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The role of plant stringent response in *Brassica napus* L. in response to biotic and abiotic factors and during seed development

Dissertation for a doctoral degree

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List of papers

This thesis is based on the following studies, which will be referred to by their Roman numerals in the text.

I. Dąbrowska G.B., **Turkan S.**, Tylman-Mojżeszek W., Mierek-Adamska A. *In silico* study of RSH (RelA/SpoT homologs) gene family and the expression analysis in response to PGPR bacteria and salinity in *Brassica napus*. International Journal of Molecular Sciences, 2021, 22(19): 10666. doi: 10.3390/ijms221910666.

II. Turkan S., Mierek-Adamska A., Głowacka K., Szydłowska-Czerniak A., Rewers M., Jędrzejczyk I., Dąbrowska G.B. Localization, and expression of *CRSH* transcript, level of calcium ions, and cell cycle activity during *Brassica napus* L. seed development. Industrial Crops & Products, 2023, 195: 116439. doi: 10.1016/j.indcrop.2023.116439.

III. Turkan S., Mierek-Adamska A., Kulasek M., Konieczna W.B., Dąbrowska G.B. New seed coating containing *Trichoderma viride* with anti-pathogenic properties. PeerJ, 2023, 11: e15392. doi: 10.7717/peerj.15392

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List of abbreviations:

ABA - abscisic acid

ATP - adenosine 5′-triphosphate

BLAST - Basic Local Alignment Search Tool

Chl - chloroplast targeting motif

CK - cytokinin

CRSH - Ca^{2+}-activated RelA/SpoT homolog

CTD - C-terminal domain

ET - ethylene

GDP - guanosine 5′-diphosphate

GMP - guanosine 5'-monophosphate

GTP - guanosine 5′-triphosphate

HPLC-MS/MS - high-performance liquid chromatography coupled to tandem mass spectrometry

JA - jasmonic acid

MeJA - methyl jasmonate

NEP - nucleus-encoded polymerase

PEP - plastid-encoded polymerase

(p)ppGpp - guanosine tetraphosphate and pentaphosphate (alarmones)

RNAP - RNA polymerase

RSH - RelA/SpoT homologue

1. Introduction

Plants are sessile organisms that to thrive and survive, must tolerate various environmental changes throughout their life cycles, such as nutrient deprivation, water scarcity, and salt stress. Salt stress is one of the major environmental stresses impairing plant growth and development. The poor plant growth under salinity stress is due to osmotic stress followed by ion toxicity (hypertonic stress), reduced nutrient mobilization, and hormonal imbalance (1). The other important factors reducing plant growth are diversified plant pathogens. However, among microorganisms, several beneficial bacteria and fungi are also found (2). Multiple microorganisms are currently used in agriculture as biocontrol agents e.g., fungi belonging to *Trichoderma* and bacteria belonging to *Serratia* (3,4). World food production must increase by 70% to meet the food requirements of a world population of 9 billion by 2050 (5). Climate change and the decreasing availability of land and water make food security the most important challenge facing humanity today (6). In response to various stresses, plants have evolved numerous intracellular and intercellular regulatory signalling pathways, which allow them to rapidly adjust gene expression and metabolism to the variable environment (7–10). The stringent response is a conserved regulatory mechanism that was initially discovered in bacteria as a mechanism for regulating gene expression and metabolic processes in response to nutrient deprivation (11,12). Subsequently, the stringent response has been shown to play a key role in regulating a range of processes in bacteria including virulence, antibiotic resistance, and biofilm formation (13–15). The effectors of the stringent response are the hyper-phosphorylated nucleotides i.e., guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), called alarmones (16). The (p)ppGpp molecule is a nucleotide consisting of guanine linked by an N-glycosidic bond to ribose, two phosphate residues linked to the 3’ carbon of the

ribose, and two (ppGpp) or three (pppGpp) residues phosphates linked to the 5' carbon the ribose (Figure 1).

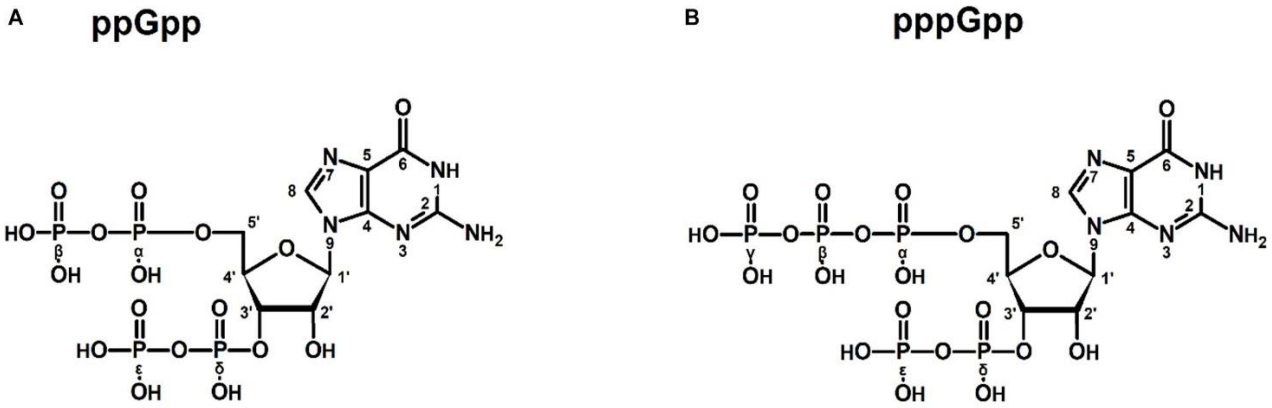

Figure 1. Structure of guanosine tetra- and pentaphosphate. A two-dimensional chemical sketch of ppGpp (A) and pppGpp (B).

The level of alarmones in *E. coli* is regulated by two enzymes, RelA and SpoT. Both RelA and SpoT synthesize (pp)pGpp by transferring the pyrophosphate group from ATP / GTP / GDP to the 3′-OH group of ribose of either GTP, GDP, or GMP. However, only SpoT hydrolyses alarmones, while RelA does not have hydrolase activity (17,18). Accumulation of alarmones in bacteria occurs under various stress conditions, including nutrient starvation (phosphate, carbon, iron, nitrogen, amino acids, fatty acids), antibiotic treatment, darkness, heat, and osmotic stresses (9,12,19–21). The relative contribution of RelA and SpoT to (p)ppGpp synthesis varies depending on the bacterial species and the specific stress condition (20) (Figure 2).

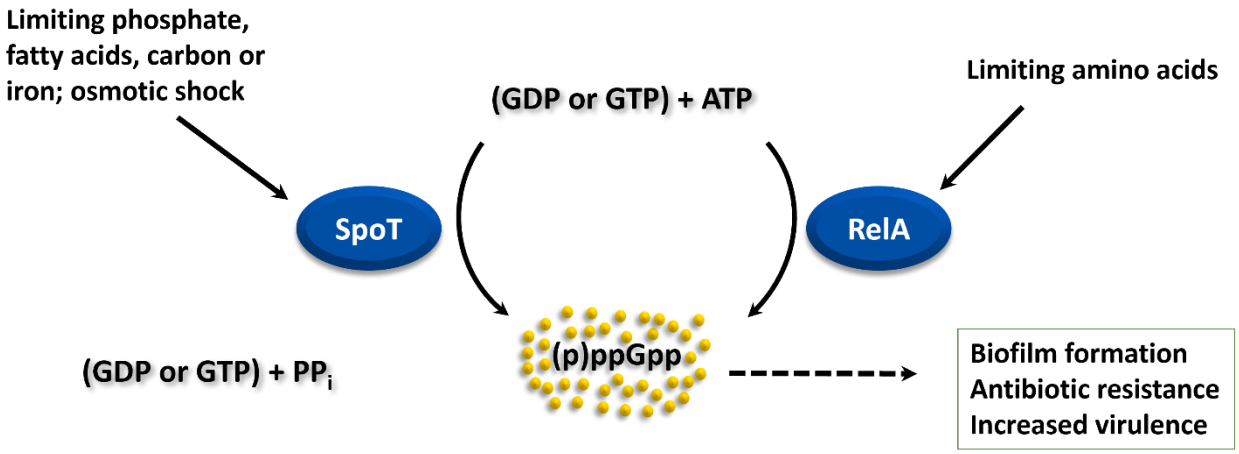

Figure 2. Schematic depiction of alarmone synthesis by SpoT and RelA in *E. coli* (Dalebroux et al. 2012, modified).

Genes encoding alarmone synthases and hydrolases have also been identified in eukaryotes (*RelA*/*SpoT* homologs – *RSH* genes), showing that the stringent response is widely evolutionarily conserved (16). The RSH protein superfamily is categorized into two types: (i) short, single-domain enzymes i.e., small alarmone hydrolases (SAHs) and small alarmone synthetases (SASs), and (ii) long RSHs that possess multidomain organization (22). In general, the N-terminus of long RSHs carries (p)ppGpp synthase and/or (p)ppGpp hydrolase domains, and the C-terminus carries regulatory domains such as the TGS (Threonyl-tRNA-synthase, GTPase, SpoT domain), ZFD (Zinc Finger Domain), ACT domain (Aspartate kinase-Chorismate mutase-TyrA), and EF-hand domain (calcium-binding domain) in case of CRSH (Ca^{2+}-dependent RSH) present in plants (17,22). While SAHs and SASs have been found in bacteria, fungi, and animals, long RSHs are widely distributed in bacteria and plants (17,22).

1.1. Alarmones synthases and hydrolases in plants

The endosymbiotic theory suggests that the chloroplasts of plants and algae evolved due to endosymbiosis between non-photosynthetic eukaryotic host cells and algal or photosynthetic

cyanobacterial endosymbionts (23). Since that initial symbiosis, the chloroplast has undergone significant evolution and adaptation, lost many bacterial genes, and transferred others to the nuclear genome of the host cell (9). Additionally, the chloroplast has taken on new roles within the eukaryotic cell, serving as a site for nitrogen and sulphur assimilation, the biosynthesis of fatty acids, amino acids, and nucleotides, and phytohormone production (24–28). The chloroplast has also emerged as a key player in plant acclimation to abiotic stressors like high/low light intensity, high/low temperature, and low water availability. The chloroplast contains a variety of sensors and signalling pathways that allow it to adjust photosynthesis rates to match changing light levels (29).

In plants, *RSH* genes were first identified in *Arabidopsis thaliana* (30), and in algae, it has been shown that *Chlamydomonas reinhardtii* has a single RSH protein, with both (p)ppGpp hydrolase and synthetase activities (31). Since then, *RSH* genes have been identified in various plant and algal species, indicating that the stringent response is conserved in photosynthetic organisms. It is now obvious that plants also possess a stringent response that is involved in stress adaptation and regulation of growth and development under stress conditions (10,16,32–36). The phylogenetic analyses of the RSH superfamily showed that *RSH* genes were introduced into an ancestral proto-plant cell through lateral gene transfer from two distinct bacterial phyla, including *Deinococcus-Thermus* bacterial phylum (22,36).

Phylogenetic analysis of plant RSH proteins showed that they could be divided into three subgroups: RSH1, RSH2/3, and CRSH (Figure 3) which share a conserved linear arrangement of domains (36). Plant RSH proteins are encoded by the nuclear genome and possess a putative chloroplast-targeting signal peptide at the N-terminus. The localization of RSH proteins in chloroplast was shown for RSH belonging to all types of plant RSH in various plant species (16,35,37–40). Members of RSH1 subgroup function as the main (p)ppGpp hydrolases. The

synthase domain of RSH1 proteins is inactive due to the substitution of glycine, essential for (p)ppGpp synthase activity, with serine. Proteins belonging to RSH2 and RSH3 show high similarity (~80% amino acid identity) and are bifunctional (p)ppGpp synthase/hydrolase enzymes. Members of the CRSH subgroup function as (p)ppGpp synthases only (10,36). CRSH is the most unique group of plant RSH since the EF-hand Ca^{2+}-binding motif is present only in plant CRSH and is not found in bacterial homologs (22,35). It was shown that EF-hand motifs are crucial for alarmone synthetic activity (35,41). Since cytosolic calcium levels change under developmental and stress-induced signals, EF-hand motif-carrying proteins can transmit information about such stimuli (35,40,42).

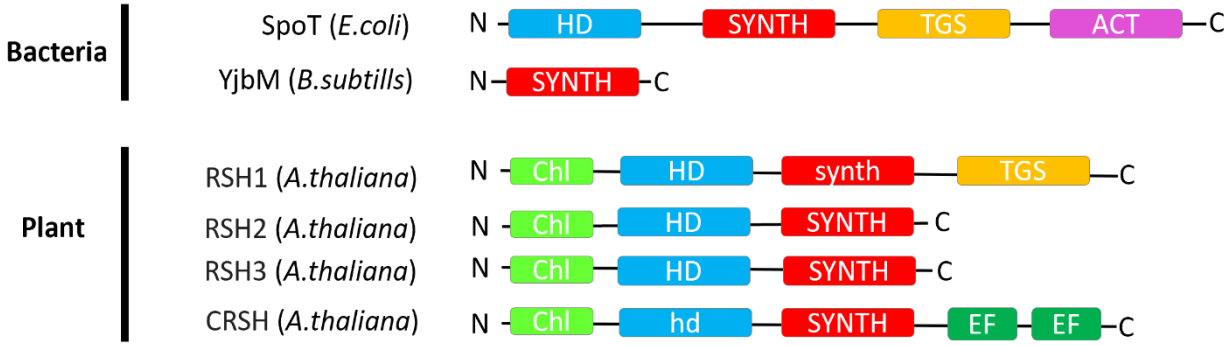

Figure 3. Schematic depiction of the predicted domain structures of RSH proteins from bacteria and plant *A. thaliana*. The putative domains and signalling peptides were identified based on the analysis of putative amino acid sequences in *E. coli*, *B. subtills*, and *A. thaliana*. The abbreviations are as follows: ACT: aspartate kinase chorismite mutase TyrA domain; Chl: chloroplast targeting motif; EF-hand: Ca^{2+}-binding motif; HD: (p)ppGpp hydrolase domain; hd: (p)ppGpp hydrolase domain lacking some critical for activity amino acids; SYNTH: (p)ppGpp synthase domain; synth: (p)ppGpp synthase domain lacking the critical for activity Gly residue; TGS: threonyl-tRNA synthetase, GTPase, SpoT domain.

1.2. The physiological functions of RSH in plants

Understanding the complex regulatory mechanisms that control (p)ppGpp synthases and hydrolases in plants is crucial for developing strategies to improve plant growth and tolerance to environmental stresses. The activity of (p)ppGpp synthases and hydrolases in plants is regulated at both the transcriptional and posttranslational levels (33,42,43). Several studies have shown that (p)ppGpp level in plants rises in response to a variety of stress factors such as darkness, UV irradiation, drought, wounding, high salinity, stress-related hormones (abscisic acid (ABA), jasmonate (JA), and ethylene (ET)), heavy metals, nitrogen limitation, heat shock, and pathogen attack (42,44–47). In addition to the increase in the level of alarmones, studies have also demonstrated a corresponding increase in the expression level of *RSH* genes in response to stress (32,33,42,45–48). Overall, the studies indicate that plants exhibit an increase in both alarmones and the expression of *RSH* genes under various stressors, highlighting the significance of the (p)ppGpp pathway in plant stress responses. Several knockdown, knockout, and overexpression lines have been characterized to determine the roles of plant RSH proteins in plant growth and development and response to stress conditions. Accumulation of alarmones in *A. thaliana* mutant overexpressing *RSH3* was found to protect plants against nitrogen deprivation (16,46,49). On the other hand, analysis of this *RSH3*-overexpression line showed that accumulation of (p)ppGpp inhibits photosynthesis, causes dwarf phenotypes, and leads to pale-green leaves (33). This mutant during nitrogen starvation did not accumulate sucrose, glucose, starch, and Rubisco (48). Under nitrogen-starvation conditions, *A. thaliana* mutant lacking all four RSHs showed leaf chlorosis, and increased levels of salicylic acid and jasmonate (50). *Arabidopsis RSH2* and *RSH3* knock-out plants (lacking the major syntheses of (p)ppGpp) produced significantly lighter seeds than wild-type plants (33). Knockdown of *CRSH* has been shown to result in abnormal flower development impeding pollination, and the production of siliques that are significantly smaller than those produced by wild-type plants (40). Sugliani et al. (2016) showed that (p)ppGpp is a potent regulator

of chloroplast gene expression. It was also shown that the (p)ppGpp accumulation inhibits the transcription of genes encoding the nucleus-encoded polymerases (NEP) and the bacterial-like plastid-encoded polymerase (PEP) in developing seedlings. This finding indicates that (p)ppGpp acts as a key regulatory molecule, modulating the activity of polymerases involved in transcribing genes related to chloroplast function and development. By regulating chloroplast gene expression, (p)ppGpp influences key physiological processes related to photosynthesis, energy metabolism, and plastid biogenesis. These findings together indicate that (p)ppGpp-dependent stringent response influences plant growth and development under favourable and adverse environmental conditions.

1.3. Cross-talk between the stringent response and other signalling pathways

The stringent response in plants interacts with a variety of other signalling pathways, including ABA, JA, and ET signalling pathways (9,34). Cross-talk between these pathways is critical for coordinating plant response to changing environmental conditions and modulating plant growth and development. A reduction in transcription rates in chloroplasts isolated from plants pre-treated with methyl jasmonate (MeJA) (51) or ABA (43) was demonstrated. Moreover, Yamburenko et al. (2015) showed that the ABA-dependent downregulation of chloroplast transcription is partly dependent on the high activity of *RSH2* and *RSH3 v*ia an increase in the synthesis of (p)ppGpp in *Arabidopsis*. Takahashi et al. (2004) demonstrated that ABA, JA, and ET can provoke rapid increases in (p)ppGpp levels in pea shoots (44).

In *Arabidopsis*, the level of the plastidial Ca^{2+} is up-regulated by the light-to-dark transition, resulting in the upregulation of CRSH-dependent (p)ppGpp synthesis (10,35,42). Increased level of (p)ppGpp inhibits the PEP-dependent transcription of plastid genes and Calvin–Benson–Bassham cycle activity. Moreover, (p)ppGpp may reduce plastidial guanylate kinase (GK) activity,

which indirectly controls the transcription of genes linked to photosynthesis by NEP, the plastid-encoded RNA polymerase (42).

1.4. Canola (*Brassica napus* L.)

Brassica napus L. (canola, rapeseed, rape) belongs to the *Brassicaceae* family. To this family belongs also other important crops like cauliflower (*Brassica oleracea* var. *botrytis*), cabbage (*Brassica oleracea* var. *capitata*), mustard (*Brassica nigra*), wild radish (*Raphanus raphanistrum*), turnip (*Brassica rapa* subsp. *rapa*), and broccoli (*Brassica oleracea* var. *italica*) (52). The model plant *A. thaliana* is also a member of the *Brassicaceae* family. Canola is an amphidiploid species (n=19, genome composition AA×CC) from the cross between the diploid *B. rapa*, (n=10, genome composition AA) and *Brassica oleracea* (wild cabbage), (n=9, genome composition BB) which both have formed the *B. napus* genome of 1,129-1,443 Mbp (53,54). Canola is one of the most important edible oil crops in the world. Increasing demand for canola is expected because it is not only a source of high-quality oil for human consumption but also a source of protein meals/cakes used in animal feedstocks and oils/fats for biofuel production (55,56).

The life cycle of the canola plant is divided into seven growth stages based on BBCH (**B**iologische Bundesanstalt, **B**undessortenamt and **CH**emical industry) decimal system i.e., germination and emergence (stage 0), leaf production (stage 1), stem extension (stage 2), flower bud development (stage 3), flowering (stage 4), pod development (stage 5), and seed development (stage 6) (57) (Table 1). The length of each phase of growth is influenced by various endo- and exogenous factors e.g., fertility, cultivar, soil moisture, nutrition, photoperiod, sunlight intensity, and air temperature.

Table 1. Stages of *B. napus* development

Growth stage	Description
0: Germination	Seed imbibition, and germination.
1: Leaf production	Cotyledons completely unfolded, the first leaf unfolded, and the stage is finished when twenty or more leaves are unfolded.
2: Stem extension	The beginning of stem elongation starts with no internodes (“rosette”), and then one visibly extended internode appears, and stages continuous until nine or more visibly extended internodes.
3: Flower bud development	This stage occurs immediately before bolting. The green flower head is enclosed by leaves but becomes visible once the leaves are rolled back.
4: Flowering	The first flowers open and full flowering is when 50% of the flowers on the main stem are open.
5: Pod development	Pods rapidly begin to develop on the lower portion of the stem while flowering continues on the top of the stem. At about 50% bloom, lower pods are beginning to elongate.
6: Seed development	Seeds are initially translucent and gradually turn green, pale yellow, brown, and finally black at maturity.

Seed development is a major focus of crop breeders as it is directly associated with grain yield. Canola embryo development can be divided into five distinct phases: ovule, globular, heart, mature green, and dry seed (58,59). The pod begins to grow once flowers are fertilised and seeds reach maturity about 80 days after flowering. The first process in seed development is the expansion of the seed coat until it reaches its maximum size (15-27 days after fertilisation). Next, the embryo grows rapidly to fill the space. The seed fill takes around 20 days and is divided into two processes: protein deposition and oil deposition. Protein accumulation rises quickly in the early

phases of seed formation. The cotyledons contain the majority of the protein in the mature seed i.e., approximately 76% of the protein is found in the cotyledons, 17% in the other parts of the embryo, and 6% in the seed coat. Most oil is synthesised between the 35th day and the 55th day after flowering. Seed development is completed around 42 days after flowering. Approximately 80 days after flowering, seeds are fully mature. Mature *B. napus* seeds contain 40% oil by weight (60,61).

2. The aim of the study

The main aim of my doctoral thesis is to determine the structure and function of *B. napus* proteins homologues to bacterial stringent response proteins, and the role of the plant stringent response in growth and development and adaptation to environmental stress in canola. In addition, the aim was to develop an innovative seed coating with antimicrobial and plant growth-promoting properties for *B. napus* seeds.

The specific objectives of the work are:

• *In silico* analysis of nucleotide and amino acid sequences of RSH representing three groups (i.e., RSH1, RSH2/RSH3, and CRSH) of plant RSH from different species of the genus *Brassica*.

• Analysis of *B. napus* C*RSH* gene expression by qPCRs and *BnCRSH* transcript localization using the fluorescence *in-situ* hybridization method during *B. napus* seed development to find out whether seed development is regulated by the calcium-dependent stringent response.

• Analysis of *BnRSHs* expression in *B. napus* seedlings, in response to salt stress, and response to the presence of plant growth-promoting rhizobacteria (PGPR), and plant growth-promoting fungi (PGPF) to elucidate the role of stringent response in the plant response to abiotic and biotic factors.

• Development of seed biocoating composed of chitin, methylcellulose, and *Trichoderma viride* spores and further evaluation of its impact on *B. napus* seed germination and seedlings growth, and the impact of this seed coating on plant metabolism, as determined by superoxide dismutase activity and expression of *RSH* genes.

The outcomes of the investigation framed above are presented in three original articles [Publication I-III] appended.

4. Discussion

More than 20 years ago, the discovery of *RSH* genes in plants demonstrated the occurrence of stringent response in plants (8,30,62). During the last 20 years, we learned that stringent response plays a crucial role in the regulation of chloroplast gene expression, photosynthesis, growth, nutrient remobilization, immunity, and adaptation to environmental changes (10,34,45,48,50,63). Alarmones are involved also in developmental processes including flowering and plant senescence. Alarmones were also proposed to regulate nutrient remobilisation and relocation from vegetative tissues into seeds during senescence (64,65). The possible role of stringent response in seed development in plants is still elusive therefore, it is important to further develop our knowledge about stringent response.

The work was focused on *B. napus*, which is the third most important source of edible vegetable oils in the world, a crop of great economic importance for food, feed, fuel, and other important commodities (55). To develop new crop cultivars via traditional breeding or transgenic techniques that can produce high yields even in adverse environmental conditions the molecular processes underlying plant stress response have to be thoroughly understood. Based on the previous results available in the literature it could be concluded that stringent response might be of crucial importance to obtain those stress-resistant cultivars of crop plants. Therefore, the RSH family was analysed in *B. napus* and other species belonging to the family *Brassicaceae.* A total of 45 *RSH* genes were identified in analysed plant specie i.e., *A. thaliana*, *B. napus*, *B. oleracea*, *B. rapa*, *C. sativa*, and *R. sativus*. *B. napus* possessed the highest number of *RSH* genes among these plants, with 14 orthologous genes found (including two pseudogenes). In comparison, the diploid plant species *A. thaliana* possesses only four *RSH* genes. *C. sativa*, which is an allohexaploid species, has 12 *RSH* genes in its genome, with three of them being pseudogenes (Publication I). In monocot

diploid *Oryza sativa* one *RSH1*, one *RSH2*, one *RSH3*, and three *CRSH* genes were identified (66). Similarly, in dicot diploid *I. nil* five *RSH* genes were identified *i.e.*, one *RSH1*, two *RSH2*, one *RSH3*, and one *CRSH* (65). In allotetraploid *Nicotiana tabacum* one *RSH1*, one *RSH2*, one *RSH3*, and three *CRSH* genes were identified (38). These findings indicate that the number of *RSH* genes in a plant genome is not necessarily correlated with the ploidy level of the species. The presence of pseudogenes in some species indicates the possibility of gene duplication and divergence during the course of evolution (67).

The distribution of *RSH* genes across the chromosomes of analysed plants from the *Brassicaceae* family was also verified (Publication I). In the genome of *B. napus*, *RSH* genes are localized on nine out of its 19 chromosomes, while *B. olearacea* and *B. rapa* had them on five out of nine and on four out of ten chromosomes. No disparities were observed between the number and localization of *RSH* genes on chromosomes in *B. oleracea* and the C-genome chromosomes in *B. napus*. However, A-genome chromosomes in *B. napus* showed additional *RSH1* genes on chromosomes A5 and A9 compared to *B. rapa* genome. Furthermore, the *CRSH* gene on chromosome A3 in *B. napus* was found to be a pseudogene. In conclusion, the distribution and characteristics of *RSH* genes vary among different *Brassica* species, with some differences in gene numbers, localization, and pseudogene occurrences.

In silico comparative analysis of the intron-exon organization of *RSH* genes in the selected *Brassicaceae* species was also performed (Publication I). The number of exons and introns, and the location of introns in different types of *RSH* genes, were preserved in the analyzed plants. The *RSH1* genes in plants have highly complex structures, with over 20 introns and exons, while other *RSH* genes in plants are much more compact. The high number of introns and exons is a common feature of *RSH1* genes from both mono- and dicotyledonous plants (Publication I). The similar

number and position of introns in orthologous genes reflect the evolutionary conservation of a gene structure (68). In genetic processes, introns have a variety of functions e.g., alternative promoters, contain regulatory components, and templates for the production of non-coding regulatory RNAs (69). Additionally, introns are essential for alternative splicing, and in plants, intron retention is commonly observed (70). Introns generally enhance the expression of genes in many organisms (71). Interestingly, in plants, genes with more and longer introns tend to exhibit higher expression levels, which is in contrast to animals (72). This indicates that introns play a crucial role in enhancing gene expression specifically in plants. The complex structure of *RSH1* genes in plants implies their potential for high expression and significant involvement in numerous metabolic pathways.

The phylogenetic analysis of *B. napus* RSH proteins showed that similarly to other plant species, they could be divided into three subgroups: RSH1, RSH2/3, and CRSH (Publication I). All analysed RSH1 proteins function as the main (p)ppGpp hydrolase since glycine which is essential for (p)ppGpp synthase activity has been replaced by serine. RSH2 and RSH3 proteins show high similarity (~80% identity) and are bifunctional (p)ppGpp synthase/hydrolase enzymes. CRSH has a functional SYNTH domain, but the hydrolytic domain lost its activity because it lacks some critical amino acids crucial for hydrolase activity. CRSH proteins contain EF-domains that are specific to only this type of RSH protein in plants (9,22,42). Moreover, analysis of plant CRSH proteins indicates that EF-hand calcium-binding motif was highly conserved in monocotyledonous and dicotyledonous plants (Publication II). Plant RSH proteins are encoded by the nuclear genome and possess a putative chloroplast-targeting signal at the N-terminus (22). The localization of plant RSH proteins which belong to various plant RSH families has been shown to be in the chloroplast (22,39).

Using the PlantCare, PlantPan, and PLACE programs, promoter sequences of 12 *BnRSHs* genes were analyzed (Publication I). In the promoter regions of the *BnRSH* genes, multiple *cis*-regulatory elements (CRE) related to plant response to light (16), plant hormones (12), abiotic (16), and biotic (3) stresses were present. The results of this study showed that the overall frequencies of different types of CRE in *BnCRSH* gene were different from the frequencies observed in *BnRSH1–3* genes, suggesting that CRSH might play a significantly different physiological role than RSH1–3. The most abundant elements in *BnRSH1-3* genes were those related to the abiotic stress response, followed by light- and hormone-responsive elements, while the most abundant elements in *BnCRSH* gene were light-responsive elements, followed by hormone-responsive elements. Previous studies showed that the expression of *Arabidopsis RSH1-3* depends on various stress factors including cold, heat, salt, and oxidative stress treatment. In the microarray experiments, cold treatment upregulates the expression of *AtRSH2* and *AtRSH3* and under heat, *AtRSH1* expression strongly decreases in shoots (73,74). Moreover, Ito et al. (2012) showed that treatment of *Arabidopsis* plants with 250 mM NaCl increased both *AtRSH2* and *AtRSH3* transcript levels. *AtRSH2* was found to be highly upregulated under oxidative stress induced with paraquat (50 μM), which also caused a significant decrease in *AtRSH1* transcripts (63). The most abundant among hormone-responsive elements in the *BnCRSH* gene promote was the ABA-response element (Publication I). Previous studies showed that the expression of *AtCRSH* was stable in response to wounding and salt stress, but it was also not changed by hormones, even ABA (39). Interestingly, the circadian rhythm of *AtCRSH* expression is also different from these observed for *AtRSH1–3*, *i.e.*, the expression peak of *AtCRSH* was observed during darkness, whereas *AtRSH1–3* genes are mostly expressed in the light (39). Ito et al. (2017) suggested that Ca^{2+}- dependent stringent response may have arisen during evolution concomitantly with Ca^{2+} signalling in chloroplasts of land plants to adapt to adverse conditions, such as physical injury, pathogen attack,

and environmental stress. Furthermore, it has been shown that calcium signalling is instrumental in developmental processes including ovule and seed development, and seed dormancy (75–78). Overall, *in silico* analyses of cis-regulatory elements in promoters of *B. napus RSH* genes provide valuable insight into the possible physiological functions and can serve as a foundation for future studies to further understand the role of *RSH* genes in plant growth, development, and stress response mechanisms.

Based on the results obtained in Publication I (i.e., there is only one *CRSH* gene in *B. napus* genome and the nature of *cis*-regulatory elements present in the promoter of *BnCRSH* is different in comparison to *BnRSH1-3*) the potential role of Ca^{2+}-dependent stringent response in *B. napus* seed development was investigated. The level and localization of *BnCRSH* mRNAs, cell cycle activity, and the level of calcium ions were examined in developing canola seeds i.e., 35, 56, 63, 70, and 80 days after flowering (DAF) (Publication II). Flow cytometry results showed that cell cycle activity in canola seeds was highest during the early stages of development, and it steadily decreased until it reached the minimal level at 70 days after flowering. Cell cycle arrest can be used as a sign of mature seeds and can be determined by flow cytometry (79,80). The expression of *BnCRSH* increased through seed development. The *BnCRSH* transcript was localized in hypocotyl, inner and outer cotyledons however it was not detected in seeds collected at 35 DAF. Those observations are important because it is the first report on *CRSH* expression and localization of *CRSH* transcript in developing seeds. Moreover, the level of calcium ions increased during the maturation of canola seeds. During seed development, the levels of the secondary messenger Ca^{2+} in immature seeds are known to increase due to developmental signals. We hypothesise that as canola seeds mature, the rising level of Ca^{2+} stimulates the activity of CRSH which results in the accumulation of (p)ppGpp. The increased concentration of alarmones inhibits the expression of

nuclear and plastidial genes, which is essential for adjusting the metabolism of developing seeds (81). The role of *CRSH* in seed development is also supported by the results showing that the knockdown mutation of *A. thaliana CRSH* led to the production of lighter seeds, probably due to the impairment of nutrient remobilization from senescent leaves (33). Interestingly, knockout mutation of *CRSH* in *A. thaliana* did not lead to abnormal flower phenotypes (42), which shows that further analyses are needed to determine the physiological role of calcium-dependent alarmone synthase in flowering and seed production. This is a promising target for plant breeders as modifications have the potential to improve seed production.

Crop yield is decreased by environmental stress conditions including salt stress. As the potential role of stringent response in adaptation to salt stress has been already proposed (82,83), we investigated the potential involvement of *BnRSH* genes in the response to salt stress (Publication I). Soil salinity stress is a significant challenge for sustainable agriculture, as it decreases crop productivity by affecting various aspects of plant metabolism (84). Although *B. napus* is considered one of the most salt-tolerant species in the genus *Brassica* the yield could be significantly affected by salt (85). Interestingly, the analysis showed that the expression of four *BnRSH* genes was not significantly affected by salt stress (50 mM, 100 mM, 150 mM, and 200 mM NaCl) (Publication I). Previous studies showed that *A. thaliana* treated with 250 mM NaCl exhibited increased *AtRSH2* expression, but NaCl had no impact on the expression of *AtRSH1*, *AtRSH3,* and *AtCRSH* (39). Similarly, Prusińska et al. (65) showed that salt stress (300 mM NaCl) stimulated *InRSH2* transcription in five-day-old *I. nil* seedlings, whereas neither *InRSH1* nor *InCRSH* showed substantial changes in response to NaCl. The stable expression of *BnRSHs* in response to salt stress may be due to the lower concentrations of NaCl used in this study, it could be also species-dependent and affected by other experimental factors.

Plant growth-promoting bacteria (PGPB) have multiple advantageous impacts on host plants by stimulating plant growth and development, even under stressful conditions (86). Several direct and indirect mechanisms of PGPR action have been described, including the synthesis of phytohormones, secondary metabolites, and antibiotics, which help in water and nutrient uptake, improve plant growth, enhance soil fertility, and abiotic and biotic stress tolerance (87–89). Halotolerant PGPB could be a crucial factor in improving plant tolerance to salt stress in an environmentally friendly way (90–92). For example, *Serratia liquefaciens* KM4 significantly reduced oxidative stress markers, but increased maize growth and biomass production, more efficient nutrient uptake, higher leaf gas exchange, and antioxidant defence systems under salt-stress conditions (3). It has been shown that *S. liquefaciens* is canola endophytic bacteria (93) therefore we analysed the expression of *BnRSH* genes in response to *S. liquefaciens*, *Serratia plymuthica*, and *Massilia timonae* (Publication I). The PGPB, *S. plymuthica*, and *S. liquefaciens*, upregulated the expression of *BnRSH1* and *BnRSH2* in cotyledons and roots, whereas *M. timonae* did not have a significant effect on *BnRSH* expression. For all *BnRSH* genes the highest level of expression was observed in plants inoculated with *S. plymuthica*. The expression of plant *RSH* genes during plant-microorganisms interactions has not yet been widely studied. Szymańska et al. (2019) showed changes in the expression of *BnRSH1* and *BnRSH3* in roots of oilseed rape growing in the presence of halotolerant PGPR *Pseudomonas stutzeri* ISE12 under salt stress (94). Our research showed changes in mRNA levels of *BnRSH*s grown in the presence of *Serratia* sp., suggesting that some PGPB bacteria might improve plant growth also *via* a stringent response pathway.

Plant growth-promoting fungi (PGPF) are non-pathogenic, common root-associated, and soil-borne fungi, which increase plant growth and protect plants from disease and abiotic stresses

through a wide variety of mechanisms (95,96). Fungi belonging to *Trichoderma* were found to increase the tolerance of canola to salinity and drought and can be used as a biological control agent for soil-borne diseases of valuable crops caused by fungi, oomycetes, bacteria, and nematodes (97–99). It has been shown that fungi belonging to *Trichoderma* promote the growth and development of canola (100). We have pioneered the development of a novel, environmentally friendly seed coating that incorporates *Trichoderma viride* spores combined with filler (chitin) and adhesive material (methylcellulose) to enhance *B. napus* germination and seedling growth and development (Publication III). Our results showed that *T. viride* inhibited the growth of tested plant pathogens, *Fusarium culmorum, Botrytis cinerea,* and *Colletotrichum* sp. The developed seed coating did not enhance seed germination, but it significantly promoted seedlings growth i.e., increased biomass and the length of shoots and roots was observed. Moreover, we checked the effect of the new seed coating on the expression of stress-related genes. The expression of *BnRSH1–3* was down-regulated, whereas the expression of *BnCRSH* was not affected in seedlings grown from coated seeds compared to seedlings that grew from uncoated seeds. The activities of the antioxidative enzyme involved in the ROS-elimination, superoxide dismutase (SOD), did not change in seedlings grown from coated seeds, compared to seedlings grown from uncoated seeds. In conclusion, the developed effective and eco-friendly seed coating promotes seedling growth limits the growth of plant pathogens and does not affect the stress-related markers in plants. The test results described in Publication III were used to prepare a patent application (Patent no: P.442362).

4. Summary of results and conclusions

- We have identified 14 *RSH* genes in the genome of the polyploid plant *B. napus*. *In silico* analysis of *B. napus* RSH amino acid sequences have revealed that they could be divided into three subgroups similar to other plant species: RSH1, RSH2/3, and CRSH. The promoter regions of these genes contain various regulatory elements that respond to different environmental cues such as light, hormones, and biotic and abiotic stresses. These findings highlight the potential roles of RSH proteins in *B. napus* and suggest their involvement in plant adaptation to stress and development. The identification of these regulatory elements provides valuable insights into the mechanisms by which these genes are controlled under different environmental conditions (Publication I).

- We showed that the EF-hand calcium-binding motif is highly conserved in plant CRSH proteins. The level of calcium ions and *BnCRSH* transcripts increased during canola seed maturation. *BnCRSH* transcripts are localized in the cotyledons and hypocotyl of developing canola seeds. The results of this study strongly suggest that calcium-dependent stringent response during late stages of plant development, by inhibiting the expression of both plastid and nuclear genes, bring seeds into a dormant state (Publication II).

- We showed that the expression of *BnRSH* genes is influenced differently by biotic and abiotic factors, suggesting that they have diverse functions in plants (Publication I and Publication III). The presence of some PGPB, particularly *Serratia* sp., significantly increased the expression of *BnRSH* genes, indicating that stringent response may be one of the pathways *via* which PGPB bacteria promote plant growth and development. On the other hand, PGPB can mitigate the harmful effects of salinity on plant growth via varied mechanisms. Analysis of salt stress showed changes in expression levels of *BnRSH*s grown

in the presence of bacteria belonging to *Serratia* genera, suggesting that some PGPB improve plant growth under salt stress also *via* a stringent response pathway (Publication I).

- We demonstrated the effectiveness of *T. viride* strains in inhibiting the growth of three important plant pathogens, particularly *Fusarium culmorum*. The new seed coating neither negatively impacts seed germination nor induces a plant stress response. The growth of *B. napus* seedlings was promoted by new seed coating which increased the length and biomass of shoots and roots. The developed seed coating is biodegradable and cost-effective and limits the growth of plant pathogens and promotes seedling growth (Publication III).

5. References

1. Kumar A, Singh S, Gaurav AK, Srivastava S, Verma JP. Plant growth-promoting bacteria: Biological tools for the mitigation of salinity stress in plants. Front Microbiol. 2020 Jul 7;11.
2. Agrios GN. Plant pathology. Fifth edition. Amsterdam: Elsevier Academic Press; 2005.
3. El-Esawi M, Alaraidh I, Alsahli A, Alzahrani S, Ali H, Alayafi A, et al. *Serratia liquefaciens* KM4 improves salt stress tolerance in maize by regulating redox potential, ion homeostasis, leaf gas exchange and stress-related gene expression. Int J Mol Sci. 2018 Oct 24;19(11):3310.
4. Rojas-Sánchez B, Guzmán-Guzmán P, Morales-Cedeño LR, Orozco-Mosqueda Ma del C, Saucedo-Martínez BC, Sánchez-Yáñez JM, et al. Bioencapsulation of microbial inoculants: Mechanisms, formulation types and application techniques. Appl. Biosci. 2022 Sep 1;1(2):198–220.
5. FAO, IFAD, UNICEF, WFP, WHO. The state of food security and nutrition in the world 2022. FAO; 2022.
6. van Dijk M, Morley T, Rau ML, Saghai Y. A meta-analysis of projected global food demand and population at risk of hunger for the period 2010–2050. Nat Food. 2021 Jul 21;2(7):494–501.
7. Ahanger MA, Akram NA, Ashraf M, Alyemeni MN, Wijaya L, Ahmad P. Plant responses to environmental stresses—from gene to biotechnology. AoB Plants. 2017 Jul;9(4).
8. Dąbrowska G, Prusińska J, Goc A. The stringent response--bacterial mechanism of an adaptive stress response. Adv. Biochem. 2006;52(1):87–93.
9. Field B. Green magic: Regulation of the chloroplast stress response by (p)ppGpp in plants and algae. J Exp Bot. 2018;69(11):2797–807.
10. Masuda S. The stringent response in phototrophs. In: Advances in photosynthesis—fundamental aspects. InTech; 2012. p. 487–500.
11. Cashel M, Kalbacher B. The control of ribonucleic acid synthesis in *Escherichia coli*. J Biol Chem. 1970 May;245(9):2309–18.
12. Cashel M, Gallant J. Two compounds implicated in the function of the *RC* gene of *Escherichia coli*. Nature. 1969;221:838–41.
13. Gaca AO, Kajfasz JK, Miller JH, Liu K, Wang JD, Abranches J, et al. Basal levels of (p)ppGpp in *Enterococcus faecalis*: the magic beyond the stringent response. mBio. 2013 Nov;4(5).
14. Kriel A, Bittner AN, Kim SH, Liu K, Tehranchi AK, Zou WY, et al. Direct regulation of GTP homeostasis by (p)ppGpp: A critical component of viability and stress resistance. Mol Cell. 2012 Oct;48(2):231–41.

15. Strugeon E, Tilloy V, Ploy MC, Da Re S. The stringent response promotes antibiotic resistance dissemination by regulating integron integrase expression in biofilms. mBio. 2016 Sep 7;7(4).

16. Maekawa M, Honoki R, Ihara Y, Sato R, Oikawa A, Kanno Y, et al. Impact of the plastidial stringent response in plant growth and stress responses. Nat Plants. 2015;1(November).

17. Hauryliuk V, Atkinson GC, Murakami KS, Tenson T, Gerdes K. Recent functional insights into the role of (p)ppGpp in bacterial physiology. Nat Rev Microbiol. 2015;13(5):298–309.

18. Steinchen W, Schuhmacher JS, Altegoer F, Fage CD, Srinivasan V, Linne U, et al. Catalytic mechanism and allosteric regulation of an oligomeric (p)ppGpp synthetase by an alarmone. Proc Natl Acad Sci U S A. 2015;112(43):13348–53.

19. Chau NYE, Ahmad S, Whitney JC, Coombes BK. Emerging and divergent roles of pyrophosphorylated nucleotides in bacterial physiology and pathogenesis. PLoS Pathog. 2021 May 13;17(5):e1009532.

20. Dalebroux ZD, Swanson MS. ppGpp: magic beyond RNA polymerase. Nat Rev Microbiol. 2012 Mar 16;10(3):203–12.

21. Ihara Y, Masuda S. Cytosolic ppGpp accumulation induces retarded plant growth and development. Plant Signal Behav. 2016;11(2):1–3.

22. Atkinson GC, Tenson T, Hauryliuk V. The RelA/SpoT Homolog (RSH) superfamily: Distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. PLoS One. 2011;6(8).

23. McFadden GI. Origin and evolution of plastids and photosynthesis in eukaryotes. Cold Spring Harb Perspect Biol. 2014 Apr 1;6(4):a016105–a016105.

24. Kmiecik P, Leonardelli M, Teige M. Novel connections in plant organellar signalling link different stress responses and signalling pathways. J Exp Bot. 2016 Jun;67(13):3793–807.

25. Chan KX, Phua SY, Crisp P, McQuinn R, Pogson BJ. Learning the languages of the chloroplast: Retrograde signaling and beyond. Annu Rev Plant Biol. 2016 Apr 29;67(1):25–53.

26. Dietz KJ, Hell R. Thiol switches in redox regulation of chloroplasts: balancing redox state, metabolism and oxidative stress. Biol Chem. 2015 May 1;396(5):483–94.

27. Spetea C, Rintamäki E, Schoefs B. Changing the light environment: chloroplast signalling and response mechanisms. 2014 Apr 19;369(1640):20130220.

28. Serrano I, Audran C, Rivas S. Chloroplasts at work during plant innate immunity. J Exp Bot. 2016 Jun;67(13):3845–54.

29. Müller-Schüssele SJ, Wang R, Gütle DD, Romer J, Rodriguez-Franco M, Scholz M, et al. Chloroplasts require glutathione reductase to balance reactive oxygen species and maintain efficient photosynthesis. Plant J. 2020 Aug 23;103(3):1140–54.

30. Van Der Biezen EA, Sun J, Coleman MJ, Bibb MJ, Jones JDG. *Arabidopsis* RelA/SpoT homologs implicate (p)ppGpp in plant signaling. Proc Natl Acad Sci U S A. 2000;97(7):3747–52.

31. Kasai K, Usami S, Yamada T, Endo Y, Ochi K, Tozawa Y. A RelA-SpoT homolog (Cr-RSH) identified in *Chlamydomonas reinhardtii* generates stringent factor in vivo and localizes to chloroplasts *in vitro*. Nucleic Acids Res. 2002;30(22):4985–92.

32. Dąbrowska G, Prusińska J, Goc A. Identification of *RSH* gene cDNA (*RelA/SpoT homolog*) involved in *Pharbitis nil* response to stress condition. Adv. Agric. Sci. Probl. Issues. 2006;509:343–51.

33. Sugliani M, Abdelkefi H, Ke H, Bouveret E, Robaglia C, Caffarri S, et al. An ancient bacterial signaling pathway regulates chloroplast function to influence growth and development in *Arabidopsis*. Plant Cell. 2016;28(3):661–79.

34. Mehrez M, Romand S, Field B. New perspectives on the molecular mechanisms of stress signalling by the nucleotide guanosine tetraphosphate (ppGpp), an emerging regulator of photosynthesis in plants and algae. New Phytol. 2023 Feb 2;237(4):1086–99.

35. Tozawa Y, Nozawa A, Kanno T, Narisawa T, Masuda S, Kasai K, et al. Calcium-activated (p)ppGpp synthetase in chloroplasts of land plants. J Biol Chem. 2007;282(49):35536–45.

36. Ito D, Ihara Y, Nishihara H, Masuda S. Phylogenetic analysis of proteins involved in the stringent response in plant cells. J Plant Res. 2017 Jul 1;130(4):625–34.

37. Sato M, Takahashi T, Ochi K, Matsuura H, Nabeta K, Takahashi K. Overexpression of RelA/SpoT homologs, PpRSH2a and PpRSH2b, induces the growth suppression of the moss *Physcomitrella patens*. Biosci Biotechnol Biochem. 2015;79(1):36–44.

38. Givens RM, Lin MH, Taylor DJ, Mechold U, Berry JO, Hernandez VJ. Inducible expression, enzymatic activity, and origin of higher plant homologues of bacrterial RelA/SpoT stress proteins in *Nicotiana tabacum*. J Biol Chem. 2004;279(9):7495–504.

39. Mizusawa K, Masuda S, Ohta H. Expression profiling of four RelA/SpoT-like proteins, homologues of bacterial stringent factors, in *Arabidopsis thaliana*. Planta. 2008;228(4):553–62.

40. Masuda S, Tozawa Y, Ohta H. Possible targets of 'magic spots' in plant signalling. Plant Signal Behav. 2008;3(11):1021–3.

41. Masuda S, Mizusawa K, Narisawa T, Tozawa Y, Ohta H, Takamiya KI. The bacterial stringent response, conserved in chloroplasts, controls plant fertilization. Plant Cell Physiol. 2008 Feb;49(2):135–41.

42. Ono S, Suzuki S, Ito D, Tagawa S, Shiina T, Masuda S. Plastidial (p)ppGpp synthesis by the Ca^{2+}-dependent RelA-SpoT homolog regulates the adaptation of chloroplast gene expression to darkness in *Arabidopsis*. Plant Cell Physiol. 2021;61(12):2077–86.

43. Yamburenko M V., Zubo YO, Börner T. Abscisic acid affects transcription of chloroplast genes via protein phosphatase 2C-dependent activation of nuclear genes: Repression by guanosine-3′-5′-bisdiphosphate and activation by sigma factor 5. Plant J. 2015;82(6):1030–41.

44. Takahashi K, Kasai K, Ochi K. Identification of the bacterial alarmone guanosine 5′-diphosphate 3′-diphosphate (ppGpp) in plants. Proc Natl Acad Sci U S A. 2004;101(12):4320–4.

45. Abdelkefi H, Sugliani M, Ke H, Harchouni S, Soubigou-Taconnat L, Citerne S, et al. Guanosine tetraphosphate modulates salicylic acid signalling and the resistance of *Arabidopsis thaliana* to *Turnip mosaic virus*. Mol Plant Pathol. 2018;19(3):634–46.

46. Honoki R, Ono S, Oikawa A, Saito K, Masuda S. Significance of accumulation of the alarmone (p)ppGpp in chloroplasts for controlling photosynthesis and metabolite balance during nitrogen starvation in *Arabidopsis*. Photosynth Res. 2018;135(1–3):299–308.

47. Romand S, Abdelkefi H, Lecampion C, Belaroussi M, Dussenne M, Ksas B, et al. A guanosine tetraphosphate (ppGpp) mediated brake on photosynthesis is required for acclimation to nitrogen limitation in *Arabidopsis*. eLife. 2022 Feb 1;11.

48. Goto M, Oikawa A, Masuda S. Metabolic changes contributing to large biomass production in the *Arabidopsis* ppGpp-accumulating mutant under nitrogen deficiency. Planta. 2022 Feb 25;255(2):48.

49. Inazu M, Nemoto T, Suzuki S, Ono S, Kanno Y, Seo M, et al. Complete loss of RelA and SpoT homologs in *Arabidopsis* reveals the importance of the plastidial stringent response in the interplay between chloroplast metabolism and plant defense response. bioRxiv. 2022 Jan 1;2022.09.20.508797.

50. Zubo YO, Yamburenko M V., Kusnetsov V V., Börner T. Methyl jasmonate, gibberellic acid, and auxin affect transcription and transcript accumulation of chloroplast genes in barley. J Plant Physiol. 2011 Aug;168(12):1335–44.

51. Šamec D, Salopek-Sondi B. Cruciferous (*Brassicaceae*) vegetables. In: Nonvitamin and Nonmineral Nutritional Supplements. Elsevier; 2019. p. 195–202.

52. Kim JS, Chung TY, King GJ, Jin M, Yang TJ, Jin YM, et al. A sequence-tagged linkage map of *Brassica rapa*. Genetics. 2006 Sep 1;174(1):29–39.

53. Snowdon R, Lühs W, Friedt W. Oilseed rape. In: Oilseeds. Berlin, Heidelberg: Springer Berlin Heidelberg; 2007. p. 55–114.

54. Raboanatahiry N, Li H, Yu L, Li M. Rapeseed (*Brassica napus*): Processing, utilization, and genetic improvement. Agronomy. 2021 Sep 3;11(9):1776.

55. Ahmed S, Warne T, Smith E, Goemann H, Linse G, Greenwood M, et al. Systematic review on effects of bioenergy from edible versus inedible feedstocks on food security. NPJ Sci Food. 2021 May 4;5(1):9.

56. Meier U. Growth stages of mono-and dicotyledonous plants. In: BBCH Monograph. Germany: Federal Biological Research Centre for Agriculture and Forestry; 2001.

57. Harada JJ. Signaling in plant embryogenesis. Curr Opin Plant Biol. 1999 Feb;2(1):23–7.

58. Siles L, Eastmond P, Kurup S. Big data from small tissues: extraction of high-quality RNA for RNA-sequencing from different oilseed *Brassica* seed tissues during seed development. Plant Methods. 2020 Dec 5;16(1):80.

59. Boyles M, Peeper T, Stamm M. 'Great plains canola production handbook'. MF-2734. Kansas State University; 2006.

60. Edwards J, Hertel K. Canola growth & development. Depi Nsw. 2011 [cited 2022 Apr 11];1–96.

61. Tozawa Y, Nomura Y. Signalling by the global regulatory molecule ppGpp in bacteria and chloroplasts of land plants. Plant Biol. 2011;13(5):699–709.

62. Ito D, Kato T, Maruta T, Tamoi M, Yoshimura K, Shigeoka S. Enzymatic and molecular characterization of arabidopsis ppGpp pyrophosphohydrolase, AtNUDX26. Biosci Biotechnol Biochem. 2012;76(12):2236–41.

63. Dąbrowska G, Prusińska J, Goc A. Plant mechanism of an adaptive stress response homologous to bacterial stringent response. Adv Biochem. 2006;52(1):94–100.

64. Prusińska JM, Boniecka J, Dąbrowska GB, Goc A. Identification and characterization of the *Ipomoea nil RelA/SpoT Homologs* (*InRSH*s) and potential directions of their transcriptional regulation. Plant Sci. 2019;284(November 2018):161–76.

65. Xiong L, Lee MW, Qi M, Yang Y. Identification of defense-related rice genes by suppression subtractive hybridization and differential screening. Mol Plant Microbe Interact. 2001;14(5):685–92.

66. Tutar Y. Pseudogenes. Comp Funct Genomics. 2012;2012:1–4.

67. Sverdlov A V. Conservation versus parallel gains in intron evolution. Nucleic Acids Res. 2005 Sep 25;33(17):5713–5713.

68. Morello L, Breviario D. Plant spliceosomal introns: Not only cut and paste. Curr Genomics. 2008 Jun 1;9(4):227–38.

69. Wang BB, Brendel V. Genomewide comparative analysis of alternative splicing in plants. Proceedings of the National Academy of Sciences. 2006 May 2;103(18):7175–80.

70. Jo BS, Choi SS. Introns: The functional benefits of introns in genomes. Genomics Inform. 2015;13(4):112.

71. Ren X, Vorst O, Fiers M, Stiekema W, Nap J. In plants, highly expressed genes are the least compact. Trends in Genetics. 2006 Oct;22(10):528–32.

72. Winter D, Vinegar B, Nahal H, Ammar R, Wilson G V., Provart NJ. An 'electronic fluorescent pictograph' Browser for exploring and analyzing large-scale biological data sets. PLoS One. 2007;2(8):1–12.

73. Kilian J, Whitehead D, Horak J, Wanke D, Weinl S, Batistic O, et al. The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. The Plant Journal. 2007 Mar 21;50(2):347–63.

74. Anil VS, Harmon AC, Rao KS. Spatio-temporal accumulation and activity of calcium-dependent protein kinases during embryogenesis, seed development, and germination in sandalwood. Plant Physiol. 2000;122(4):1035–43.

75. Ghosh S, Bheri M, Pandey GK. Delineating calcium signaling machinery in plants: Tapping the potential through functional genomics. Curr Genomics. 2021 Dec 30;22(6):404–39.

76. Edel KH, Marchadier E, Brownlee C, Kudla J, Hetherington AM. The evolution of calcium-based signalling in plants. Current Biology. 2017 Jul;27(13):R667–79.

77. Pang X, Halaly T, Crane O, Keilin T, Keren-Keiserman A, Ogrodovitch A, et al. Involvement of calcium signalling in dormancy release of grape buds. J Exp Bot. 2007 Jul 13;58(12):3249–62.

78. Sliwinska E. Nuclear DNA replication and seed quality. Seed Sci Res. 2009;19(1):15–25.

79. Rewers M, Sliwinska E. Endoreduplication intensity as a marker of seed developmental stage in the Fabaceae. Cytometry Part A. 2012;81 A(12):1067–75.

80. Boniecka J, Prusińska J, Dąbrowska GB, Goc A. Within and beyond the stringent response-RSH and (p)ppGpp in plants. Planta. 2017 Nov [cited 2019 Oct 21];246(5):817–42.

81. Yamada A, Tsutsumi K, Tanimoto S, Ozeki Y. Plant RelA/SpoT homolog confers salt tolerance in *Escherichia coli* and *Saccharomyces cerevisiae*. Plant Cell Physiol. 2003;44(1):3–9.

82. Kim TH, Ok SH, Kim D, Suh SC, Byun MO, Shin JS. Molecular characterization of a biotic and abiotic stress resistance-related gene *RelA/SpoT homologue (PepRSH)* from pepper. Plant Science. 2009;176(5):635–42.

83. Etesami H, Noori F. Soil salinity as a challenge for sustainable agriculture and bacterial-mediated alleviation of salinity stress in crop plants BT - saline soil-based agriculture by halotolerant microorganisms. In: Kumar M, Etesami H, Kumar V, editors. Singapore: Springer Singapore; 2019. p. 1–22.

84. Ashraf M, McNeilly T. Salinity tolerance in Brassica oilseeds. CRC Crit Rev Plant Sci. 2004 Mar;23(2):157–74.

85. Etesami H, Maheshwari DK. Use of plant growth promoting rhizobacteria (PGPRs) with multiple plant growth promoting traits in stress agriculture: Action mechanisms and future prospects. Ecotoxicol Environ Saf. 2018 Jul;156:225–46.

86. Vaghela N, Gohel S. Medicinal plant-associated rhizobacteria enhance the production of pharmaceutically important bioactive compounds under abiotic stress conditions. J Basic Microbiol. 2023 Mar 6;63(3–4):308–25.

87. Backer R, Rokem JS, Ilangumaran G, Lamont J, Praslickova D, Ricci E, et al. Plant growth-promoting rhizobacteria: Context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. Front Plant Sci. 2018 Oct 23;9.

88. Tsukanova KA, Chebotar VK, Meyer JJM, Bibikova TN. Effect of plant growth-promoting Rhizobacteria on plant hormone homeostasis. South African Journal of Botany. 2017 Nov;113:91–102.

89. Bal HB, Nayak L, Das S, Adhya TK. Isolation of ACC deaminase producing PGPR from rice rhizosphere and evaluating their plant growth promoting activity under salt stress. Plant Soil. 2013 May;366(1–2):93–105.

90. Dąbrowska G, Zdziechowska E, Hrynkiewicz K. Evaluation of potential suitability of rhizobacteria for phytodesalination of soils. Ochrona Srodowiska. 2016;38(3):9–14.

91. Shilev S. Plant-growth-promoting bacteria mitigating soil salinity stress in plants. Appl. Sci. 2020;10(20):1–20.

92. Kloepper J, Hume D, Scher F, Singleton C, Tipping B, Laliberte M, et al. Plant growth-promoting rhizobacteria on canola (rapeseed). Plant Dis. 1988;72:42–6.

93. Szymańska S, Dąbrowska GB, Tyburski J, Niedojadło K, Piernik A, Hrynkiewicz K. Boosting the *Brassica napus* L. tolerance to salinity by the halotolerant strain *Pseudomonas stutzeri* ISE12. Environ Exp Bot. 2019;163(April):55–68.

94. de Souza R, Meyer J, Schoenfeld R, da Costa PB, Passaglia LMP. Characterization of plant growth-promoting bacteria associated with rice cropped in iron-stressed soils. Ann Microbiol. 2015 Jun 22;65(2):951–64.

95. Naziya B, Murali M, Amruthesh KN. Plant growth-promoting fungi (PGPF) instigate plant growth and induce disease resistance in *Capsicum annuum* L. upon infection with *Colletotrichum capsici* (Syd.) Butler & Bisby. Biomolecules. 2019 Dec 26;10(1):41.

96. Safari Motlagh MR, Abolghasemi M. The effect of *Trichoderma* spp. isolates on some morphological traits of canola inoculated with *Sclerotinia sclerotiorum* and evaluation of their efficacy in biological control of pathogen. J Saudi Soc Agric Sci. May;21(4):217–31.

97. Poveda J. *Trichoderma parareesei* favors the tolerance of rapeseed (*Brassica napus* L.) to salinity and drought due to a chorismate mutase. Agronomy. 2020 Jan 13;10(1):118.

98. Tyśkiewicz R, Nowak A, Ozimek E, Jaroszuk-Ściseł J. *Trichoderma*: The current status of its application in agriculture for the biocontrol of fungal phytopathogens and stimulation of plant growth. Int J Mol Sci. 2022 Feb 19;23(4):2329.

99. Znajewska Z, Dąbrowska GB, Narbutt O. *Trichoderma viride* strains stimulating the growth and development of winter rapeseed (*Brassica napus* L.). Prog. Plant Prot. 2018;58(4):264–9.

6. Abstracts

Abstract of PhD thesis entitled:" The role of plant stringent response in *Brassica napus* L. in response to biotic and abiotic factors and during seed development".

The regulatory nucleotides (p)ppGpp (alarmones) were originally identified in *E. coli* and control several aspects of cell metabolism. The alarmone dependent control of cell metabolism is referred to as the stringent response. At the beginning of the 21st century, genes responsible for the synthesis and degradation of alarmones were identified in plants, indicating that the stringent response is a widely evolutionarily conserved regulatory mechanism. The plant RelA/SpoT Homolog (*RSH*) genes have been identified and characterized in various plant species. Moreover, alarmones have been shown to accumulate mainly in chloroplasts. The stringent response in plants homologous to bacterial stringent response plays a very important role in plant growth, development, and adaptation to the environmental changes, and is not just a leftover from the plastid endosymbiotic ancestor. During evolution, the *RSH* ancestor genes were sustained in the host plant cells and further diversified. The evolutionary aspect of the plant mechanism homologous to the bacterial stringent response raises the question about the complexity of the plant RSH families including the number, structure, and functions in different plant species.

A total number of 14 *RSH* genes was identified in the genome of *Brassica napus* L., and further examination of their amino acid sequences revealed that they could be categorized into three subgroups: RSH1, RSH2/3, and CRSH, similar to other plant species. Notably, the promoter regions of these genes were found to harbour diverse regulatory elements, indicating their responsiveness to various environmental cues, including light, phytohormones, and both biotic and abiotic stresses (Publication I). In CRSH proteins EF-hand calcium-binding motif in present which is highly conserved in these plant proteins. During canola seed maturation, an increase in calcium ions and the amount of *BnCRSH* transcripts was observed, suggesting a that calcium-dependent

stringent response is involved in seed maturation and condition seed dormancy (Publication II). The expression of *BnRSH* genes was influenced by biotic and abiotic factors. Plant growth-promoting bacteria (PGPB), belonging to *Serratia* sp., were found to significantly increase the expression of *BnRSH* genes, not only in control condition but also under salinity suggesting the involvement of the stringent response in plant response to biotic and abiotic factors (Publication I). Moreover, the expression of BnRSH was affected in canola seedlings grown in the presence of plant growth-promoting fungi (PGPF) *Trichoderma viride*. Furthermore, the antagonisms of T. viride towards plant pathogens, especially *Fusarium culmorum* was demonstrated. New biodegradable seed coating containing the spores of *T. viride* was developed. It promoted the growth of *B. napus* seedlings, did not negatively affect seeds germination, did not induce plant stress responses, and limit pathogen growth (Publication III).

Overall, this research has brought new knowledge about the potential roles of RSH proteins in *B. napus*, particularly in plant growth and development, adaptation to stress, and interactions plant-microorganisms. In addition, new biocoating has been developed which might be used for increasing crop yield and phytopathogens biocontrol. The insights into the stringent response provided in this study significantly contribute to developing our knowledge of plant adaptation to stress and plant development and offer potential strategies for enhancing crop productivity and biological control of plant pathogens. Understanding the mechanisms by which RSH controlled plant metabolisms in changeable environmental conditions is of utmost importance for the survival of not only a single organism but also the whole species.

Streszczenie rozprawy doktorskiej pt.: „Rola odpowiedzi ścisłej w reakcji na czynniki biotyczne i abiotyczne oraz w czasie rozwoju nasion *Brassica napus* L.”

Nukleotydy regulatorowe, zwane alarmonami ((p)ppGpp), pierwotnie zidentyfikowano u *E. coli*, gdzie odpowiadają za regulację metabolizmu komórkowego. Zależna od alarmonów kontrola metabolizmu nazywana jest odpowiedzą ścisłą. Na początku XXI wieku u roślin zidentyfikowano geny odpowiedzialne za syntezę i degradację alarmonów, co wskazuje, że odpowiedź ścisła jest konserwowana ewolucyjnie. Roślinne geny *RSH* (*RelA*/*SpoT* Homolog) zostały zidentyfikowane i scharakteryzowane u różnych gatunków roślin. Ponadto wykazano, że alarmony, produkty aktywności katalitycznej białek RSH, gromadzą się głównie w chloroplastach. Mechanizm roślinny, homologiczny do bakteryjnej odpowiedzi ścisłej, odgrywa istotną rolę we wzroście, rozwoju i adaptacji roślin do zmian środowiskowych i nie stanowi jedynie pozostałości po plastydowym endosymbiotycznym przodku. W drodze ewolucji geny *RSH* utrzymywały się w komórkach roślinnych i zostały przeniesione do genomu jądrowego, a następnie podlegały różnicowaniu. Ewolucyjny aspekt mechanizmu roślinnego, homologiczny do bakteryjnej odpowiedzi ścisłej nasuwa pytanie o złożoność roślinnych rodzin RSH, uwzględniającą liczbę, strukturę i funkcję u różnych gatunków roślin.

W przeprowadzonych badaniach w genomie rzepaku (*Brassica napus* L.) zidentyfikowano łącznie 14 genów *RSH*, a dalsze badanie sekwencji aminokwasowych wykazało, że geny te można zaklasyfikować, podobnie jak u innych gatunków roślin, do trzech podgrup: *RSH1*, *RSH2/3* i *CRSH*. Stwierdzono, że regiony promotorowe tych genów zawierają sekwencje regulatorowe wskazujące na ich zaangażowanie w odpowiedzi na różne bodźce środowiskowe, w tym światło, fitohormony oraz inne czynniki powodujące stresy biotyczne lub abiotyczne (publikacja I). W podgrupie CRSH obecny jest motyw EF, odpowiadający za wiązanie jonów wapnia, który jest

wysoce konserwowany w tych roślinnych białkach. Zaobserwowano, że podczas dojrzewania nasion rzepaku wzrastają zawartość jonów wapnia i poziom transkryptów genu *BnCRSH,* co sugeruje, że odpowiedź ścisła zależna od jonów wapnia uczestniczy w dojrzewaniu nasion i warunkuje ich spoczynek (publikacja II). Stwierdzono, że bakterie promujące wzrost roślin (ang. *Plant Growth-Promoting Bacteria*, PGPB), należące do rodzaju *Serratia* sp., znacząco zwiększały ekspresję genów *BnRSH,* nie tylko w warunkach kontrolnych, ale także w warunkach podwyższonego zasolenia, co sugeruje udział odpowiedzi ścisłej w reakcji rośliny na czynniki biotyczne i abiotyczne (publikacja I). Ponadto wykazano, że ekspresja genów *BnRSH* zmienia się w siewkach rzepaku rosnących w obecności grzybów PGPF (ang. *Plant Growth-Promoting Fungi*) *Trichoderma viride*. Wykazano antagonizm *T. viride* w stosunku do patogenów roślin, w szczególności do *Fusarium culmorum*. Opracowano nową biodegradowalną otoczkę nasion zawierającą zarodniki *T. viride*, która sprzyjała wzrostowi siewek *B. napus* bez negatywnego wpływu na kiełkowanie nasion i indukowanie reakcji stresowych u roślin, jednocześnie ograniczała wzrost fitopatogenów (publikacja III).

Podsumowując, badania te wniosły nowe informacje na temat roli białek RSH u rzepaku podczas wzrostu i rozwoju oraz adaptacji roślin do stresu i w interakcjach między roślinami a mikroorganizmami. Ponadto, opracowano nowe rozwiązanie w postaci otoczki nasion, które może znaleźć zastosowanie do zwiększania produktywności upraw i biokontroli patogenów. Zrozumienie mechanizmów, za pomocą których odpowiedź ścisła kontroluje metabolizm roślin w zmieniających się warunkach środowiskowych, ma ogromne znaczenie nie tylko dla przetrwania danego organizmu, ale także całego gatunku.

Publication list

1.	Dąbrowska G.B., **Turkan S.,** Tylman-Mojżeszek W., Mierek-Adamska A. *In silico* study of RSH (RelA/SpoT homologs) gene family and the expression analysis in response to PGPR bacteria and salinity in *Brassica napus*. International Journal of Molecular Sciences, 2021, 22(19): 10666. Doi: 10.3390/ijms221910666. Contribution: 35%	IF – 6.208 $IF_{5\text{-year}}$ – 6.628 MNiSW – 140
2.	**Turkan S.**, Mierek-Adamska A., Głowacka K., Szydłowska-Czerniak A., Rewers M., Jędrzejczyk I., Dąbrowska G.B. Localization, and expression of CRSH transcript, level of calcium ions, and cell cycle activity during *Brassica napus* L. seed development. Industrial Crops & Products, 2023, 195: 116439. Doi: 10.1016/j.indcrop.2023.116439 Contribution: 70%	IF – 6.449 $IF_{5\text{-year}}$ – 5.645 MNiSW – 200
3.	**Turkan S.**, Mierek-Adamska A., Kulasek M., Konieczna W.B., Dąbrowska G.B. New seed coating containing *Trichoderma viride* with anti-pathogenic properties. PeerJ, 2023, 11: e15392. doi: 10.7717/peerj.15392. Doi: 10.1016/j.indcrop.2023.116439 Contribution: 65%	IF – 3.061 $IF_{5\text{-year}}$ – 2.929 MNiSW – 100
		Summarized **IF** –15.718 Summarized **MNiSW points** - 440

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In Silico Study of the *RSH* (*RelA/SpoT* Homologs) Gene Family and Expression Analysis in Response to PGPR Bacteria and Salinity in *Brassica napus*

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Abstract: Among several mechanisms involved in the plant stress response, synthesis of guanosine tetra and pentaphosphates (alarmones), homologous to the bacterial stringent response, is of crucial importance. Plant alarmones affect, among others, photosynthetic activity, metabolite accumulation, and nutrient remobilization, and thus regulate plant growth and development. The plant *RSH* (*RelA/SpoT* homolog) genes, that encode synthetases and/or hydrolases of alarmones, have been characterized in a limited number of plant species, e.g., *Arabidopsis thaliana*, *Oryza sativa*, and *Ipomoea nil*. Here, we used dry-to-wet laboratory research approaches to characterize *RSH* family genes in the polyploid plant *Brassica napus*. There are 12 *RSH* genes in the genome of rapeseed that belong to four types of *RSH* genes: 6 *RSH1*, 2 *RSH2*, 3 *RSH3*, and 1 *CRSH*. *BnRSH* genes contain 13–24 introns in *RSH1*, 2–6 introns in *RSH2*, 1–6 introns in *RSH3*, and 2–3 introns in the *CRSH* genes. In the promoter regions of the *RSH* genes, we showed the presence of regulatory elements of the response to light, plant hormones, plant development, and abiotic and biotic stresses. The wet-lab analysis showed that expression of *BnRSH* genes is generally not significantly affected by salt stress, but that the presence of PGPR bacteria, mostly of *Serratia* sp., increased the expression of *BnRSH* significantly. The obtained results show that *BnRSH* genes are differently affected by biotic and abiotic factors, which indicates their different functions in plants.

Keywords: rapeseed; RelA/SpoT homolog; RSH; alarmones; salinity; stringent response; PGPR

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1. Introduction

Several species belonging to the *Brassicaceae* Burnett family are economically important plants, i.e., oil and fodder plants in agriculture, vegetables in horticulture including herbal species, and plants used in floriculture. The model plant *A. thaliana* also belongs to this plant family. The genus *Brassica* contains 37 species; the most extensively cultivated are *B. rapa* L., *B. juncea* L. Czernj & Cosson (mustard plant), *B. napus* L. (oilseed rape, rape, rapeseed, canola), and *B. carinata* A. Braun (Abyssinian cabbage) [1]. Rapeseed is a crop plant cultivated in temperate and subtropical regions, mainly for oil production purposes, as seeds of this plant are rich in fat (40–49%). The rapeseed oil is used in both the food industry, as it is one of the healthiest oils, and the energy industry, to produce biofuel. Rape oil by-products are utilised for the production of fodder due to their high protein content [2]. Rapeseed is cultivated all over the world, depending on climatic conditions and latitudes; three types, i.e., the winter, semi-winter, and spring types, are cultivated with varying intensity [3]. *B. napus* is an allopolyploid plant ($A_rA_rC_oC_o$); its genome is a result of *B. oleracea* (Mediterranean cabbage, C_oC_o) and *B. rapa* (A_rA_r) genome hybridization, followed by duplication. The genome of *B. napus* has already been sequenced [4].

The crop yield depends strictly on the ability of plants to adapt to adverse and changeable environmental conditions, which is especially important during seed germination,

and during the first stages of plant growth and development. Soil salinity is one of the crucial environmental stresses that have severely decreased crop productivity all over the world. It negatively affects plant physiology and metabolism, including photosynthesis, lipid metabolism, protein synthesis, and nitrogen fixation [5]. The abundance of Na^+ and Cl^- inhibits absorption of other macronutrients causing nutritional imbalance. Moreover, salinity leads to water stress, increased reactive oxygen species production, and oxidative stress [6,7].

Plant growth-promoting rhizobacteria (PGPR) exert several beneficial effects on host plants by promoting plant growth and development, including in stress conditions, via varied mechanisms, such as the production of phytohormones, secondary metabolites, and antibiotics [8–10]. Plant growth promoting bacteria, especially halotolerant bacteria, could be a crucial factor for improving plant tolerance to salt stress in an environmentally friendly way [8,9]. PGPR isolated from the rice rhizosphere improved the growth of rice plants exposed to salt stress by lowering the level of ethylene [10]. *Serratia liquefaciens* KM4 increased the growth and biomass of maize grown in salt-stress conditions, and the increased expression of plant stress-related genes has been observed [6]. The inoculation of lettuce with *Pseudomonas mendocina* has a greater effect on plant growth in salt stress conditions than inoculation with arbuscular mycorrhizal fungi. In the presence of analysed PGPR the induction of a plant antioxidant system was observed, even in severe salinity conditions [11]. The inoculation of tomato with PGPR, especially *Arthobacter* sp. and *Pseudomonas* sp., under salinity stress outperformed chemical fertilization [12].

Organisms living in a fluctuating environment have evolved a range of mechanisms to respond to various stress conditions. Among several other mechanisms in bacteria, one of the most important is the stringent response. It was first described in *Escherichia coli* in response to the absence of amino acids [13]. The response is based on the synthesis of the atypical signalling nucleotides, guanosine tetraphosphates (ppGpp) and guanosine pentaphosphates (pppGpp), called alarmones. The increased amount of alarmones in response to stress conditions leads to the immediate arrest of rRNA, tRNA, and ribosomal protein gene expression, followed by the induction of expression of genes encoding proteins involved in adaptation to unfavourable conditions [14,15]. The metabolism of (p)ppGpp in *E. coli* is regulated by RelA and SpoT enzymes encoded by paralogous genes. RelA is a (p)ppGpp synthetase, whereas SpoT is mainly a (p)ppGpp hydrolase, however, in certain conditions it exhibits low activity of alarmone synthetase. Most bacteria possess only one bifunctional Rel enzyme [16–19].

The presence of (p)ppGpp in photosynthetic *Eucaryota* was first confirmed in the alga *Chlamydomonas reinhardtii*, where the accumulation of alarmones in response to amino acid starvation was observed [20]. Homologs of the bacterial genes *RelA/SpoT* called *RSH* (*RelA/SpoT* Homologs) were first identified in *A. thaliana* [21] and, in subsequent years, *RSH* genes have been identified in other plant species [22–25]. RSH proteins have been divided into three groups, i.e., *RSH1*, *RSH2/3*, and *CRSH*, based on their primary structure and domain structure [26]. In *A. thaliana*, there are four genes encoding RSH proteins, namely *RSH1*, *RSH2*, *RSH3*, and *CRSH* (Ca^{2+}-activated *RSH*). RSH1 exhibits only (p)ppGpp hydrolytic activity due to the substitution, critical for (p)ppGpp synthase activity, of glycine by serine in the RSD domain. Proteins belonging to the RSH2/3 group (AtRSH2 and AtRSH3) can both synthesize and hydrolase alarmones, whereas CRSH proteins do not possess a functional hydrolytic domain (HD domain) and are (p)ppGpp synthases [26,27,30]. Members of the RSH1 group possess a TGS domain which has been proposed to play a regulatory role in ligand binding [27], and a role in establishing the RSH-ribosome interaction in chloroplasts [28–30]. Moreover, RSH1 as the only group of plant RSH proteins that possess the ACT domain [30], recently described as an RNA recognition motif (RRM) domain [28]. CRSH group proteins also contain the EF-hand motif at the C-terminus of the protein. Interestingly, this Ca^{2+}-binding motif has not been identified in any bacterial or plant homolog [26,31]. It was confirmed in vitro that, for (p)ppGpp synthase activity, *CRSH* requires Ca^{2+} [32]. The plant stringent response has

been implicated in the stress response, flowering, seed development, photosynthesis, plant senescence, and nutrient remobilization [27].

In animals, homologs of bacterial SpoT have been identified (Mesh1) with alarmone hydrolysing activity [33]. However, until quite recently, the existence of (p)ppGpp in metazoa has been questioned. Last year the presence of ppGpp in *Drosophila* and human cells was shown [34], opening a new chapter in the discussion about the origin and functions of alarmones.

In the present study, we attempt to answer the question about the complexity of the plant RSH groups in representatives of the *Brassicaceae* family via the in silico analysis of *RSH* genes and RSH proteins from selected species of this plant family. Inspired by the postulated role of RSH in the plant response to varied abiotic and biotic factors, we also examined *B. napus RSH* gene expression in response to salinity. Moreover, we analysed the expression of *BnRSHs* in the presence of *Serratia liquefaciens*, *S. plymuthica,* and *Massilia timonae*, PGPR bacteria for which the ability to promote the growth of rape has been confirmed. To pinpoint other potential regulators of *RSH* gene expression, we revealed the presence of multiple putative regulatory *cis*-elements in the promoter regions of *BnRSH* genes.

2. Results and Discussion

2.1. In Silico Analysis of RSH Genes and Proteins in B. napus and Selected Close Relatives from the Brassicaceae Family

Over 20 years ago, *RelA/SpoT* homologs (*RSH*) were discovered in plants [21], and the occurrence of the stringent response in plants was also proposed. Subsequently, *RSH* genes have been characterized in other plant species, and it has been shown that the stringent response plays a critical role in the regulation of plant growth and development, and in adaptation to different environmental niches [23,24]. The nature of the evolutionary basis of the stringent response raises questions regarding the complexity of plant *RSH* gene families including their number, and the structure of plant *RSH* proteins in various plant species. The plant *RSH* proteins have been divided into three groups (*RSH1*, *RSH2/3*, and *CRSH*), based mostly on protein primary structure. The members of these three groups of *RSH* proteins vary in their expression patterns and catalytic activities and, therefore, they probably fulfil distinct physiological roles. It seems that the diversification in plant *RSH* genes occurred when plants adapted to terrestrial conditions, and resulted either in the loss or acquisition of some structural and functional features [35,36]. Here, in order to reveal the complexity of the *RSH* gene family, and to further predict relations between sequence and function, we have analysed in silico *RSH* genes and RSH proteins in *B. napus*, and in selected relatives from the *Brassicaceae* family.

2.1.1. Characteristics of Selected *Brassicaceae RSH* Genes

In silico studies are often used as a preliminary means of analysis of plant gene families that enable the capturing of the phylogenetic relationships within a family of genes in one species, as well as between species [36–40]. A total of 45 *RSH* genes that were identified were selected for this study of *Brassicaceae* (*B. napus*, *B. olearacea*, *B. rapa*, *Camelina sativa*, and *Raphanus sativus*) plants are shown in Table 1. *B. napus* is an allotetraploid species and thus, as expected, has more *RSH* orthologous genes (14 in total, including 2 pseudogenes) than *A. thaliana*, where only 4 *RSH* genes have been described [35,41]. Four *RSH* genes are present also in the *B. rapa* genome, whereas in the genome of *B. oleracea* 6 *RSH* genes occur, and in the genome of *R. sativus* 8 *RSH* genes are present, though all these plants are diploids. In the allohexaploid genome of *C. sativa* 12 *RSH* genes are present, however, 3 of them are pseudogenes. In *O. sativa*, one gene in each of the *RSH1*, *RSH2*, and *RSH3* subgroups, and three *CRSH* genes were identified [42]. In *I. nil*, five *RSH* genes were identified, i.e., 1 *RSH1*, 2 *RSH2*, 1 *RSH3*, and 1 *CRSH* [25]. Genes encoding *RSH* were described also in *Capsicum annum* [43], *Nicotiana tabacum* [44], and *Suaeda japonica* [45].

Table 1. *RSH* genes present in the genomes of *A. thaliana*, *B. napus*, *B. oleracea*, *B. rapa*, *C. sativa*, and *R. sativus*. As a comparison, bacterial proteins of the stringent response for *E. coli*, RelA and SpoT, and for *Streptomyces coelicolor*, Rel, were included. The number of exons and introns, the length of CDS, the length, molecular weight, pI, and predicted subcellular localization of putative RSH proteins, are also given. Asterisks (*) indicate the *B. napus* (*RSH1_b*, *RSH2_b*, *RSH3_a*, and *CRSH*) genes that were further analysed for their expression level (vide infra).

Species	Genes	Gene ID	Transcript ID	CDS (bp)	Chromosome Location	Protein ID	AA	pI	Mw (kD)	Introns	Exons	Predicted Transfer Peptide (Probability)
A. thaliana	RSH1	828096	NM_116459.4	2655	4	NP_567226.1	883	6.65	98.58	23	24	cTP (0.455), mTP (0.0002), tlTP (0.0051), other (0.5393)
	RSH2	820619	NM_112259.5	2130	3	NP_188021.1	709	6.89	79.05	5	6	cTP (0.6081), mTP (0.0003), tlTP (0.0846), other (0.3047)
	RSH3	841853	NM_104291.8	2148	1	NP_564652.2	715	6.66	79.72	5	6	cTP (0.7887), mTP (0.0024), tlTP (0.0397), other (0.1669)
	CRSH	821012	NM_001338291.1	1752	3	NP_001327079.1	598	6.14	68.28	3	4	cTP (0.0708), mTP (0.1949), tlTP (0.0002), other (0.7341)
B. napus	RSH1_a	106345251	XM_013784481.2	2652	unknown	XP_013639935.1	883	6.64	98.56	23	24	cTP (0.3786), mTP (0.0002), tlTP (0.0059), other (0.6149)
	RSH1_b *	106399012	XM_013839498.2	2565	A5	XP_013694952.1	854	6.60	95.88	22	23	cTP (0.4978), mTP (0.0025), tlTP(0.004), other (0.4957)
	RSH1_c	106436227	XM_013877186.2	2652	A8	XP_013732640.1	883	6.64	98.58	23	24	cTP (0.3786), mTP (0.0002), tlTP(0.0059), other (0.6149)
	RSH1_d	106365508	XM_013804925.2	2652	A9	XP_013660379.1	883	6.48	98.55	23	24	cTP (0.5232), mTP (0.0005), tlTP(0.0171), other (0.459)
	RSH1_e	106362473	XM_013802370.2	1860	A9	XP_013657824.1	619	6.62	69.26	19	20	cTP (0.5232), mTP (0.0005), tlTP(0.0171), other (0.459)
	RSH1_f	106381614	XM_013821535.2	2640	C2	XP_013676989.1	879	6.60	98.44	23	24	cTP (0.2021), mTP (0.0011), tlTP(0.0043), other (0.7924)
	RSH2_a	106452255	XM_013894318.2	2055	A5	XP_013749772.2	684	6.67	77.13	5	6	cTP (0.2425), mTP (0.0001), tlTP(0.0058), other (0.7498)
	RSH2_b *	111206471	XM_022703426.1	2091	C5	XP_022559147.1	696	6.56	77.98	5	6	cTP (0.4948), mTP (0.0003), tlTP(0.0816), other (0.4229)
	RSH3_a *	106345829	XM_013785013.2	861	A6	XP_013640467.1	286	6.04	31.11	1	2	cTP (0.6255), mTP (0.0001), tlTP(0.0181), other (0.3557)
	RSH3_b	106431664	XM_013872470.2	2133	C6	XP_013727924.1	710	6.50	78.78	5	6	cTP (0.7621), mTP (0.0005), tlTP(0.0889), other (0.1473)
	RSH3_c	106348454	XM_022704818.1	2109	C6	XP_022560539.1	702	6.77	78.07	6	7	cTP (0.5288), mTP (0.0001), tlTP(0.0244), other (0.446)

Table 1. *Cont.*

Species	Genes	Gene ID	Transcript ID	CDS (bp)	Chromosome Location	Protein ID	AA	pI	Mw (kD)	Introns	Exons	Predicted Transfer Peptide (Probability)
	RSH3 _pseudo	106345828	-	-	A6	-	-	-	-	-	-	-
	CRSH *	106389210	XM_013829418.2	1743	C3	XP_013684872.1	580	6.03	65.83	3	4	cTP (0.2211), mTP (0.0648), tlTP(0.0095), other (0.7045)
	CRSH _pseudo	106439579	-	-	A3	-	-	-	-	-	-	-
B. oleracea	RSH1_a	106327624	XM_013765826.1	2628	C2	XP_013621280.1	875	6.52	97.87	23	24	cTP (0.1379), mTP (0.0016), tlTP(0.0142), other (0.8462)
	RSH1_b	106318815	XM_013756949.1	2652	C9	XP_013612403.1	883	6.64	98.55	23	24	cTP (0.3786), mTP (0.0002), tlTP(0.0059), other (0.6149)
	RSH2	106295267	XM_013731123.1	2091	C5	XP_013586577	696	6.56	77.98	5	6	cTP (0.4948), mTP (0.0003), tlTP(0.0816), other (0.4229)
	RSH3_a	106300657	XM_013736852.1	2109	C6	XP_013592306.1	702	6.77	78.07	5	6	cTP (0.5288), mTP (0.0001), tlTP(0.0244), other (0.446)
	RSH3_b	106300381	XM_013736509.1	2133	C6	XP_013591963.1	710	6.50	78.78	5	6	cTP (0.7621), mTP (0.0005), tlTP(0.0889), other (0.1473)
	CRSH	106334911	XM_013773298.1	1743	C3	XP_013628752.1	580	6.11	65.88	2	3	cTP (0.2263), mTP (0.0536), tlTP(0.0104), other (0.7096)
B. rapa	RSH1	103836764	XM_033278751.1	2685	A9	XP_033134642.1	894	6.38	100.02	23	24	cTP (0.5255), mTP (0.0005), tlTP(0.0172), other (0.4566)
	RSH2	103870072	XM_009148172.3	2064	A5	XP_009146420.1	687	6.67	77.3	5	6	cTP (0.2318), mTP (0.0001), tlTP(0.0048), other (0.7612)
	RSH3	103871068	XM_009149293.3	2091	A6	XP_009147541.1	696	6.30	77.87	5	6	cTP (0.6833), mTP (0.0001), tlTP(0.0135), other (0.3026)
	CRSH	103859710	XM_009137283.3	1731	A3	XP_009135531.1	576	5.99	65.47	2	3	cTP (0.3013), mTP (0.0173), tlTP(0.0221), other (0.6593)

Table 1. *Cont.*

Species	Genes	Gene ID	Transcript ID	CDS (bp)	Chromosome Location	Protein ID	AA	pI	Mw (kD)	Introns	Exons	Predicted Transfer Peptide (Probability)
C. sativa	RSH1_a	104747094	XM_010468670.2	2664	2	XP_010466972.1	887	6.66	98.83	24	25	cTP (0.5423), mTP (0.0003), tlTP(0.0096), other (0.4475)
	RSH1_b	104737555	XM_010457755.2	2655	13	XP_010456057.1	884	6.56	98.56	25	26	cTP (0.3518), mTP (0.0012), tlTP(0.0168), other (0.6298)
	RSH1 _pseudo	104707874	-	-	8	-	-	-	-	-	-	-
	RSH2_a	104778842	XM_010503267.2	2154	1	XP_010501569.1	717	6.57	79.77	6	7	cTP (0.4377), mTP (0.0002), tlTP(0.0256), other (0.5339)
	RSH2_b	104788263	XM_010513997.2	630	5	XP_010512299.1	209	7.72	23.53	3	4	cTP (0), mTP (0), tlTP(0), other (0.9999)
	RSH2_c	104745674	XM_010466982.2	2148	15	XP_010465284.1	715	6.42	79.56	6	7	cTP (0.3945), mTP (0.0001), tlTP(0.0761), other (0.5286)
	RSH3_a	104778355	XM_010502782.2	2151	3	XP_010501084.1	716	6.19	80.09	5	6	cTP (0.8885), mTP (0.002), tlTP(0.0436), other (0.0634)
	RSH3_b	104758764	XM_010481702.2	2151	17	XP_010480004.1	716	6.77	79.75	5	6	cTP (0.7837), mTP (0.0027), tlTP(0.0167), other (0.1939)
	RSH3 _pseudo 1	104742935	-	-	14	-	-	-	-	-	-	-
	RSH3 _pseudo 2	104761544	-	-	18	-	-	-	-	-	-	-
	CRSH_a	104782095	XM_010506922.2	1764	1	XP_010505224.1	587	6.20	66.97	3	4	cTP (0.2705), mTP (0.0903), tlTP(0.0037), other (0.6353)
	CRSH_b	104765592	XM_010489335.2	1758	19	XP_010487637.1	585	6.07	66.89	3	4	cTP (0.0869), mTP (0.1501), tlTP(0.0008), other (0.7621)

Table 1. *Cont.*

Species	Genes	Gene ID	Transcript ID	CDS (bp)	Chromosome Location	Protein ID	AA	pI	Mw (kD)	Introns	Exons	Predicted Transfer Peptide (Probability)
R. sativus	RSH1_a	108828360	XM_018602017.1	2640	unknown	XP_018457519.1	879	6.78	97.76	23	24	cTP (0.6503), mTP (0.0013), tlTP(0.0287), other (0.3196)
	RSH1_b	108843457	XM_018616659.1	2601	unknown	XP_018472161.1	866	6.96	97	23	24	cTP (0.1051), mTP (0.0009), tlTP(0.0005), other (0.8934)
	RSH1_c	108834481	XM_018607822.1	1290	unknown	XP_018463324.1	429	7.56	48.26	13	14	cTP (0.1051), mTP (0.0009), tlTP(0.0005), other (0.8934)
	RSH2	108863143	XM_018637469.1	2037	unknown	XP_018492971.1	678	6.55	76.31	5	6	cTP (0.2086), mTP (0), tlTP(0.0096), other (0.7815)
	RSH3	108862601	XM_018636787.1	2121	unknown	XP_018492289.1	706	6.44	78.36	6	7	cTP (0.6764), mTP (0.0001), tlTP(0.1784), other (0.1413)
	RSH3 _pseudo	108815328	-	-	unknown	-	-	-	-	-	-	-
	CRSH_a	108857634	XM_018631638.1	1749	unknown	XP_018487140.1	582	6.06	66.11	3	4	cTP (0.1098), mTP (0.0301), tlTP(0.0031), other (0.857)
	CRSH_b	108857621	XM_018631622.1	1749	unknown	XP_018487124.1	582	6.06	66.11	3	4	cTP (0.1098), mTP (0.0301), tlTP(0.0031), other (0.857)
	CRSH_c	108857284	XM_018631245.1	1749	unknown	XP_018486747.1	582	6.06	66.08	3	4	cTP (0.1098), mTP (0.0301), tlTP(0.0031), other (0.857)
E. coli	RelA	947244	-	2235	-	NP_417264.1	744	6.29	83.89	-	-	-
	SpoT	948159	-	2109	-	NP_418107.1	702	8.89	79.34	-	-	-
S. coelicolor	Rel	1096939	-	2544	-	WP_003977314.1	847	9.36	94.2	-	-	-

Gene ID, transcript ID, protein ID—accession numbers from NCBI GenBank, cTP—chloroplast transit peptide, mTP—mitochondrial transit peptide, tlTP—tonoplast transit peptide, other—most probable cytoplasmic protein.

B. napus RSH genes are distributed in 9 out of 19 chromosomes (Figure 1), but one of the *BnRSH1* genes has not yet been assigned to any chromosome. In *B. oleracea*, *RSH* genes are located on 5 out of 9 chromosomes, and in *B. rapa* the *RSH* genes are located on 4 out of 10 chromosomes. There are no differences between the number and the localization of *RSH* genes on chromosomes in *B. oleracea* and on C-genome chromosomes in *B. napus*. In the case of A-genome chromosomes, there are additional *RSH1* genes on chromosome A5 and A9 in comparison with the genome of. *B. rapa*. Moreover, the *CRSH* gene located on chromosome A3 is a pseudogene in *B. napus*. The presence of an *RSH3* pseudogene located on chromosome A6 could be caused by genome assembly errors since both genes lies in proximity and are separated by an unknown sequence.

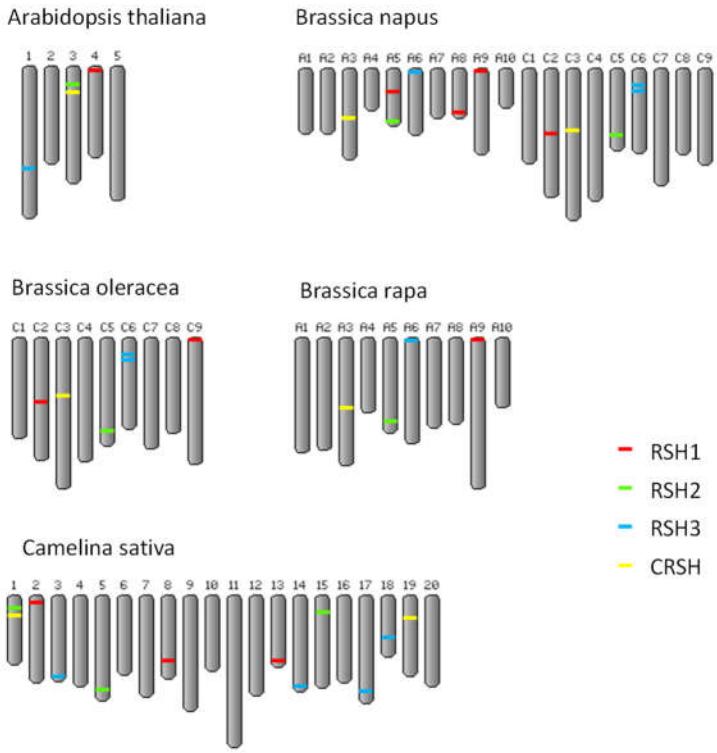

Figure 1. Chromosomal localization of *RSH* genes in *A. thaliana*, *B. napus*, *B. oleracea*, *B. rapa* and *C. sativa*.

Further in silico comparative analysis of the intron-exon organization of *RSH* genes in selected *Brassicaceae* species (Figure 2 and Supplementary Figure S1) showed that the number of exons and introns, and the location of introns in different types of *RSH* genes, was preserved in the plants analysed. Plant *RSH1* genes are characterized by very complex structures, with over 20 introns and exons in each analysed gene, except for *BnRSH1_e* and *RsRSH1_c* (Table 1). The high number of introns and exons is a common feature of *RSH1* genes from both mono- and di-cotyledonous plants [25] (data from the NCBI Gene Database). The average number of introns per gene in plants is about 4 [46,47], which raises a question about the possible role of such great complexity in the *RSH1* gene. It is widely accepted that introns fulfil different roles, i.e., introns may contain regulatory elements, they may serve as alternative promoters, or they may be a template for synthesis of non-coding regulatory RNAs [46]. Moreover, introns are crucial for alternative splicing and, in plants, intron retention is a widely observed phenomenon [48]. The presence of introns enhances the expression of genes in varied organisms [49]; however, interestingly, in plants in contrast to animals, higher expression is observed for genes containing more and longer introns [50]. The highly complex structure of *RSH1* genes in plants may suggest their high expression and important roles in many metabolic pathways. Other *RSH* genes

in plants are much more compact than *RSH1*, containing approximately 5 introns in *RSH2/3* genes, and 2–3 introns in *CRSH* genes (Table 1, Figure 2 and Supplementary Figure S1).

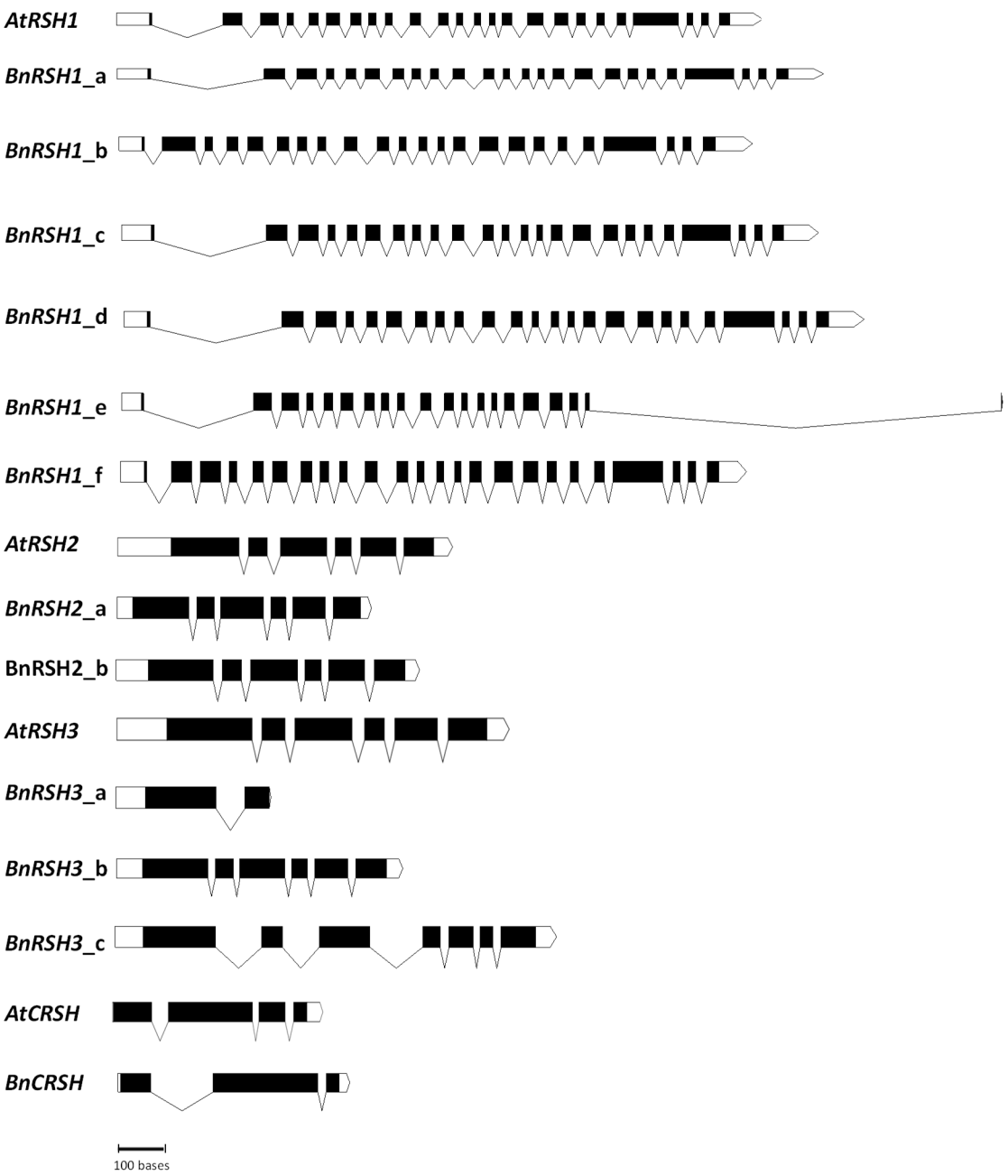

Figure 2. Intron-exon structure of *RSH* genes in *A. thaliana* and *B. napus*. White rectangles indicate UTRs, and black rectangles indicate coding sequence. Intron positions are marked by lines. The analysis was performed using the CIWOG tool.

2.1.2. Characteristic of Selected *Brassicaeae* RSH Proteins

In silico studies have shown that all analysed RSH proteins contain the (p)ppGpp hydrolase (HD) and (p)ppGpp synthetase (SYNTH) domains (Figures 3 and S2). RSH1 proteins also possess a TGS domain that is also present in bacterial stringent-response proteins. CRSH proteins contain an EF domain which is specific only for plant CRSH. On the other hand, bacterial RelA and SpoT proteins contain an ACT domain that is not present in any group of plant RSH proteins (Figure 3).

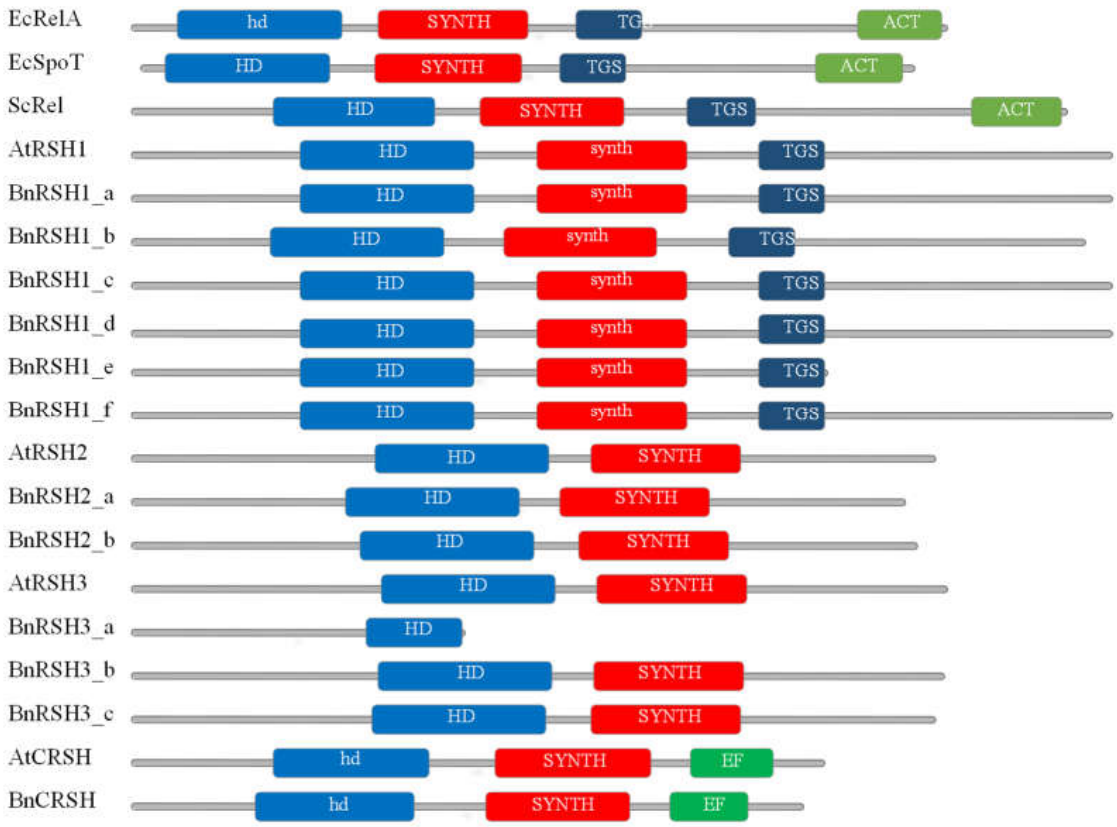

Figure 3. Predicted primary structures of RSH1, RSH2/RSH3, and CRSH proteins from *A. thaliana* and *B. napus*. HD (hd contains HD-SE substitution) (p)ppGpp hydrolase domain; SYNTH (synth contains G-S substitution) (p)ppGpp synthase domain; ACT aspartate kinase chorismate mutase TyrA domain; EF Ca^{2+}-binding domain; TGS: threonyl-tRNA synthetase, GTPase, SpoT domain.

The analysed plant RSH1 proteins contain a functional HD domain, i.e., proteins belonging to this group possess alarmone hydrolytic activity, whereas they do not have a functional (p)ppGpp synthesis domain due to the substitution of functional glycine with serine (Figures 4 and S3). The proteins belonging to RSH2/3 have both (p)ppGpp hydrolase and synthetase activity. CRSH has a functional SYNTH domain, but the hydrolytic domain has lost its activity because of the substitution, conserved in bacterial and plant proteins, of histidine (H) and aspartic acid (D) with serine and glutamic acid, respectively. The *E. coli* RelA protein is also characterized by the lack of a functional HD domain due to the substitution of His and Asp with phenylalanine and proline, respectively (Supplementary Figure S3). The catalytic activity of plant RSH proteins predicted by the in silico analysis of amino acid sequences could be confirmed by a complementation test in *E. coli relA*$^-$ and *relA*$^-$/*spoT*$^-$ mutants. It was shown that RSH1 proteins from *A. thaliana* and *I. nil* do not possess (p)ppGpp synthase activity, whereas AtRSH2, AtRSH3, and InRSH2, are able to synthesise and hydrolyse alarmones [25,26,41]. The (p)ppGpp synthesis activity was confirmed also for RSH2/3 from *Suaeda japonica* [45], and for *Nicotiana tabacum* RSH2 alarmone synthesis and hydrolysis activity was shown [44]. Interestingly, AtCSRH has only (p)ppGpp synthase activity, as expected based on amino acid sequence analysis [51], whereas InCRSH complements both mutations suggesting that this protein is able also to hydrolyse alarmones, despite the crucial His and Asp in HD domain in InCRSH being substituted with Arg and Gln [25].

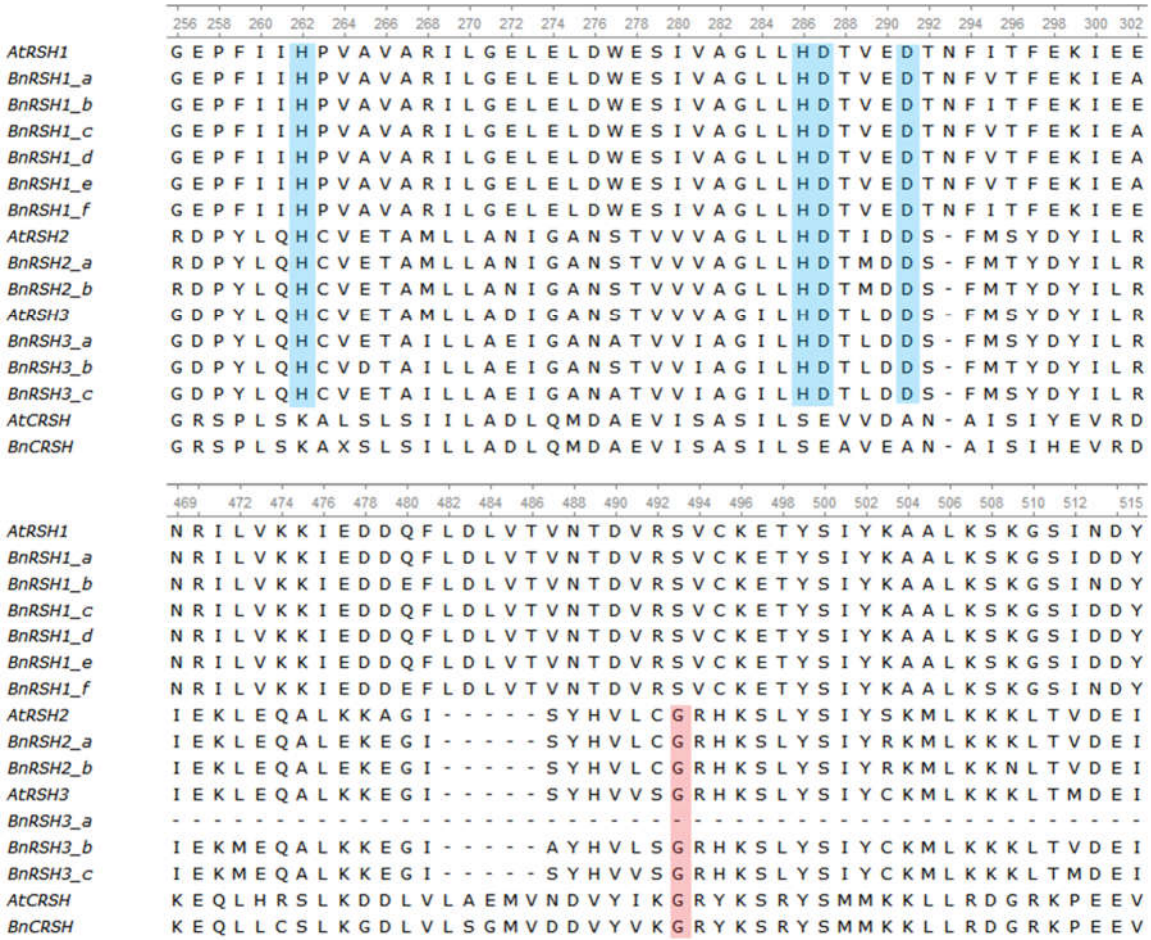

Figure 4. Amino acid alignments for the (p)ppGpp hydrolase HD (upper part) and synthetase SYNTH (lower part) domains of the RSH1, RSH2, RSH3, and CRSH proteins in *A. thaliana* and *B. napus*. In the HD domain the His (H) and Asp (D), important for its hydrolysis activity, are highlighted in blue. In the SYNTH domain the Gly (G), important for its synthetase activity, is highlighted in pink.

Plant RSH proteins such as bacterial Rel, RelA, and SpoT proteins belong to the so-called "long RSH" group. However, there are also "short RSH" proteins containing either a SYNTH domain (SAS) or an HD domain (SAH), without any regulatory domains. SAS and SAH are present in some bacteria together with long RSH. It was hypothesised that "short RSH" proteins allow different lineages of bacteria to expeditiously adapt to fluctuating environments, increasing their chance to survive harsh environmental conditions [36]. In metazoa, SpoT homolog 1 (Mesh) is a class of SAH and contains only (p)ppGpp hydrolytic domains [34]. However, in plants no representatives of "short" RSH proteins have been identified. In some plant species the degradation of the HD domain has been shown, however mostly in algae species [36]. Interestingly, one of the RSH3 proteins in *B. napus* (Figure 3) contains only an HD domain that is an unprecedented feature of plant RSH. However, the functionality of this truncated protein remains to be confirmed. The degradation of the HD or SYNTH domains in plant RSH proteins suggests subfunctionalization similar to that found in bacteria specialised RSHs which may be needed to strengthen the stringent response [24].

Although plant RSHs are nuclear-encoded proteins they contain chloroplast transit peptides at their N-terminus [31]. In silico analysis of putative amino acid sequences of RSH proteins from the *Brassicaceae* family also showed that the chloroplast is the most probable subcellular localisation (Table 1). Interestingly, in the case of CRSH, the presence of a chloroplast signal peptide is less probable than for other types of RSH protein. In fact, the chloroplast localization has been shown for many of these proteins belonging to all types of

plant RSH groups [26,31,44,51–53]. There is a paucity of reports of the direct measurement of (p)ppGpp in whole plants, and in particular, in isolated chloroplasts. Takahashi et al. [54] showed that the level of ppGpp in pea chloroplasts is 13 times higher than in shoots, which confirmed, that the majority of alarmones in plants are localized in chloroplasts. Later reports have determined the level of (p)ppGpp only in whole plants [41,56,57].

The phylogenetic analysis of RSH proteins from selected *Brassicaceae* species (Figure 5) showed the presence of three separate RSH groups. RSH2 and RSH3 could be distinguished but, due to sequence similarity, they are grouped on one branch of the phylogenetic tree. In *A. thaliana*, true *RSH3* homologs are missing since AtRSH2 and AtRSH3 are the result of recent duplication of the ancestral *RSH2* gene with a 75% amino acid sequence similarity [36]. True RSH3 homologs are, however, present in other plants. Interestingly, the amino acid sequence similarities between RSH2 and RSH3 in other plant species analysed in this study are very high (ranging from 74% to even 80%), which may suggest that, similar to *A. thaliana*, a true RSH3 homolog is also missing from other plants belonging to the *Brassicaceae* family.

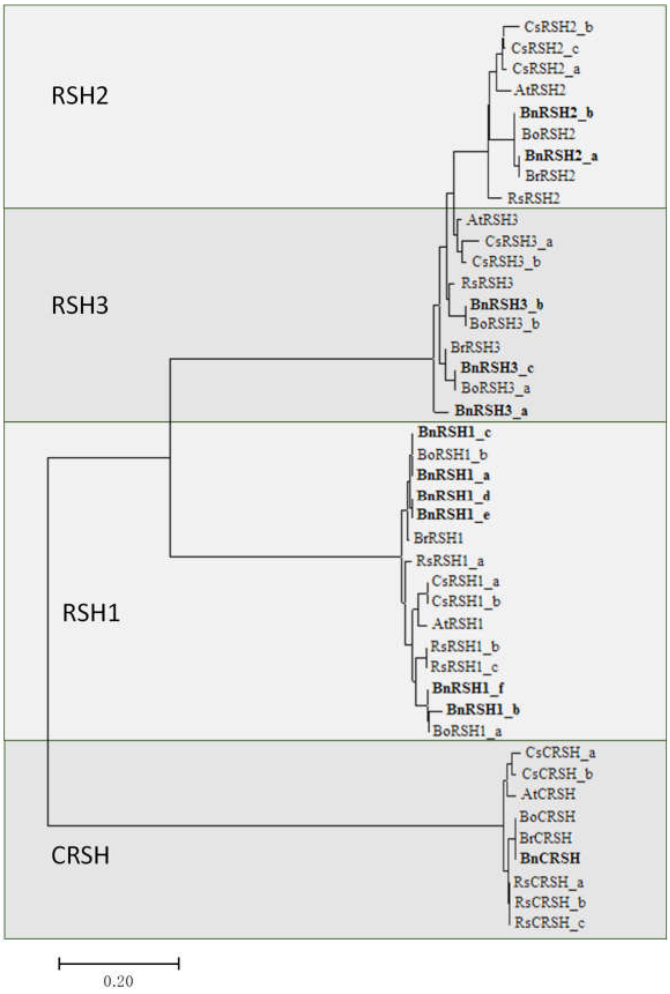

Figure 5. The phylogenetic analysis of RSH proteins based on predicted amino acid sequences given in Table 1. The evolutionary history was inferred using the Neighbor–Joining method by MEGA7.0 software. The optimal tree, with the sum of branch length = 3.01054035, is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson-correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. *B. napus* RSH sequences are indicated in bold.

2.2. Regulatory Elements Present in Promoter Regions of B. napus RSH Genes

The expression of plant *RSH* genes is tissue/organ-dependent; it depends on the stage of development as well as on the type of the *RSH* gene. It is generally thought, that (p)ppGpp affects gene expression in chloroplasts at transcriptional, translational, and post-translational level, and thus alarmones regulate plant growth and development, and response to stress stimuli [55]. In fact, the expression of *RSH* genes, and thus the level of alarmones, is up-regulated by different factors, including abscisic acid [53,56], salt stress [5,25,26,59], oxidative stress [57], drought [25], and the presence of plant growth promoting bacteria [5]. Interestingly, it was also shown that the overaccumulation of (p)ppGpp in plants has some negative effects. For instance, *Arabidopsis* plants overexpressing *RSH2* and *RSH3* were smaller, contained less chlorophyll, and their seeds had lower vigour [41]. The increased level of (p)ppGpp in *Arabidopsis* led to dwarf chloroplasts, and reduction of metabolites, however, the mutant plants were more tolerant to nutrient-deficient conditions than wild-type plants [52]. Moreover, the increased level of alarmones increased the susceptibility of plants to turnip mosaic virus, whereas for plants with a decreased level of (p)ppGpp, reduced susceptibility was observed [58]. These results clearly show that the level of (p)ppGpp is tightly controlled, since alarmones are critical not only for plastid development and metabolism, but also for the fine-tuning of plant growth and development.

Promoters are responsible for controlling the efficiency, timing, and location of gene expression via clusters of short sequences, including *cis*-regulatory elements (CREs). CREs provide binding sites for transcription factors [37,62,63] and their presence may reflect multiple pathways of gene expression regulation. In order to gain some insight into the putative roles of *BnRSH*, in silico analysis of promoter regions, using the PlantCare database, was performed. This kind of bioinformatical analysis provides a background for further research [59–62]. A promoter analysis of the *BnRSH* genes revealed the presence of several putative *cis*-acting elements involved in light signalling, in plant development, in response to plant hormones, as well as in plant response to abiotic and biotic stress (Supplementary Table S1). The most abundant elements in all *BnRSH* genes were those related to the abiotic stress response, followed by light- and hormone-responsive elements. Only 1% of all identified CREs in *BnRSH* genes were related to the biotic stress response, and this kind of element was not identified in *BnRSH2/3* genes (Figure 6 and Supplementary Table S2). The highest number of elements was identified in the *BnRSH3_b* gene (69), and the lowest in the *BnRSH1_b* gene (25) (Supplementary Table S1 and Supplementary Figure S4). The most abundant of the abiotic stress response elements were the drought and ABA response element MYB (41), followed by MYC (27), which is a drought, ABA, and cold response element, and the general stress-response element, STRE (22). Among hormone-responsive elements the ethylene response element was the most frequently occurring (36) (Supplementary Table S1). The frequencies of the types of CRE in *BnCRSH* genes were different from the frequencies observed in *BnRSH1–3* genes. In the promoter region of *BnCRSH*, the most abundant elements were those related to response to light, followed by hormone responsive elements. Only 9% of CREs were abiotic stress response elements (Figure 6). This observation may imply that CRSH plays a significantly different physiological role than RSH1–3. In fact, the expression of *CRSH* was not changed by salt stress, osmotic stress, or drought in *I. nil* [25]. The expression of *AtCRSH* was also stable in response to wounding and NaCl, however, it was also not changed by hormones, even ABA [26], and the ABA-response element is the most abundant among hormone responsive elements in the *BnCRSH* gene promoter (Supplementary Table S1). Interestingly, the circadian rhythm of *AtCRSH* expression is also different to that of *AtRSH1–3*, i.e., the expression peak of *AtCRSH* is during darkness whereas *AtRSH1–3* genes are mostly expressed in the light [26].

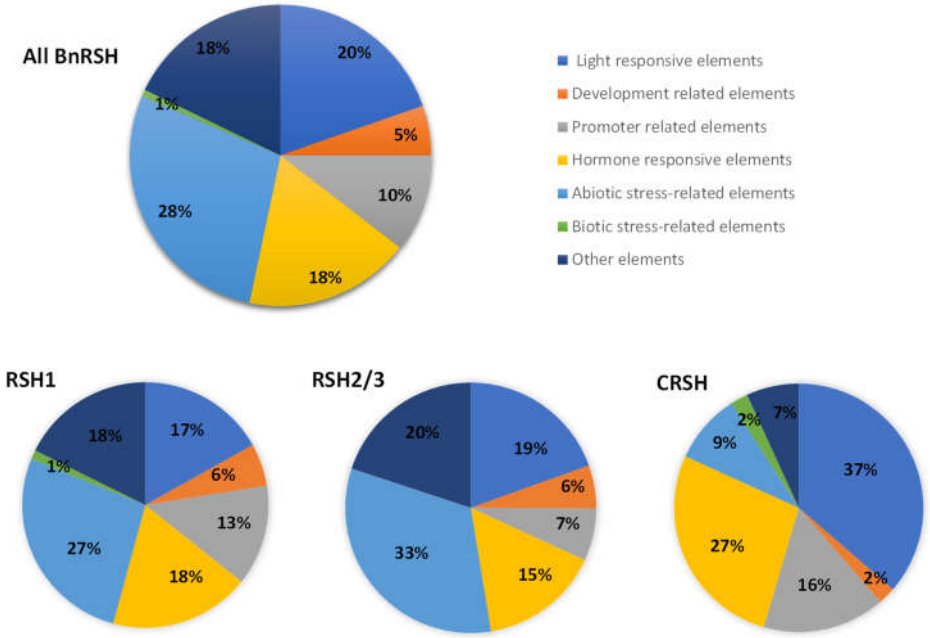

Figure 6. Frequencies of putative *cis*-regulatory elements in *B. napus RSH* genes (upper pie chart), and in *BnRSH1*, *BnRSH2/3*, and *CRSH* genes (lower pie charts). Pie charts depict the *cis*-regulatory elements categorized in seven types according to their predicted functions.

The presence of multiple putative regulatory elements involved in the light response in promoter regions of *BnRSH* genes suggests that the potential roles of corresponding proteins may not be restricted to the stress response but are also important for plant growth and developmental programs. Additionally, promoters of *BnRSH1_a*, *BnRSH1_c*, *BnRSH1_d*, *BnRSH1_e*, and *BnCRSH* genes contain motifs involved in the control of the circadian cycle (Supplementary Table S1). It was shown that the mRNA level of *RSH* genes and alarmone levels are light dependent. The expression of all *RSH* genes in *Arabidopsis* fluctuated during the diurnal time course [26]. Takahashi et al. [54] showed that prolonged darkness (12 h) reduced ppGpp levels, whereas abrupt changes to *Pisum sativum* plants, from prolonged light (12 h) to dark, caused a substantial elevation in ppGpp levels. Similarly, alarmone concentration altered in 12-h light/12-h dark cycling conditions, with increasing alarmone levels at the beginning, and its highest peak during the dark time period [63]. The functionality of the identified potential *cis*-elements needs to be further confirmed.

2.3. Effect of Salinity and Rhizobacteria on the Expression of BnRSH Genes

Soil salinity stress mitigates crop productivity and is an important challenge for global sustainable agriculture [64]. It affects several aspects of plant metabolism leading to significant decreases in plant growth and yield [6]. *B. napus* is considered one of the most saline-resistant species in the genus *Brassica*, being more tolerant not only than its diploid ancestors, but also than other polyploid species [65]. Salinity had a visible impact on *B. napus* seed germination (Supplementary Figure S5) and the growth of 6-day-old rapeseed seedlings (Supplementary Figure S6). The germination ratio was visibly decreased even in 50 mM NaCl whereas in the presence of 200 mM NaCl less than half of the seeds germinated in comparison to the control (seeds germinated in water). The length of root and hypocotyl, as well as the fresh and dry biomass of *B. napus* seedlings, significantly decreased in the presence of salt (Supplementary Figure S6) and the most affected by NaCl was hypocotyl growth (Supplementary Table S3).

The potential involvement of *RSH* genes and alarmones in the plant response to salt stress has been shown previously [26,43,44,60]. In order to gain more insight into the possible physiological roles of *BnRSHs*, the expression of four selected *B. napus RSH* genes (*RSH1_b*, *RSH2_b*, *RSH3_a*, *CRSH*) was analysed using sqRT-PCR in seedling organs

(Figure 7) in response to salt stress, and in response to the presence of PGPR bacteria (Figure 8). *BnRSH* genes were differentially expressed in cotyledons and roots, i.e., *BnRSH1* and *BnRSH2* genes were highly expressed, while *BnRSH3* and *BnCRSH* mRNAs were expressed at a lower level in both organs.

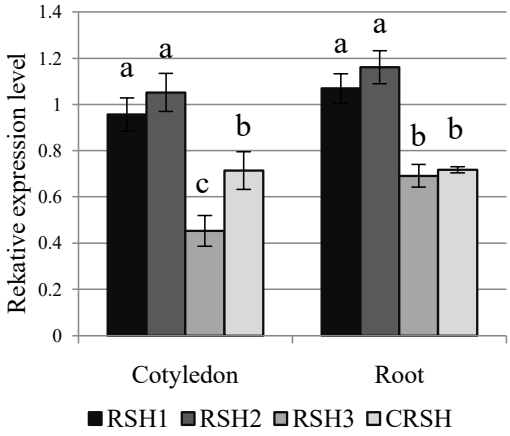

Figure 7. Expression analysis of *BnRSH* in the *B. napus* cotyledons and roots of 6-day-old seedlings. Chart shows the relative transcript level of analysed genes (*BnRSH1_b*, *BnRSH2_b*, *BnRSH3_a*, and *BnCRSH*) with respect to the expression of the reference gene (*BnAc*). Different letters indicate statistically significant changes according to one-way ANOVA test at $p < 0.05$. Bars represents means ± SD.

Using histochemical staining of GUS activity, it was shown that in *Arabidopsis*, *AtRSH1* and *AtRSH3* were highly expressed in hypocotyls and leaves, whereas *AtRSH2* and *AtCRSH* were expressed in leaves. In the roots of seedlings only *AtRSH2* was expressed, whereas in the roots of mature plants, *AtRSH3* was also expressed [26]. Using RT-PCR, high expression of *AtRSH1* and *AtRSH3*, and low expression of *AtRSH2* in shoots, were also shown. In the roots, *AtRSH2* and *AtRSH3* were highly expressed, whereas *AtRSH1* was expressed at a low level. *AtCRSH* was not tested in this study [66]. In rice, *OsCRSH* was expressed both in roots and shoots, however, in roots at a lower level than in shoots [31]. In contrast, in the cotyledons of *I. nil* seedlings, *RSH1*, *RSH2*, and *CRSH* were equally highly expressed, whereas in roots, *RSH2* was highly expressed, *RSH1* was expressed at the low level, and no expression of *CRSH* was detected [25].

In general, salinity stress had no significant effect on the expression of *BnRSH* genes in cotyledons and roots (Figure 8). The levels of *BnRSH2* and *BnCRSH* transcripts in cotyledons, and the levels of *BnRSH1* and *BnCRSH* in roots, slightly increased under salinity stress as compared with control plants, and the differences were statistically significant (Supplementary Tables S5, S6, S10 and S11). Interestingly, previous studies showed that *A. thaliana* treated with 250 mM NaCl exhibited increased *AtRSH2* expression, but that salt had no impact on the expression of *AtRSH1*, *AtRSH3*, and *AtCRSH* [26], whereas, in another study, treatment with 250 mM NaCl significantly increased both *AtRSH2* and *AtRSH3* transcript levels, decreased the amount of *AtCRSH* mRNA, and had no impact on *AtRSH1* expression [57]. Similarly, Prusińska et al. [25] showed that salt stress (300 mM NaCl) elevated the *InRSH2* transcript level, whereas both *InRSH1* and *InCRSH* did not show substantial changes in 5-day-old *I. nil* seedlings. Although, in promoters of *BnRSH* genes, several putative regulatory *cis*-elements involved in response to varied abiotic stresses, possibly including salinity stress, have been identified (Supplementary Table S1), the stable expression of *BnRSHs* in response to salt has been observed. This may be due to the concentrations of NaCl used in this study. Using an NaCl solution, up to 200 mM mimics non saline, slightly saline, and medium saline soils, whereas a concentration above 250 mM is typical for highly saline soils [67]. Moreover, the observed, almost changeless expression of *BnRSH* genes in response to NaCl, and the differences in expression of *RSH*

genes response to salinity among plants, might be caused by the different developmental stages of the analysed plants, and/or varied sampling time points.

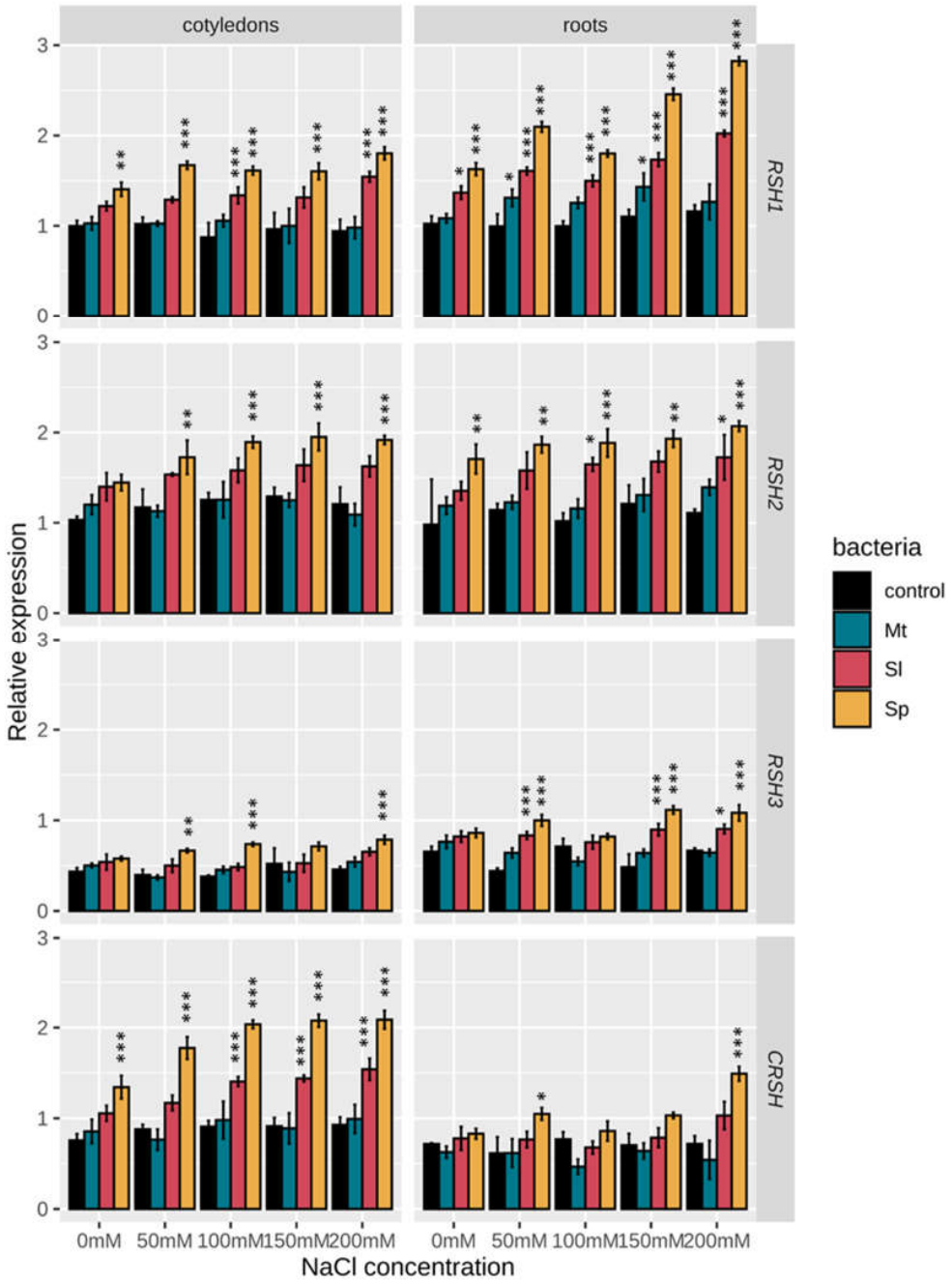

Figure 8. Expression analysis of *BnRSHs* in salt stress and in the presence of PGPR bacteria using sqRT-PCR. Charts show the relative transcript level of *BnRSH1_b*, *BnRSH2_b*, *BnRSH3_a*, and *BnCRSH* genes with respect to the expression of a reference gene (*BnAc*). Bars represent means ± SD. Control (black bars) are plants grown in in different NaCl concentrations (0 mM, 50 mM, 100 mM, 150 mM, and 200 mM NaCl) but without inoculation with bacteria. Mt (green bars)—*M. timonae*, Sl (red bars)—*S. liquefaciens*, Sp (yellow bars)—*S. plymuthica*. Statistical analysis was performed using two-way ANOVA followed by Scheffe post-hoc test. Asterisks indicate statistically significant differences in comparison to the control (i.e., expression of a particular *RSH* gene in plants grown in the same concentration of salt but without bacteria—black bars) at *p*-value < 0.001 (***), *p*-value < 0.01 (**), and *p*-value < 0.05 (*). Full statistical analysis data are available as Supplementary Materials (Supplementary Tables S4–S11).

The effects of the rhizobacteria, *S. plymuthica*, *S. liquefaciens*, and *M. timonae*, on the expression of *BnRSHs* in leaves and roots was investigated (Figure 8). Using plant growth-promoting bacteria to improve plant tolerance to environmental stresses, including salt stress, in order to obtained a high yield even in adverse environmental conditions, is considered an economically and environmentally friendly approach [9]. Earlier reports have shown that PGPR bacteria mitigate salt stress via varied mechanisms including the production of indole acetic acid (IAA) [6], induction of potassium and calcium accumulation in plants, increased content of osmolytes including proline [68], and activation of plant antioxidant enzymes [69]. Using two-way ANOVA, significant interactions between salt concentration and species of bacteria for all analysed genes, besides *BnRSH2* in roots, has been found (Supplementary Tables S4–S11). Therefore, we examined the bacteria simple main effect, i.e., the differences between the expression of *BnRSHs* in plants inoculated with different bacteria, for each salt concentration. Among all analysed bacteria *S. plymuthica* had the greatest impact on the expression of all *BnRSH* genes in all tested salt concentrations, both in cotyledons and roots. The expression of *BnRSH1* was upregulated by *S. plymuthica* and *S. liquefaciens* in both cotyledons and roots, whereas *M. timonae* increased the expression of *BnRSH1* in roots only (Figure 8). *S. plymuthica* increased the expression of *BnRSH2* and *BnRSH3* in cotyledons and roots, while *S. liquefaciens* increased the expression of *BnRSH2* in roots only. The expression of *BnCRSH* in roots is mostly unaffected by PGPR bacteria, whereas *S. plymuthica* and *S. liquefaciens* induced the expression of *BnCRSH* in cotyledons (Figure 8). In response to salt stress, *BnRSH* gene expression is elevated in *S. plymuthica* and *S. liquefaciens* inoculated plants, whereas *M. timonae* inoculated plants did not show substantial changes as compared with control plants (without bacteria but treated with NaCl at the same concentration). For all *BnRSH* genes the highest level of expression was observed in plants inoculated with *S. plymuthica* (Figure 8). There is little data in the literature about the possible relation between (p)ppGpp and PGPR bacteria. Szymańska et al. [5] showed changes in the expression of *BnRSH1* and *BnRSH3* in roots of oilseed rape growing in the presence of the halotolerant PGPR bacterium *Pseudomonas stutzeri* ISE12 under salt stress. Increased expression of plant *RSH* genes was also demonstrated in response to pathogen attack. It was found that the infection of tobacco plants with the bacterial *Erwinia carotovora* pathogen leads to a 10-fold increase in the *NtRSH2* protein level [44].

S. plymuthica used in this study is characterized by high metabolic activity; it is able to biodegrade plastic in compost and agricultural soil and stimulate the growth of *B. napus*, *Miscanthus x giganteus*, and *Salix viminalis* [70,71]. It was shown that several salt-tolerant strains of *S. plymuthica* improved cucumber biomass and yield via synthesis of IAA [72]. *S. liquefaciens* improved salt stress tolerance and plant growth in maize and rape [6]. *M. timonae* colonizes the rhizosphere, roots and leaves, and is a growth promoter via the production of IAA and siderophores in various plant species [73]. Our research clearly showed changes in mRNA levels of *BnRSHs* grown in the presence of the strains *S. liquefaciens* and *S. plymuthica*, but not in the presence of *M. timonae* which suggests that some PGPR bacteria might also improve plant growth under salt stress via the stringent response pathway.

3. Materials and Methods

3.1. In Silico Analysis of B. napus, B. olearacea, B. rapa, C. sativa, and R. sativus RSH Genes and Proteins

The *RSH1*, *RSH2*, *RSH3,* and *CRSH* in the plant genomes selected for this study from *Brassicaceae* family genes have been identified using *A. thaliana RSH* cDNA sequences (*AtRSH1*, *AtRSH2*, *AtRSH3*, and *AtCRSH*) as queries. A search was performed using BLASTN (Basic Local Alignment Search Tool) using the NCBI (ncbi.nlm.nih.gov, accessed on 10 May 2021) nucleotide database. The analysis of the intron-exon organisation was carried out using the CIWOG tool (http://peroxibase.toulouse.inra.fr/tools/ciwog_search_form, accessed on 15 May 2021) [74]. The putative amino acid sequences were then obtained from the NCBI protein database. For primary and secondary structure predictions of RSH

proteins InterProScan (https://www.ebi.ac.uk/interpro/search/sequence/, accessed on 23 May 2021), Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 23 May 2021), and PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/, accessed on 24 May 2021) were utilized. Clustal Omega was used for multiple sequence alignments (http://www.clustal.org/omega/, accessed on 27 May 2021) [75]. For calculation of molecular mass and pI of putative RSH proteins the Compute pI/Mw tool (https://web.expasy.org/compute_pi/, accessed on 13 June 2021) was utilised. TargetP (http://www.cbs.dtu.dk/services/TargetP/, accessed on 14 June 2021) [76,77] was used to predict subcellular localization of analysed RSH proteins. The phylogenetic analysis was caried out in MEGA7 software [78,79] using the neighbour-joining method [80].

The promoter regions of *BnRSH* genes were analysed using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 10 May 2021) [59]. For each *BnRSH* gene a 1500-bp long fragment including promoter and 5′UTR of genomic DNA was retrieved from the NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/, accessed on 5 May 2021).

3.2. Bacterial Strains

Three bacterial strains: *Massilia timonae* [81], *Serratia liquefaciens* [82], and *Serratia plymuthica* [71,83], obtained from the collection of Professor Katarzyna Hrynkiewicz from the Department of Microbiology at the Nicolaus Copernicus University in Toruń, were used in the experiments. Bacteria were grown in R2A (Difco, Franklin Lakes, NJ, USA) liquid medium (18 g/L) at 24 °C for 24 h. The optical density of bacterial culture was checked spectrophotometrically at λ = 600 nm (SmartSpec Plus, BioRad, Hercules, CA, USA) and adjusted to the value of 5×10^6 c.f.u./cm^3 [8].

3.3. Plant Material

Seeds of the *B. napus* L. winter cultivar 'Harry' (Obrol Company, Kruszewnia, Poland) were surface sterilized with a mixture of 30% hydrogen peroxide and 96% ethanol (1:1, *v*/*v*) for 3 min and rinsed at least six times with sterile distilled water. The seeds were inoculated with a bacterial suspension, prepared as described above, and incubated for 10 min, with shaking, at room temperature. Non-inoculated (control) and inoculated seeds were placed in Petri dishes on filter paper moistened with 5 mL of sterile water (control) and 50, 100, 150, and 200 mM NaCl.

To analyse the impact of NaCl on *B. napus* seed germination and seedling growth, seeds were incubated in 16 h darkness/8 h light photoperiod at 24 °C for 6 days. The number of germinated seeds was checked after 14 h, 17 h, 20 h, 24 h, and 48 h of the start of experiment. The length of the hypocotyl and roots of 6-day-old seedlings were measured. Moreover, the fresh mass of 10 6-day-old seedlings was determined, and after drying (80 °C for 24 h) the dry mass of 10 seedlings was determined.

For *BnRSH* gene expression analysis, seeds were incubated for a 16 h darkness/8 h light photoperiod at 24 °C for 6 days. Cotyledons and roots of 6-day-old seedlings were frozen in liquid nitrogen and stored at −80 °C until RNA isolation was performed. The experiments were performed in triplicates.

3.4. Expression Analysis of BnRSH Genes

Total RNA was extracted from the *B. napus* organs using TRI Reagent (Sigma-Aldrich, Poznań, Poland), according to the manufacturer's protocol. RNA was analysed by spectrophotometric measurement and gel electrophoresis in 1% agarose gel in 1x TAE (Tris-Acetate-EDTA) buffer stained with ethidium bromide. Prior cDNA synthesis from 1 µg of RNA genomic DNA was removed using RNAse free DNase I (Thermo Fisher Scientific, Waltham, MA, USA). Further oligo(dT)$_{18}$ primer and RevertAid reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) were used for cDNA synthesis, in accordance with the protocol described in [84].

Semi-quantitative RT-PCR (sqRT-PCR) assays were performed to evaluate the effects of NaCl and/or the presence of PGPR on mRNA level of *RSH* genes. For each pair of primers, the PCR conditions, including the concentration of primers, DNA polymerase, and Mg^{2+}, annealing temperature, and the number of cycles, were optimised according to [85]. The relative expression level of *BnRSH1*, *BnRSH2*, *BnRSH3*, and *CRSH* genes, was expressed as a ratio of the amount of PCR product for analysed gene to the amount of PCR product for the reference gene. *B. napus* actin-7 (*BnAc*, NCBI GenBank accession no. XM_013858992.2) was used as a reference gene. The PCR reaction mixture contained: 1.25 U of Opti*Taq* DNA polymerase (EURx, Gdańsk, Poland), 1.5 µL of cDNA as the template, 0.15 µM of each primer, and 1.5 mM $MgCl_2$, in a total reaction volume of 20 µL. Primers are listed in Table 2. The thermal cycling conditions were as follows: 95 °C for 30 s, 54 °C (*BnRSH2*, *BnRSH3*), 52 °C (*BnCRSH*), or 58 °C (*BnRSH1*) for 40 s, and 72 °C for 40 s for 26 cycles (*BnAc*), 39xcycles (*BnRSH1*), 33 cycles (*BnRSH2*, *BnRSH3*), and 37 cycles (*BnCRSH*). Products of sqRT-PCR were separated on a 1.5% agarose gel with EtBr in TAE buffer and quantified by intensity using the ImageGauge 3.46. software (FujiFilm, Tokyo, Japan). Each reaction was repeated three times.

Table 2. Sequences of primers used for expression analysis of *B. napus RSH* genes.

Primer Name	Sequence of Primers 5′–3′	Analysed Gene and Amplicon Length [bp]
BnRSH1_f	GGAGGTTCAGATCAGAACGG	*BnRSH1*
BnRSH1_r	CCATTCACCTTCGCTGCTAC	396
BnRSH2_f	GCAAGATGTTGAAGAATCTAACG	*BnRSH2*
BnRSH2_r	GCACAGACATCTTGTCATTTTCG	534
BnRSH3_f	CCGAAACTTTCCGATTTCAA	*BnRSH3*
BnRSH3_r	TCGTAGTCAACGCACGAGTC	524
BnCRSH_f	AAGTGATGGAGGAGCTTGGA	*BnCRSH*
BnCRSH_r	CCATTTACTGGAACGCAACA	263
BnAc_f	CTCACGCTATCCTCCGTCTC	*BnAc*
BnAc_r	TTGATCTTCATGCTGCTTGG	469

3.5. Statistical Analysis

Statistical differences of *BnRSH* gene expression data were assessed using one-way ANOVA followed by Tukey's honest significance test (for comparison of *BnRSHs* expression in cotyledons and roots) or two-way ANOVA test followed by Scheffe post-hoc test (for comparison of *BnRSHs* expression in response to salt, and the presence of PGPR bacteria). Results are means ± SD. For one way ANOVA, a p-value < 0.05 was considered statistically significant. For two-way ANOVA, p-values < 0.05 (*), < 0.01 (**), and < 0.001 (***), were considered statistically significant. Statistical analyses were performed using R version 4.1.1 and packages DescTools and ggplot2 (r-project.org, accessed on 15 September 2021).

4. Conclusions

Our results suggest that in plants belonging to the *Brassicaceae* family the stringent response is coordinated by numerous isoforms of RSH proteins. There is a high level of conservancy between the respective orthologs of *RSH* genes and proteins analysed in the study plant species. Plants possess higher number of genes encoding synthetases and/or hydrolases of alarmones than bacteria, which is especially apparent for polyploid plants, e.g., *B. napus*. The presence of multiple isoforms that underwent subfunctionalization highlights the need of rigorous control of (p)ppGpp-dependent pathways in plants. The mechanisms of the plant stringent response are beginning to emerge, but the specific roles of RSH isoforms are still puzzling. An in silico promoter analysis of *BnRSH* genes revealed the presence of several putative regulatory elements, and indicated that, (i) *RSH* gene expression might be regulated by multiple abiotic and biotic factors, (ii) *RSH* proteins

might be involved in varied metabolic pathways, (iii) the possible roles of *RSH1*, *RSH2/3*, and *CRSH*, seems to be diversified. The wet-lab expression analysis of selected *B. napus RSH* genes in response to salt stress supported the idea of different physiological roles of plant RSH isoforms. Moreover, we showed that the plant stringent response might be one of the pathways via which PGPR bacteria promote plant growth and development; however this seems to be bacteria species-dependent.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms221910666/s1.

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References

1. Gupta, S.K. *Breeding Oilseed Crops for Sustainable Production: Opportunities and Constraints*; Elsevier Inc.: Amsterdam, The Netherlands, 2015; ISBN 9780128014691.
2. Warner, D.J.; Lewis, K.A. Evaluation of the risks of contaminating low erucic acid rapeseed with high erucic rapeseed and identification of mitigation strategies. *Agriculture* **2019**, *9*, 190. [CrossRef]
3. Wu, D.; Liang, Z.; Yan, T.; Xu, Y.; Xuan, L.; Tang, J.; Zhou, G.; Lohwasser, U.; Hua, S.; Wang, H.; et al. Whole-genome resequencing of a worldwide collection of rapeseed accessions reveals the genetic basis of ecotype divergence. *Mol. Plant* **2019**, *12*, 30–43. [CrossRef] [PubMed]
4. Chalhoub, B.; Denoeud, F.; Liu, S.; Parkin, I.A.P.; Tang, H.; Wang, X.; Chiquet, J.; Belcram, H.; Tong, C.; Samans, B.; et al. Early allopolyploid evolution in the post-neolithic *Brassica napus* oilseed genome. *Science* **2014**, *345*, 950–953. [CrossRef] [PubMed]
5. Szymańska, S.; Dąbrowska, G.B.; Tyburski, J.; Niedojadło, K.; Piernik, A.; Hrynkiewicz, K. Boosting the *Brassica napus* L. tolerance to salinity by the halotolerant strain *Pseudomonas stutzeri* ISE12. *Environ. Exp. Bot.* **2019**, *163*, 55–68. [CrossRef]
6. El-Esawi, M.A.; Alaraidh, I.A.; Alsahli, A.A.; Alzahrani, S.M.; Ali, H.M.; Alayafi, A.A.; Ahmad, M. *Serratia liquefaciens* KM4 improves salt stress tolerance in maize by regulating redox potential, ion homeostasis, leaf gas exchange and stress-related gene expression. *Int. J. Mol. Sci.* **2018**, *19*, 3310. [CrossRef]
7. Isayenkov, S.V.; Maathuis, F.J.M. Plant salinity stress: Many unanswered questions remain. *Front. Plant Sci.* **2019**, *10*, 80. [CrossRef]
8. Dąbrowska, G.B.; Zdziechowska, E.; Hrynkiewicz, K. Evaluation of potential suitability of *Rhizobacteria* for phytodesalination of soils. *Environ. Pollut. Control* **2016**, *38*, 9–14.
9. Shilev, S. Plant-growth-promoting bacteria mitigating soil salinity stress in plants. *Appl. Sci.* **2020**, *10*, 7326. [CrossRef]
10. Bal, H.B.; Nayak, L.; Das, S.; Adhya, T.K. Isolation of ACC deaminase producing PGPR from rice rhizosphere and evaluating their plant growth promoting activity under salt stress. *Plant Soil* **2013**, *366*, 93–105. [CrossRef]
11. Kohler, J.; Hernández, J.A.; Caravaca, F.; Roldán, A. Induction of antioxidant enzymes is involved in the greater effectiveness of a PGPR versus AM fungi with respect to increasing the tolerance of lettuce to severe salt stress. *Environ. Exp. Bot.* **2009**, *65*, 245–252. [CrossRef]
12. Cordero, I.; Balaguer, L.; Rincón, A.; Pueyo, J.J. Inoculation of tomato plants with selected PGPR represents a feasible alternative to chemical fertilization under salt stress. *J. Plant Nutr. Soil Sci.* **2018**, *181*, 694–703. [CrossRef]
13. Cashel, M.; Gallant, J. Two compounds implicated in the function of the RC gene of *Escherichia coli*. *Nature* **1969**, *224*, 177–178. [CrossRef]
14. Cashel, M.; Gentry, D.; VJ, H.; Vinella, D. The stringent response in: *Escherichia coli* and *Salmonella typhimurium*. *Cell. Mol. Biol.* **1996**, *2*, 1458–1496.

15. Dabrowska, G.; Prusinska, J.; Goc, A. The stringent response-the mechanism of bacterial adaptive response to stress conditions. *Adv. Biochem.* **2006**, *52*, 87–93.
16. Xiao, H.; Kalman, M.; Ikehara, K.; Zemel, S.; Glaser, G.; Cashel, M. Residual guanosine 3′,5′-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. *J. Biol. Chem.* **1991**, *266*, 5980–5990. [CrossRef]
17. Berdychowska, J.; Boniecka, J.; Dąbrowska, G.B. The stringent response and its involvement in the actions of bacterial cells to stress. *Adv. Microbiol.* **2019**, *58*, 127–142. [CrossRef]
18. Irving, S.E.; Choudhury, N.R.; Corrigan, R.M. The stringent response and physiological roles of (pp)pGpp in bacteria. *Nat. Rev. Microbiol.* **2020**, *19*, 256–271. [CrossRef]
19. Mittenhuber, G. Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). *J. Mol. Microbiol. Biotechnol.* **2001**, *3*, 585–600.
20. Heizmann, P.; Howell, S.H. Synthesis of ppGpp and chloroplast ribosomal RNA in *Chlamydomonas reinhardi*. *Biochim. Biophys. Acta Nucleic Acids Protein Synth.* **1978**, *517*, 115–124. [CrossRef]
21. Van der Biezen, E.A. Arabidopsis RelA/SpoT homologs implicate (p)ppGpp in plant signaling. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 3747–3752. [CrossRef]
22. Dąbrowska, G.; Prusińska, J.; Goc, A. Identification of *RSH* gene cDNA (*RelA/SpoT homolog*) involved in *Pharbitis nil* response to stress condition. *Adv. Agric. Sci. Probl. Issues* **2006**, *509*, 333–341.
23. Dabrowska, G.; Prusińska, J.; Goc, A. Plant mechanism of an adaptive stress response homologous to bacterial stringent response. *Adv. Biochem.* **2006**, *52*, 94–100.
24. Boniecka, J.; Prusińska, J.; Dąbrowska, G.B.; Goc, A. Within and beyond the stringent response-RSH and (p)ppGpp in plants. *Planta* **2017**, *246*, 817–842. [CrossRef]
25. Prusińska, J.M.; Boniecka, J.; Dąbrowska, G.B.; Goc, A. Identification and characterization of the *Ipomoea nil* RelA/SpoT Homologs (*InRSHs*) and potential directions of their transcriptional regulation. *Plant Sci.* **2019**, *284*, 161–176. [CrossRef]
26. Mizusawa, K.; Masuda, S.; Ohta, H. Expression profiling of four RelA/SpoT-like proteins, homologues of bacterial stringent factors, in *Arabidopsis thaliana*. *Planta* **2008**, *228*, 553–562. [CrossRef]
27. Wolf, Y.I.; Aravind, L.; Grishin, N.V.; Koonin, E.V. Evolution of aminoacyl-tRNA synthetases-analysis of unique domain architectures and phylogenetic trees reveals a complex history of horizontal gene transfer events. *Genome Res.* **1999**, *9*, 689–710. [CrossRef]
28. Brown, A.; Fernández, I.S.; Gordiyenko, Y.; Ramakrishnan, V. Ribosome-dependent activation of stringent control. *Nature* **2016**, *534*, 277–280. [CrossRef]
29. Loveland, A.B.; Bah, E.; Madireddy, R.; Zhang, Y.; Brilot, A.F.; Grigorieff, N.; Korostelev, A.A. Ribosome-RelA structures reveal the mechanism of stringent response activation. *eLife* **2016**, *5*, 1–23. [CrossRef]
30. Ito, D.; Ihara, Y.; Nishihara, H.; Masuda, S. Phylogenetic analysis of proteins involved in the stringent response in plant cells. *J. Plant Res.* **2017**, *130*, 625–634. [CrossRef]
31. Tozawa, Y.; Nozawa, A.; Kanno, T.; Narisawa, T.; Masuda, S.; Kasai, K.; Nanamiya, H. Calcium-activated (p)ppGpp synthetase in chloroplasts of land plants. *J. Biol. Chem.* **2007**, *282*, 35536–35545. [CrossRef]
32. Ono, S.; Suzuki, S.; Ito, D.; Tagawa, S.; Shiina, T.; Masuda, S. Plastidial (p)ppGpp synthesis by the Ca^{2+}-dependent RelA–SpoT homolog regulates the adaptation of chloroplast gene expression to darkness in *Arabidopsis*. *Plant Cell Physiol.* **2020**, *61*, 2077–2086. [CrossRef] [PubMed]
33. Sun, D.; Lee, G.; Lee, J.; Kim, H.; Rhee, H.; Park, S.; Kim, K.; Kim, Y.; Kim, B.; Hong, J.; et al. A metazoan ortholog of SpoT hydrolyzes ppGpp and functions in starvation responses. *Nat. Struct. Mol. Biol.* **2010**, *17*, 1188–1194. [CrossRef] [PubMed]
34. Ito, D.; Kawamura, H.; Oikawa, A.; Ihara, Y.; Shibata, T.; Nakamura, N.; Asano, T.; Kawabata, S.-I.; Suzuki, T.; Masuda, S. ppGpp functions as an alarmone in metazoa. *Commun. Biol.* **2020**, *3*, 1–11. [CrossRef] [PubMed]
35. Masuda, S. The stringent response in phototrophs. In *Advances in Photosynthesis—Fundamental Aspects.*; Najfpour, M., Ed.; InTech: Shanghai, China, 2012; pp. 487–500. ISBN 978-953-307-928-8.
36. Atkinson, G.C.; Tenson, T.; Hauryliuk, V. The RelA/SpoT Homolog (RSH) superfamily: Distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. *PLoS ONE* **2011**, *6*, e23479. [CrossRef]
37. Dabrowska, G.; Mierek-Adamska, A.; Goc, A. Plant metallothioneins: Putative functions identified by promoter analysis in silico. *Acta Biol. Crac. Ser. Bot.* **2012**, *54*, 109–120. [CrossRef]
38. Pan, Y.; Zhu, M.; Wang, S.; Ma, G.; Huang, X.; Qiao, C.; Wang, R.; Xu, X.; Liang, Y.; Lu, K.; et al. Genome-wide characterization and analysis of metallothionein family genes that function in metal stress tolerance in *Brassica napus* L. *Int. J. Mol. Sci.* **2018**, *19*, 2181. [CrossRef]
39. Chen, J.; Gao, T.; Wan, S.; Zhang, Y.; Yang, J.; Yu, Y.; Wang, W. Genome-wide identification, classification and expression analysis of the HSP gene superfamily in tea plant (*Camellia sinensis*). *Int. J. Mol. Sci.* **2018**, *19*, 2633. [CrossRef]
40. Yuan, D.; Li, W.; Hua, Y.; King, G.J.; Xu, F.; Shi, L. Genome-wide identification and characterization of the aquaporin gene family and transcriptional responses to boron deficiency in *Brassica napus*. *Front. Plant Sci.* **2017**, *8*, 1336. [CrossRef]
41. Sugliani, M.; Abdelkefi, H.; Ke, H.; Bouveret, E.; Robaglia, C.; Caffarri, S.; Field, B. An ancient bacterial signaling pathway regulates chloroplast function to influence growth and development in *Arabidopsis*. *Plant Cell* **2016**, *28*, 661–679. [CrossRef]
42. Xiong, L.; Lee, M.W.; Qi, M.; Yang, Y. Identification of defense-related rice genes by suppression subtractive hybridization and differential screening. *Mol. Plant Microbe Interact.* **2001**, *14*, 685–692. [CrossRef]

43. Kim, T.H.; Ok, S.H.; Kim, D.; Suh, S.C.; Byun, M.O.; Shin, J.S. Molecular characterization of a biotic and abiotic stress resistance-related gene RelA/SpoT homologue (*PepRSH*) from pepper. *Plant Sci.* **2009**, *176*, 635–642. [CrossRef]
44. Givens, R.M.; Lin, M.H.; Taylor, D.J.; Mechold, U.; Berry, J.O.; Hernandez, V.J. Inducible expression, enzymatic activity, and origin of higher plant homologues of bacterial RelA/SpoT stress proteins in *Nicotiana tabacum*. *J. Biol. Chem.* **2004**, *279*, 7495–7504. [CrossRef]
45. Yamada, A.; Tsutsumi, K.; Tanimoto, S.; Ozeki, Y. Plant RelA/SpoT homolog confers salt tolerance in *Escherichia coli* and *Saccharomyces cerevisiae*. *Plant Cell Physiol.* **2003**, *44*, 3–9. [CrossRef]
46. Morello, L.; Breviario, D. Plant spliceosomal introns: Not only cut and paste. *Curr. Genom.* **2008**, *9*, 227–238. [CrossRef]
47. Frey, K.; Pucker, B. Animal, fungi, and plant genome sequences harbor different non-canonical splice sites. *Cells* **2020**, *9*, 458. [CrossRef]
48. Wang, B.-B.; Brendel, V. Genomewide comparative analysis of alternative splicing in plants. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 7175–7180. [CrossRef]
49. Jo, B.-S.; Choi, S.S. Introns: The functional benefits of introns in genomes. *Genom. Inform.* **2015**, *13*, 112. [CrossRef]
50. Ren, X.Y.; Vorst, O.; Fiers, M.W.; Stiekema, W.J.; Nap, J.P. In plants, highly expressed genes are the least compact. *Trends Genet.* **2006**, *22*, 528–532. [CrossRef]
51. Masuda, S.; Mizusawa, K.; Narisawa, T.; Tozawa, Y.; Ohta, H.; Takamiya, K.I. The bacterial stringent response, conserved in chloroplasts, controls plant fertilization. *Plant Cell Physiol.* **2008**, *49*, 135–141. [CrossRef]
52. Maekawa, M.; Honoki, R.; Ihara, Y.; Sato, R.; Oikawa, A.; Kanno, Y.; Ohta, H.; Seo, M.; Saito, K.; Masuda, S. Impact of the plastidial stringent response in plant growth and stress responses. *Nat. Plants* **2015**, *1*, 1–7. [CrossRef]
53. Sato, M.; Takahashi, T.; Ochi, K.; Matsuura, H.; Nabeta, K.; Takahashi, K. Overexpression of RelA/SpoT homologs, *PpRSH2a* and *PpRSH2b*, induces the growth suppression of the moss *Physcomitrella patens*. *Biosci. Biotechnol. Biochem.* **2015**, *79*, 36–44. [CrossRef] [PubMed]
54. Takahashi, K.; Kasai, K.; Ochi, K. Identification of the bacterial alarmone guanosine 5′-diphosphate 3′-diphosphate (ppGpp) in plants. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 4320–4324. [CrossRef] [PubMed]
55. Field, B. Green magic: Regulation of the chloroplast stress response by (p)ppGpp in plants and algae. *J. Exp. Bot.* **2018**, *69*, 2797–2807. [CrossRef] [PubMed]
56. Yamburenko, M.V.; Zubo, Y.O.; Börner, T. Abscisic acid affects transcription of chloroplast genes via protein phosphatase 2C-dependent activation of nuclear genes: Repression by guanosine-3′-5′-bisdiphosphate and activation by sigma factor 5. *Plant J.* **2015**, *82*, 1030–1041. [CrossRef]
57. Ito, D.; Kato, T.; Maruta, T.; Tamoi, M.; Yoshimura, K.; Shigeoka, S. Enzymatic and molecular characterization of *Arabidopsis* ppGpp pyrophosphohydrolase, AtNUDX26. *Biosci. Biotechnol. Biochem.* **2012**, *76*, 2236–2241. [CrossRef]
58. Abdelkefi, H.; Sugliani, M.; Ke, H.; Harchouni, S.; Soubigou-Taconnat, L.; Citerne, S.; Mouille, G.; Fakhfakh, H.; Robaglia, C.; Field, B. Guanosine tetraphosphate modulates salicylic acid signalling and the resistance of *Arabidopsis thaliana* to Turnip mosaic virus. *Mol. Plant Pathol.* **2018**, *19*, 634–646. [CrossRef]
59. Lescot, M.; Déhais, P.; Thijs, G.; Marchal, K.; Moreau, Y.; Van De Peer, Y.; Rouzé, P.; Rombauts, S. PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res.* **2002**, *30*, 325–327. [CrossRef]
60. Hernandez-Garcia, C.M.; Finer, J.J. Identification and validation of promoters and *cis*-acting regulatory elements. *Plant Sci.* **2014**, *217–218*, 109–119. [CrossRef]
61. Chang, W.-C.; Lee, T.-Y.; Huang, H.-D.; Huang, H.-Y.; Pan, R.-L. PlantPAN: Plant promoter analysis navigator, for identifying combinatorial *cis*-regulatory elements with distance constraint in plant gene groups. *BMC Genom.* **2008**, *9*, 1–14. [CrossRef]
62. Yamaguchi-Shinozaki, K.; Shinozaki, K. Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci.* **2005**, *10*, 88–94. [CrossRef]
63. Ihara, Y.; Ohta, H.; Masuda, S. A highly sensitive quantification method for the accumulation of alarmone ppGpp in *Arabidopsis thaliana* using UPLC-ESI-qMS/MS. *J. Plant Res.* **2015**, *128*, 511–518. [CrossRef]
64. Etesami, H.; Noori, F. *Soil Salinity as a Challenge for Sustainable Agriculture and Bacterial-Mediated Alleviation of Salinity Stress in Crop Plants BT-Saline Soil-based Agriculture by Halotolerant Microorganisms*; Kumar, M., Etesami, H., Kumar, V., Eds.; Springer Singapore: Singapore, 2019; pp. 1–22. ISBN 978-981-13-8335-9.
65. Ashraf, M.; McNeilly, T. Salinity tolerance in *Brassica* oilseeds. *Crit. Rev. Plant Sci.* **2004**, *23*, 157–174. [CrossRef]
66. Chen, J.; Bang, W.Y.; Lee, Y.; Kim, S.; Lee, K.W.; Kim, S.W.; Son, Y.S.; Kim, D.W.; Akhter, S.; Bahk, J.D. AtObgC-AtRSH1 interaction may play a vital role in stress response signal transduction in *Arabidopsis*. *Plant Physiol. Biochem.* **2014**, *74*, 176–184. [CrossRef]
67. Brouwer, C.; Goffeau, A.; Heibloem, M. *Irrigation Water Management: Training Manual No. 1—Introduction to Irrigation*; FAO: Rome, Italy, 1985.
68. Nawaz, A.; Shahbaz, M.; Asadullah; Imran, A.; Marghoob, M.U.; Imtiaz, M.; Mubeen, F. Potential of salt tolerant PGPR in growth and yield augmentation of wheat (*Triticum aestivum* L.) under saline conditions. *Front. Microbiol.* **2020**, *11*, 2019. [CrossRef]
69. Hashem, A.; Abd_Allah, E.F.; Alqarawi, A.A.; Al-Huqail, A.A.; Wirth, S.; Egamberdieva, D. The interaction between arbuscular mycorrhizal fungi and endophytic bacteria enhances plant growth of *Acacia gerrardii* under salt stress. *Front. Microbiol.* **2016**, *7*, 1089. [CrossRef]

70. Janczak, K.; Hrynkiewicz, K.; Znajewska, Z.; Dąbrowska, G. Use of rhizosphere microorganisms in the biodegradation of PLA and PET polymers in compost soil. *Int. Biodeterior. Biodegrad.* **2018**, *130*, 65–75. [CrossRef]
71. Janczak, K.; Dąbrowska, G.B.; Raszkowska-Kaczor, A.; Kaczor, D.; Hrynkiewicz, K.; Richert, A. Biodegradation of the plastics PLA and PET in cultivated soil with the participation of microorganisms and plants. *Int. Biodeterior. Biodegrad.* **2020**, *155*, 105087. [CrossRef]
72. Egamberdieva, D.; Wirth, S.J.; Alqarawi, A.A.; Abd-Allah, E.F.; Hashem, A. Phytohormones and beneficial microbes: Essential components for plants to balance stress and fitness. *Front. Microbiol.* **2017**, *8*, 2104. [CrossRef]
73. Miguel, P.S.B.; de Oliveira, M.N.V.; Delvaux, J.C.; de Jesus, G.L.; Borges, A.C.; Tótola, M.R.; Neves, J.C.L.; Costa, M.D. Diversity and distribution of the endophytic bacterial community at different stages of *Eucalyptus* growth. *Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **2016**, *109*, 755–771. [CrossRef]
74. Wilkerson, M.D.; Ru, Y.; Brendel, V.P. Common introns within orthologous genes: Software and application to plants. *Brief. Bioinform.* **2009**, *10*, 631–644. [CrossRef]
75. Sievers, F.; Wilm, A.; Dineen, D.; Gibson, T.J.; Karplus, K.; Li, W.; Lopez, R.; McWilliam, H.; Remmert, M.; Söding, J.; et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **2011**, *7*. [CrossRef]
76. Emanuelsson, O.; Nielsen, H.; Brunak, S.; Von Heijne, G. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* **2000**, *300*, 1005–1016. [CrossRef]
77. Emanuelsson, O.; Brunak, S.; von Heijne, G.; Nielsen, H. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat. Protoc.* **2007**, *2*, 953–971. [CrossRef]
78. Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **2007**, *24*, 1596–1599. [CrossRef]
79. Kumar, S.; Nei, M.; Dudley, J.; Tamura, K. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief. Bioinform.* **2008**, *9*, 299–306. [CrossRef]
80. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425. [CrossRef] [PubMed]
81. Hrynkiewicz, K.; Złoch, M.; Kowalkowski, T.; Baum, C.; Niedojadło, K.; Buszewski, B. Strain-specific bioaccumulation and intracellular distribution of Cd^{2+} in bacteria isolated from the rhizosphere, ectomycorrhizae, and fruitbodies of ectomycorrhizal fungi. *Environ. Sci. Pollut. Res.* **2015**, *22*, 3055–3067. [CrossRef]
82. Hrynkiewicz, K.; Baum, C.; Leinweber, P. Density, metabolic activity, and identity of cultivable rhizosphere bacteria on S*alix viminalis* in disturbed arable and landfill soils. *J. Plant Nutr. Soil Sci.* **2010**, *173*, 747–756. [CrossRef]
83. Janczak, K.; Dąbrowska, G. Bacteria able to polylactide and polycaprolactone biodegradation. *Przem. Chem.* **2018**, *97*, 435–438. [CrossRef]
84. Mierek-Adamska, A.; Kotowicz, K.; Goc, A.; Boniecka, J.; Berdychowska, J.; Dąbrowska, G.B. Potential involvement of rapeseed (*Brassica napus* L.) metallothioneins in the hydrogen peroxide-induced regulation of seed vigour. *J. Agron. Crop Sci.* **2019**, *205*, 598–607. [CrossRef]
85. Marone, M.; Mozzetti, S.; De Ritis, D.; Pierelli, L.; Scambia, G. Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcriptsfrom the same sample. *Biol. Proced. Online* **2001**, *3*, 19. [CrossRef] [PubMed]

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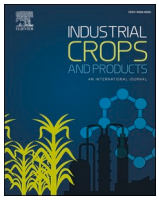

Localization and expression of *CRSH* transcript, level of calcium ions, and cell cycle activity during *Brassica napus* L. seed development

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ABSTRACT

Seeds are used as human food and animal feed, but the seed is also the onset of a new life. Therefore, seeds are a crucial agricultural product for global food security. Among several mechanisms involved in seed formation and maturation, the stringent response seems to be of great but rather overlooked significance. Plant homologs of bacterial RelA/SpoT proteins, called RSH, metabolize hyperphosphorylated regulatory nucleotides guanosine tetra- and pentaphosphate ((p)ppGpp, alarmones) which are involved in varied aspects of plant growth and development. The role of plant RSHs and alarmones in seed development remains elusive. In this study, the possible function of Ca^{2+}-dependent RSH (CRSH) proteins during seed formation and maturation was verified. In silico analysis of plant CRSH proteins showed that EF-hand calcium-binding motif was highly conserved in monocotyledonous and dicotyledonous plants. Remarkably, this motif is present only in plant CRSH and has not been found in bacterial homologs. In developing *Brassica napus* (canola) seeds we analyzed the level and the localization of *BnCRSH* mRNAs by RT-qPCR and in situ localization, respectively. Further, we examined the cell cycle activity via flow cytometry, and the level of calcium ions using scanning electron microscopy. Our results showed that DNA replication intensity was at the highest level in seeds at the early stages of development, and then constantly decreased to the minimal level reached 70 days after flowering. In contrast, the level of calcium ions and *BnCRSH* transcript increased during canola seed maturation. The results of this study strongly suggest that calcium-dependent stringent response plays a significant role during canola seed development.

1. Introduction

Seeds are the most relevant agricultural product, providing over 70% of the human caloric intake through food and feed (Sreenivasulu and Wobus, 2013). Seeds have a high content of storage proteins, carbohydrates (especially starch), and lipids. Moreover, seeds are used not only as food and animal feed, but also for the manufacturing of biofuels, plastics, and lubricants. Seeds are complex structures that are composed of three genetically distinct sections i.e., the embryo, the endosperm, and the seed coat. In each part of the seed extensive alterations in transcript abundance from fertilization through seed maturity are observed (Bewley et al., 2013). Seed development is a complex physiological process that is tightly coordinated by various physiological, metabolic, genetic factors, and is affected by environmental signals.

Brassica napus L. (known as canola, rapeseed, and oilseed rape), belongs to the family *Brassicaceae* and is cultivated almost all over the world. *B. napus* pod begins to grow once flowers are fertilized and seed reaches the maturity about 80 days after flowering. The first step of seed development is the expansion of the seed coat until it reaches its maximum size (15–27 days after fertilization). Next, the embryo grows rapidly to fill the space. The seed fill lasts around 20 days and is divided into two phases: protein deposition and oil deposition. Protein

Abbreviations: pppGpp, Guanosine penta- and tetraphosphates; RSH, RelA/SpoT Homolog; CRSH, Ca^{2+} -activated RSH.

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Table 1
The content of genetic material in seeds of *B. napus* collected at 35, 56, 63, 70, and 80 days after flowering (DAF).

DAF	Percentage of nuclei with particular DNA content			(Σ>2C)/2C ratio*
	2C	4C	8C	
35	88.2	8.7	3.1	0.12 a
56	94.2	5.8	0	0.06 b
63	96.2	3.8	0	0.04c
70	97.4	2.6	0	0.03c
80	97.4	2.6	0	0.03c

* values (in the column) followed by different letters are significantly different at $p < 0.05$

expression rises quickly in the early phases of seed formation. The cotyledons contain the majority of the protein in the mature seed; approximately 76% of the protein is found in the cotyledons, 17% in the other parts of the embryo, and 6% in the seed coat. Most of the oil is synthesized between the 35th day and the 55th day after flowering. Around 42 days after flowering seed development is completed. At this stage seeds are still green and during subsequent stages seeds will dehydrate and change into black. Approximately 80 days after flowering, seeds are fully mature (Edwards and Hertel, 2011). A mature canola embryo consists of a central embryonic axis (which will grow into the hypocotyl and root) surrounded by two cotyledons. During seed development, the two cotyledons bend toward the embryonic axis with one remaining on the external part of the seed (outer cotyledon) and the other positioned in the internal part of the seed (inner cotyledon) (Woodfield et al., 2017). During the early stages of seed development embryo and endosperm cells rapidly divide, whereas later the cell cycle activity gradually decreases. A typical proliferative cell undergoes four sequential phases i.e., phase G1 during which DNA content is 2C; phase S when DNA synthesis occurs and the amount of DNA increases to 4C; phase G2 during which the DNA level remains 4C; and phase M, mitosis, when a cell nucleus divides into two identical daughter nuclei (4C→2C) (Śliwińska, 2009).

Plants, as sessile organisms, have evolved several mechanisms to cope with and/or adapt to adverse environmental conditions. One such mechanism, found initially in bacteria, is the stringent response (Berdychowska et al., 2019; Dąbrowska et al., 2006). The stringent response is characterized by the production of hyperphosphorylated nucleotides called alarmones - guanosine tetra- and pentaphosphate ((p)ppGpp), which were discovered in *E. coli* (Cashel and Gallant, 1969). In plants, the genes involved in (p)ppGpp metabolism (RelA/SpoT Homolog; RSH) were identified first in *Arabidopsis thaliana* (L.) Heynh (van der Biezen, 2000). Plant RSH proteins are classified into three distinct groups i.e., RSH1, RSH2/3, and Ca^{2+}-dependent RSH (CRSH). Enzymes belonging to RSH1 lack a conserved glycine residue in (p)ppGpp synthase domain and act in plants as an alarmones hydrolase. Proteins representing RSH2 and RSH3 are bifunctional (p)ppGpp synthase/hydrolase enzymes. Proteins belonging to CRSH lack a conserved histidine and aspartate necessary for (p)ppGpp hydrolase activity and function as an alarmones synthase (Atkinson et al., 2011; Boniecka et al., 2017). Several studies have shown that (p)ppGpp level rises in response to a variety of abiotic and biotic stresses such as darkness, drought, wounding, high salinity, stress-related hormones (e.g. abscisic acid, jasmonate, and ethylene), heavy metals, nitrogen limitation, heat shock, and pathogen attack (Abdelkefi et al., 2018; Honoki et al., 2018; Ono et al., 2021; Romand et al., 2022; Takahashi et al., 2004). It is now widely accepted that plant stringent response occurs mainly in chloroplasts (Boniecka et al., 2017). Alarmones have been found to regulate chloroplast transcription, translation, and the production of many metabolites. Alarmones are involved also in developmental processes including flowering and plant senescence. These hyperphosphorylated nucleotides are also proposed to play a role in nutrient remobilization from vegetative tissues to seeds during seed development (Dąbrowska et al., 2006a; Prusińska et al., 2019).

B. napus genome was sequenced in 2014 (Chalhoub et al., 2014), which expedites studies of canola genes and gene families (Dąbrowska et al., 2021) and allows for comparison canola genome with genomes of model plants especially closely related *A. thaliana*. *B. napus* is an allotetraploid and thus it possesses more *RSH* genes than diploid species i.e., 12 *RSH* genes. *A. thaliana* (diploid species) has four *RSH* genes (*AtRSH1, AtRSH2, AtRSH3*, and *AtCRSH*). Proteins encoded by *AtRSH2*, *AtRSH3*, and *AtCRSH* are (p)ppGpp synthetase, whereas AtRSH1 is responsible for (p)ppGpp hydrolysis (Sugliani et al., 2016). *B. napus* genome contains six genes belonging to RSH1, two genes belonging to RSH2, three genes belonging to RSH3, and interestingly only one encoding CRSH (Dąbrowska et al., 2021).

Calcium ions (Ca^{2+}) are key secondary messengers in plant growth and development. Vacuoles, together with the apoplast/cell wall and endoplasmic reticulum are the main Ca^{2+} storage compartments in plant cells. Furthermore, it has been shown that chloroplasts and mitochondria function as Ca^{2+} sinks. The concertation of free Ca^{2+} ions in the cytosol and chloroplasts changes during development and in response to environmental conditions (Costa et al., 2018; Nomura and Shiina, 2014). Calcium ions affect almost every aspect of plant growth and

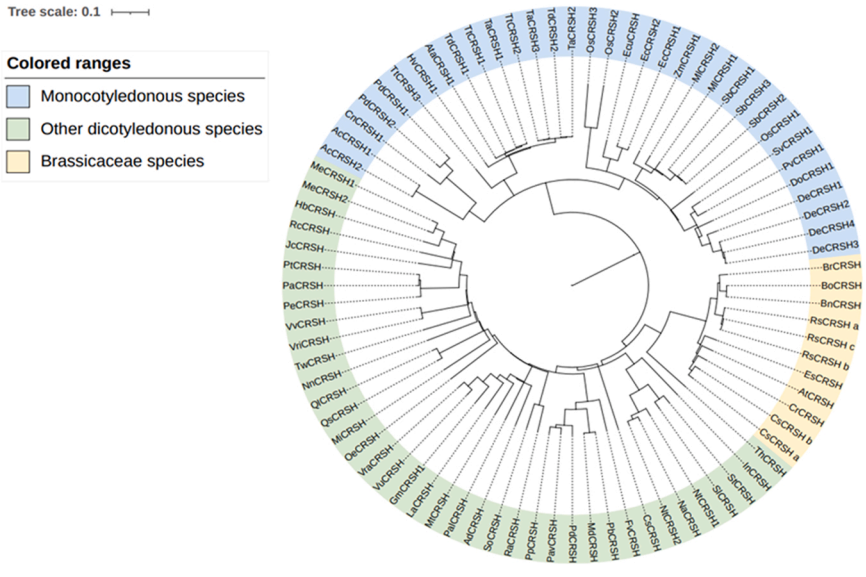

Fig. 1. The phylogenetic tree of plant CRSH proteins constructed from 84 full-length CRSH amino acid sequences obtained from 63 plant species including 18 monocot and 45 dicot plants. Monocot CRSHs are highlighted in blue, CRSHs from *Brassicaceae* family are highlighted in yellow, and CRSHs from other dicot plants are highlighted in green. The phylogenetic tree was constructed by Clustal Omega and visualized by iTOL. The amino acid sequences of plant CRSHs were retrieved from the NCBI Protein database, and the accession numbers of amino acid sequences are listed in Supplementary Table1.

A

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                E helix     Ca2+ loop      F helix       E helix     Ca2+ loop      F helix
                ••••hh•hh X Y Z -Y -X -Z h••hh••h•    Dh••hh•hh X Y Z -Y -X -Z h••h•••h•
MdCRSH   470 QNNRVFRLL DKNGDG RI NIEE LMEVMEELGAPGEDACEMMQLL DANSDG SL SSDE FELFQKQVG
PbCRSH   470 QNNRAFRLL DKNGDG RI NIEE LMEVMEELGAPGEDACEMMQLL DANSDG SL SSDE FELFQKQVG
PdCRSH   472 QSDRVFRLL DKNGDG RI NIEE LMEVMEELGAPGEDAREMMQLL DANSDG SL SSDE FDFFQRQVE
PpCRSH   479 QSDRVFRLL DKNGDG RI NIEE LMEVMEELGAPGEDAREMMQLL DANSDG SL SSDE FDFFQRQVE
PavCRSH  500 QSDRVFRLL DKNGDG RI NIEE LMEVMEELGAPGEDAREMMQLL DANSDG SL SSDE FDLFQRQVE
OeCRSH   484 CRDRVFRLL DKNGDG KI SIEE LMEVMDELGAQGEDAREMMRLL DANSDG SL SSDE FDLFQKQVE
SoCRSH   485 RRDRVFCLL DKNGDG RI SIEE LTEVMEELGAPGDDAREMMQLL DANSDG SL SSDE FDAFQKQVE
RaCRSH   479 RRDRVFRLL DKNGDG RI SIEE LMEVMEELGAPGDDAREMMQLL DANSDG SL SCDE FDVFQNQVE
MiCRSH   484 RRNRIFRLL DKNGDG RI SIEE LTEVMEELGAKGEDAREMMQLL DANSDG SL SSDE FDLFQKQVE
QlCRSH   478 HRNRVFRLL DKNGDG RI SIEE LMEVMEELGAPGEDACEMMQLL DSNSDG SL SSDE FDLFQKQVE
QsCRSH   478 HRNRVFRLL DKNGDG RI SIDE LMEVMEELGAPGEDACEMMQLL DSNSDG SL SSDE FDLFQKQVE
PalCRSH  486 QRNRVFYLL DKNGDG RI SIEE LTEVMEELGAPGEDAREMMLLL DSNSDG SL SSDE FDKFQQQVE
HbCRSH   467 QRDRVFRLL DKNGDG QI SIEE LMEVMEELGAPGEDAREMMQLL DANSDG SL SSDE FDTFQKQVE
PeCRSH   463 QRDQVFQLL DKNGDG RI SIEE LMEVMEELGAPGEDAREMMQLL DSNSDG SL SPDE FSTFQKQVE
PtCRSH   463 QRDRVFQLL DKNGDG RI SIEE LMEVMEELGAPGEDAREMMQLL DSNSDG SL SPDE FDTFQKQVE
PaCRSH   463 QRDRVFQLL DKNGDG RI SIEE LMEVMEELGAPGEDAREMMQLL DSNSDG SL SPDE FNTFQKQVE
                :: * ** ****** :* .*:* * ***:**** *:** *** ** *:**** ** * ** *. **.**
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B

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                E helix     Ca2+ loop      F helix       E helix     Ca2+ loop      F helix
                ••••hh•hh X Y Z -Y -X -Z h••hh••h•    Dh••hh•hh X Y Z -Y -X -Z h••h•••h•
RsCRSH_a 473 QRDRVFCLL DKNGDG MI SIEE LMEVMEELGAPGEDAEEMMQLL DSNSDG SL SSDE FDTFQKQVE
RsCRSH_b 473 QRDRVFCLL DKNGDG MI SIEE LMEVMEELGAPGEDAEEMMQLL DSNSDG SL SSDE FDTFQKQVE
RsCRSH_c 473 QRDRVFCLL DKNGDG MI SIEE LMEVMEELGAPGEDAEEMMQLL DSNSDG SL SSDE FDTFQKQVE
BrCRSH   467 QRDRVFCLL DKNGDG MI SIEE LMEVMEELGAPGEDAEEMMQLL DSNSDG SL SSDE FDTFQKQVE
BnCRSH   471 QRDRVFCLL DKNGDG MI SIEE LMEVMEELGAPGEDAEEMMQLL DSNSDG SL SSDE FDTFQKQVE
BoCRSH   471 QRDRVFCLL DKNGDG MI SIEE LMEVMEELGAPGEDAEEMMQLL DSNSDG SL SSDE FDTFQKQVE
EsCRSH   477 QRDRVFCLL DKNGDG MI SIEE LMEVMEELGAPGEDAEEMMQLL DSNSDG SL SSDE FDTFQKQVE
AtCRSH   489 QRDRVFCLL DKNGDG MI SIEE LMEVMEELGAPGEDAEEMMQLL DSNSDG SL SSDE FDTFQKQVE
CrCRSH   474 QRDRVFCLL DKNGDG MI SIEE LMEVMEELGAPGEDAEEMMQLL DSNSDG SL SSEE FDTFQKQVE
CsCRSH_a 476 QRDRVFCLL DKNGDG MI SIEE LMEVMEELGAPGEDAEEMMQLL DSNSDG SL SSDE FDTFQKQVE
CsCRSH_b 478 QRDRVFCLL DKNGDG MI SIEE LMEVMEELGAPGEDAEEMMQLL DSNSDG SL SSDE FDTFQKQVE
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Fig. 2. The alignment of the EF-hand motif from plant CRSHs performed in Clustal Omega. The consensus sequence is shown above each alignment and conserved residues in the Ca^{2+}- binding loops are shown in bold. Abbreviations: h - hydrophobic amino acid; • - any amino acid; X, Y, Z, -Y, -X, and - Z - amino acids that chelate Ca^{2+}. The numbers on the left indicate the position of the first amino acid in the row. Asterisks (*) show fully conserved amino acid residues; colons (:) indicate different amino acids with strongly similar properties; dots (.) indicate different amino acids with weakly similar properties. (A) CRSHs from dicot tree species, (B) CRSHs from plants belonging to the *Brassicaceae* family, (C) CRSHs from other dicot plant species, (D) CRSHs from monocot plant species. The amino acid sequences of plant CRSHs were retrieved from the NCBI Protein database, and the accession numbers of amino acid sequences are listed in Supplementary Table 1.

development and are the crucial regulator of seed formation, embryogenesis, seed development, and germination (Anil et al., 2000; Anil and Sankara Rao, 2001; Chen et al., 2019; Hepler, 2005). The positive effect of calcium on seed production and seed quality has been observed in various plant species including bean (*Phaseolus vulgaris* L.), soybean (*Glycine max* L.) Merr.), corn (*Zea mays* L.), pomegranate (*Punica granatum* L.), and cauliflower (*Brassica oleracea* L.) (Nakada-Freitas et al., 2021). Chloroplasts are an important part of intracellular Ca^{2+} signaling and homeostasis. Tight control of the level of calcium ions in the chloroplast is essential for their proper functioning. The stromal level of Ca^{2+} affects also intracellular calcium signaling. Therefore, chloroplast can receive and transmit Ca^{2+} signals inside the whole plant cell (Navazio et al., 2020; Reddy and Reddy, 2004; White and Broadley, 2003). Plant CRSHs have two Ca^{2+}-binding EF-hand motifs at the C-terminus of the protein and require Ca^{2+} for (p)ppGpp synthesis activity (Masuda et al., 2008a). EF-hand motif, which is the most common calcium-binding motif, is a conserved helix-loop-helix structure that can bind a single Ca^{2+} ion (Day et al., 2002). Remarkably, the EF-hand motif is specific only for the CRSH proteins from plants and algae, and has not been found in their bacterial homologs (Dąbrowska et al., 2021; Ito et al., 2017). In general, proteins that contain EF-hand e.g., calmodulins, calcineurin B-like proteins, and calcium-dependent protein kinases, can transmit information about varied stimuli when cytosolic calcium levels alter in response to stress and developmental signals (Day et al., 2002; Zeng et al., 2017).

It is plausible to hypothesize that a relationship between Ca^{2+} signaling pathways and Ca^{2+}-dependent (p)ppGpp accumulation in plants exists. The involvement of stringent response in regulating plant growth and development is now widely accepted however the possible role of this mechanism in seed development remains elusive. During seed development not only dehydration occurs but also the amount of available light is reduced. We have shown previously that light- and dehydration-responsive elements are present in promoters of all *B. napus RSH* genes (Dąbrowska et al., 2021). Inspired by the well-known role of calcium during fertilization (Dumas and Gaude, 2006) and seed development (Burton et al., 2000) and by the observation that in *A. thaliana* the accumulation of alarmones after the light-to-dark transition is due to the activity of CRSH (Ono et al., 2021) in this study we focused on the possible role of the Ca^{2+}-dependent RSH in *B. napus* seed development. To gain some insight into CRSH-mediated (p)ppGpp synthesis during canola seed development we verified the changes in the cell cycle activity and the level of Ca^{2+} at different stages of seed development in parallel with the analysis of the amount and the localization of the *BnCRSH* transcripts in developing seeds. A comprehensive understanding of the involvement of stringent response in seed development from the ovule to a mature seed in crops will allow not only to optimize seed

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                    E helix      Ca2+ loop      F helix       E helix     Ca2+ loop      F helix
                    ••••hh•hh X Y Z -Y -X -Z h••hh••h•     Dh••hh•hh X Y Z -Y -X -Z h••h•••h•
InCRSH      469 NRDMAFHLL DKNGDG KI SIEE LMEVMEELGAQGEDAREMMQLL DSNNDS LL SSDE FDLFQKQIE
NtCRSH1     471 KRGRVFRLL DKNGDG KL SIDE LMEVMEELGAPGDDAREMMQLL DSNSDG FL SSDE FDLFQDQVE
NtCRSH2     476 KRGRVFRLL DKNGDG KL SIDE LMEVMEELGAPGDDAREMMQLL DSNSDG FL SSDE FDLFQDQIE
NaCRSH      475 KRGRVFRLL DKNGDG KL SIDE LMEVMEELGAPGDDAREMMQLL DSNSDG FL SSDE FDLFQDQIE
SlCRSH      476 KRGRVFRLL DKNGDG KI SIDE LMEVMEELGAPGDDAREMMQLL DSNSDG FL SSDE FDIFQNQVE
StCRSH      476 KRGRVFRLL DKNGDG KI SIGE LMEVMEELGAPGDDAREMMQLL DSNSDG FL SSDE FDIFQNQVE
ThCRSH      626 EGEGVFSLL DKNGDG RI SIEE LIEVMEELGAPGEDAEEMMQLL DLNNDG SL SCDE FDNFQKQVE
CsCRSH      487 QQNRVFRLL DKNGDG RI SIEE LMEVMEELGAPGDDAREMMKLL DSNSDG SL SSDE FDLFQQQVE
AdCRSH      485 QRDRVFRLL DKNGDG KI SIEE LTEVIEELGAPGEDAREMMQLL DSNSDG SL SSDE FHMFQKQVE
MtCRSH      475 QRDRVFRLL DKNGDG KI SIEE LTEVIEELGAPGEDAHDMMLLL DSNSDG SL SSDE FQMFQKQVE
LaCRSH      468 QRDRVFRLL DKNGDG KI SIEE LTEVMEELGAPGEDAREMMQLL DSNSDG SL SSDE FEMFQKQVE
GmCRSH1     474 QRDRVFRLL DKNGDG KI SIEE LTEVMEELGAPGEDAREMMQLL DSNSDG SL SSDE FHMFQEQVE
VraCRSH     471 QRDRVFRLL DKNGDG KI SIEE LTEVMEELGAPGEDAREMMQLL DSNSDG SL SCDE FHMFQEQVE
VuCRSH      471 QRDRVFRLL DKNGDG KI SIEE LTEVMEELGAPGEDAREMMQLL DSNSDG SL SCDE FHMFQEQVE
FvCRSH      473 KSDRVFRLL DKNGDG RI NFEE LKEVMEELGAPGEDACEMMQLL DANSDG SL SSDE FDLFQKQVE
NnCRSH      479 QRDRIFRLL DKNGDG RI SMEE LKEVMEELGAEREDAEEMMRLL DANSDG SL SSDE FDLFQKQVE
TwCRSH      491 QRDRVFRLL DKNGDG RI SIEE LMEVMEELGAPGEDAQEMMQLI DSNSDG SL STDE FDLFQKQVE
VvCRSH      474 QRDRAFCLL DKNGDG RI SIEE LMEVMEELGAQGEDAREMMQLL DSNSDG SL SSDE FDLFQKQVE
VriCRSH     474 QRDQAFCLL DKNGDG RI SIEE LMEVMEELGAQGEDAREMMQLL DSNSDG SL SSDE FDLFQKQVE
JcCRSH      469 QRDRVFRLL DKNGDG QI SIEE LMEVMEELGAPGEDAREMMQLL DSNSDG SL SSDE FNTFQKQVE
RcCRSH      464 RNDRVFCLF DKNGDG RI SIEE LMEVMEELGAPGEDAREMMQLL DSNSDG SL SSDE FDTFQKQVE
MeCRSH1     345 QRERVFRLF DKNGDG QI SIDE LMEVMEELGAPGEDAREMMQLL DANSDG SL SSDE FDTFQKQVE
MeCRSH2     487 HRERVFRLF DKNGDG QI SIDE LMEVMEELGAPGEDAREMMQLL DANSDG SL SSDE FDTFQKQVE
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D

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                    E helix      Ca2+ loop      F helix       E helix     Ca2+ loop      F helix
                    ••••hh•hh X Y Z -Y -X -Z h••hh••h•     Dh••hh•hh X Y Z -Y -X -Z h••h•••h•
OsCRSH1     477 CTNRAFCQL DKNGDG RI SIEE LTEVMEDLGAGGKDAKELMHLL DANSDG SL SSDE FEAFQRQIE
ZmCRSH1     466 CKNPAFCQL DKNGDG RI SIEE LTEVMEDLGAGGEDATELMHLL DANSDG SL SSDE FESFQRQIE
OsCRSH2     453 NIERAFRLL DKNGDG RI SMEE LTELMEDLGAGGKDAEELMRLL DDNNDG SL SSDE FALFQKRVE
OsCRSH3     473 NVERAFQLL DKNGDG RI SMEE LTEIMEDLGAGGHDAEELMRLL DANSDG SL SSDE FