpp targets in chloroplasts remain largely unknown.

*Arabidopsis* *RSH2* and *RSH3* knock-out plants (lacking the major synthases of (p)ppGpp) produced seeds significantly lighter in weight than the seeds of wild-type plants (Sugliani et al. 2016). Our results extend on these findings by showing that the stringent response is actively involved in seed development and maturation. We showed that ppGpp levels started to increase at 63 DAF and reached the highest level at the last seed development stage (80 DAF) (Fig. 2B,C). This was consistent with the increase in *BnRSH2* and *BnRSH3* expression levels that started at 56



DAF (Fig. 2A). The correlation was not significant (Fig. 5), likely due to some delaying events between mRNA synthesis and accumulation of active proteins in chloroplasts, or the involvement of other *BnRSH* genes not included in this study. Our recent study showed a similar trend of *BnCRSH* expression during seed development, and the transcript localised in all embryonic tissues. Simultaneously, the ploidy of seed cells drops during seed maturation, suggesting a possible involvement of the stringent response in cell cycle arrest, which is essential for seed maturity (Turkan et al. 2023a). Interestingly, a comparable phenomenon was observed in the diatom *Phaeodactylum tricornutum*, which showed a severe reduction in proliferation in response to high ppGpp levels (Avilan et al. 2021). Taken together, our results suggest that ppGpp and *RSH* play a vital role in the late stages of seed development. Presumably, alarmone accumulation is a prerequisite to seed maturity and determines seed germination capacity. This hypothesis is worth investigating in the future. On the one hand, it would shed light on the process that ensures the continuity of the species, and, on the other hand, the knowledge gained may potentially lead to the development of more productive crops, seeds with higher nutritional quality and the improvement of methods for long-term seed storage.

In bacteria, (pp)pGpp binds a large range of effector proteins to influence cellular metabolism and proliferation (Mehrez et al. 2023; Kanjee et al. 2012). This includes enzymes involved in lipid and protein biosynthesis. Therefore, in addition to *RSH* gene expression and alarmone levels, we also investigated the elements of possible downstream pathways: antioxidant system capacity (Fig. 3) as well as sugar (Fig. 4A), and protein content (Fig. 4B), and performed an extensive analysis of oil quality and quantity (Fig. 4C-F) during rape seed development. All the results are summarised in Fig. 6.



**Figure 6.** Scheme depicting trends in analysed parameters during rape seed development.

Chloroplasts are the main source of reactive oxygen species (ROS) in plant cells (Domínguez and Cejudo 2021). Under standard growth conditions, alarhone and *RSH* genes are required to adjust the architecture of photosynthetic complexes (Ihara and Masuda 2016; Sugliani et al. 2016). Interestingly, *Arabidopsis RSH3* overexpressing lines adapted to nitrogen-deficient conditions displayed higher energy dissipation at the expense of photochemistry (Honoki et al. 2018). This phenotype was further explained by Romand *et al.* (2022), whose extensive studies based on the *RSH1* mutants showed that ppGpp is required to remodel the photosynthetic electron transport chain by downregulating photosynthetic activity and thus protecting against oxidative stress (Romand et al. 2022). ROS levels are elevated in *Arabidopsis* wild type in response to nitrogen deprivation, and the increase was even higher in plant lines displaying defects in ppGpp biosynthesis, triggering cell death (Romand et al. 2022). These results indicate that alarhone signalling is required to prevent ROS overaccumulation, oxidative stress, and tissue damage.

ROS are produced in seeds and are involved in all the stages of seed development, from embryogenesis to germination (Pehlivan 2017). ROS can control plant growth and defence in both a negative and a positive way. Excessive accumulation of ROS in seeds not only diminishes their antioxidant capacity but also lowers their nutritional value by altering amino acid ratios, triggering protein carbonylation and increasing the levels of oxidized FAs (Granado-Rodríguez et al. 2022; Li et al. 2022b). Homeostasis of ROS at safe levels, neither too high (oxidative stress) nor too low (reductive stress), is part of managing stress tolerance (Nadarajah 2020). Furthermore, adjusting ROS levels could facilitate improved stress tolerances of crop plants under various environmental stresses (You and Chan 2015). An efficiently functioning antioxidant system is characterised by optimal POD, CAT, and SOD activity that prevents ROS overaccumulation (Liu et al. 2014). SOD is one group of enzymes in the antioxidant system that catalyses free superoxide radicals to highly diffusible hydrogen peroxide ( $H_2O_2$ ) and oxygen ( $O_2$ ) in chloroplasts and mitochondria (Gallie and Chen 2019). Moreover, a high abundance of *SOD* transcripts was found in embryo axes and cotyledons of pea (*Pisum sativum* L.) seeds, and it was suggested that ROS homeostasis promotes cellular extension, thereby, embryo axis expansion and seedling growth (Yao et al. 2012; Kranner et al. 2010). *SOD* genes showed high expression during seeds development in maize (*Zea mays* L.) (Liu et al. 2021), tobacco (*Nicotiana tabacum* L.) (Lee et al. 2010) and mung bean (*Vigna radiata* L.) (Singh et al. 2014). In our study, SOD activity levels were relatively low, and the changes during seed development were not significant (Fig. 3A). This observation does not exclude the role of SOD in embryo development or its desiccation tolerance, and their activity may be restricted to a local group of cells, e.g. meristematic tissue. We showed that the activity of CAT was constantly high (significantly higher than SOD and POD) (Fig. 3B), indicating its involvement along the whole process of seed development and maturation. POD activity was the most dynamic among all studied antioxidant enzymes, and its level decreased successively throughout rape seed development (Fig. 3C), suggesting its involvement in the initial steps of seed maturation. This parameter was positively correlated with pigment content (Figs 1C and 5), which suggests that POD plays a role in protecting cells during the degreening process. A heterogeneous trend in the activity of antioxidant enzymes was also observed in bean seeds, which may result from the distinct cellular compartmentalisation of these proteins (Bailly et al. 2001).

Successful degreening of seeds is required to ensure the good quality of oils. This process is regulated by abscisic acid through a nuclear signal transduction pathway that involves ABI3 (Delmas et al. 2013), ABI4 and ABI5 (Zinsmeister et al. 2023) transcription factors, which bind to ABRE elements during the late stages of seed maturation (Guerriero et al. 2009). *In silico* analysis of *B. napus* RSH genes revealed the presence of multiple ABREs within the promoter regions (Dąbrowska et al. 2021), which suggests that RSH/ppGpp might be downstream effectors of ABA during chlorophyll degradation. Another premise that supports this hypothesis is the significant negative correlation between chlorophyll *a/b* ratio and ppGpp levels (Fig. 5). In moss, ppGpp accumulation in chloroplasts caused the dramatic decrease in this parameter, accompanied by a substantial remodelling of chloroplast proteome (Harchouni et al. 2022). This phenomenon also occurs in *Arabidopsis*, though is less striking (Sugliani et al. 2016). This suggests that the function of alarmone during degreening might be not only fine-tuning of antioxidative enzymes but also the regulation of the order and rate of the dismantling of pigment-binding proteins. This probably allows the maintenance of photosynthetic activity at some level until the end of this process and ensures that the accumulation of chlorophylls and their intermediates are manageable for pigment-degrading enzymes. Moreover, it is speculated that in rice (Ito et al. 2022) alarmone oversynthesis can severely impair chloroplast biogenesis by reducing the GTP pool. This suggests that alarmone may coordinate degreening *via* multiple parallel pathways, such as downregulating chloroplast transcription and altering GTP metabolism. Interestingly, at the earliest developmental stage studied here, the chlorophyll *a/b* ratio was 1.4 (Fig. 1C), far lower than that observed in leaves (Lin et al. 2022). This can be explained by the fact that the colour change in rape seeds starts at 25<sup>th</sup> DAF (Lorenz et al. 2014). Thus, at the 36<sup>th</sup> DAF, seeds are already in an advanced stage of degreening.

The simultaneous increase of ppGpp, protein and sugar levels in seeds (Fig. 2 and 4A,B) and unaffected oil quality and quantity (Fig. 4C-F) appears to contradict the literature data showing the inhibition of the enzymes involved in amino acid, sugar, and oil biosynthesis. However, this study covers the late stages of seed development, where oils are already built-up (Shen et al. 2018) (see also Fig. 4C). Moreover, so far ppGpp-signalling dependent regulation of these processes was proven only for bacteria (Kanjee et al. 2012; Steinchen et al. 2020) or is bioinformatic-based (Kushwaha et al. 2020; Kanjee et al. 2012). More compelling evidence on the impact of ppGpp on sugar, amino acid and FA metabolism was provided by *in planta* studies. Maekawa *et al.* (2015) provided metabolomic-based evidence of the effect of ppGpp on a wide range of metabolic processes. In their research, ppGpp-overproducing plants showed lower protein content and starch deposition during N deficiency conditions and displayed differences in the composition of polar glycerolipids. In the studied developmental variants, ppGpp could potentially reduce the quality of deposited oils. The FA profile seems to be affected in an RSH3 overexpression line, however, since levels of all the components were lower, the differences are probably due to the overall reduction in FA content in this line (Maekawa et al. 2015). In the moss *Physcomitrium patens*, the FA profile showed only minor changes after dramatic remodelling of chloroplast structure and protein composition triggered by the artificial accumulation of ppGpp (Harchouni et al. 2022). Moreover, Honoki *et al.* (2018) showed that ppGpp-overproducing mutant *A. thaliana* lines (*RSH3* overexpression in *rsh2 rsh3* background) do not accumulate sucrose, glucose starch, or Rubisco and keep green leaves during nitrogen starvation (Honoki et al. 2018). Li *et al.* (2022a) showed that during N starvation, higher (p)ppGpp levels in the *are2-2* rice mutant lacking the alarmone hydrolase RSH1 were accompanied by a higher accumulation of amino acids probably used as a source of N (Li et al. 2022a). Romand *et al.* (2022) demonstrated the significance of the synthesis

of (p)ppGpp by RSH2 and RSH3 and its accumulation in tissues for acclimation to nitrogen starvation in *Arabidopsis* (Romand et al. 2022). Our results surprisingly showed that total sugar, protein and oil quantity and quality (Fig. 4) did not decrease with the increase of ppGpp level during seed development (Fig. 2B,C). This can be explained by two scenarios: that ppGpp at the observed levels in seeds is not able to inhibit sugar and protein metabolism nor oil quality, or that the accumulation of alarmone and seed storage substances are spatially separated.

The subcellular organisation of the cell allows for maintaining the specialised environments where specific biochemical reactions can occur without interfering with other processes within the cell. During seed filling and desiccation, sugars, proteins, and oils are deposited in specialised structures, which are formed independently of chloroplasts. In *B. napus* L. seeds, sugars are preferentially accumulated in starch granules. Storage starch is deposited in heterotrophic plastids called amyloplasts, unlike in the leaves during prolonged darkness, where it transiently accumulates in chloroplasts (Tetlow 2011). In fully desiccated rape seeds, oil and protein bodies are present in embryo and endosperm (Fig. 4E). These structures are produced during seed desiccation in the form of vessels originating from the endoplasmic reticulum (Frey-Wyssling et al. 1963; Chrispeels and Herman 2000). RSH localisation experiments indicate that in plants the stringent response mainly occurs in chloroplasts (Maekawa et al. 2015; Sato et al. 2015; Masuda et al. 2008a, 2008b; Tozawa et al. 2007). However, direct evidence for the subcellular localisation of ppGpp, or its presence in other plastid types is limited. Further experiments should be conducted to verify the hypothesis on the spatial separation of ppGpp signalling and the deposition of storage substances. Alarmone content was analysed in isolated rice chloroplasts (Ito et al. 2022), and in the future could be implemented for ppGpp analysis in other organelles. However, this approach has complications due to the high dynamics in organelle number and volume, which is observed during stress and development and could lead to ambiguous results. Further development of methods enabling *in vivo* detection of alarmone at subcellular resolution would be another milestone in understanding the spacio-temporal context of the stringent response in plants. Already, such a sensor was developed for the selective detection of alarmones in bacteria (Sun et al. 2021).

## 5. Conclusion

In this study, we explored the accumulation and possible influence of ppGpp on biochemical composition of rape seeds. We show that despite ppGpp and *RSH* upregulation during seed development, sugars and proteins simultaneously accumulate, and the quality of lipids does not change. We suggest that the plant stringent response may therefore be separated from these processes and instead plays a role in orchestrating degreening and subsequent desiccation probably *via* regulating the antioxidant system during seed development. A better understanding of the activity and localisation of the stringent response at particular stages of seed development will allow the fine-tuning of (p)ppGpp content as a tool to improve seed nutritional value. This can be achieved either through localization experiments or by using mutants for validation.

## 6. Competing Interest Statement

The authors declare no competing interests.

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## 9. CRediT authorship contribution statement

S.T. – Investigation; Writing - original draft; Final approval of the manuscript.

M.K. – Data curation; Formal analysis; Investigation; Visualisation; Writing - review & editing; Final approval of the manuscript.

A.Z. - Data curation; Formal analysis; Investigation; Visualisation; Writing - review & editing; Final approval of the manuscript.

A.M.-A. – Investigation; Writing - review & editing; Final approval of the manuscript.

E.S. – Investigation; Writing - review & editing; Final approval of the manuscript.

M.W. – Investigation; Writing - review & editing; Final approval of the manuscript.

A.S.-C. – Investigation; Visualization; Writing - review & editing; Final approval of the manuscript.

J.B. – Investigation; Writing - review & editing; Final approval of the manuscript.

B.F. – Investigation; Writing - review & editing; Final approval of the manuscript.

G.D. – Conceptualisation; Funding acquisition; Project administration; Supervision; Writing - review & editing; Final approval of the manuscript.

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### **CRedit authorship contribution statement**

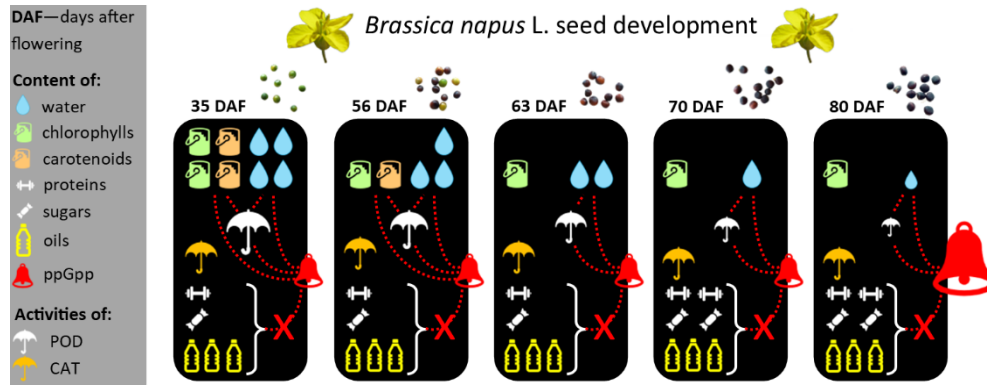
**Sena Turkan:** Investigation, Writing - original draft, Final approval of the manuscript. **Milena Kulasek:** Data curation, Formal analysis, Investigation, Visualisation, Writing - review & editing, Final approval of the manuscript. **Agnieszka Zienkiewicz:** Data curation, Formal analysis, Investigation, Visualisation, Writing - review & editing, Final approval of the manuscript. **Agnieszka Mierek-Adamska:** Investigation, Writing - review & editing, Final approval of the manuscript. **Edyta Skrzypek:** Investigation, Writing - review & editing, Final approval of the manuscript. **Marzena Warchol:** Investigation, Writing - review & editing, Final approval of the manuscript. **Aleksandra Szydłowska-Czerniak:** Investigation, Visualization, Writing - review & editing, Final approval of the manuscript. **Julia Bartoli:** Investigation, Writing - review & editing, Final approval of the manuscript. **Ben Field:** Investigation; Writing - review & editing; Final approval of the manuscript. **Grażyna B. Dąbrowska:** Conceptualisation; Funding acquisition; Project administration; Supervision; Writing - review & editing; Final approval of the manuscript.

### **Guanosine tetraphosphate (ppGpp) is a new player in *Brassica napus* L. seed development**

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#### **Highlights**

- Alarmone ppGpp was detected in the seed of *B. napus* L., non-leaf organs.
- ppGpp accumulation peaked at the last stage of seed development.
- Alarmone accumulation did not prevent build-up of storage compounds.
- ppGpp concentration negatively correlates with chlorophyll content in seeds.



### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: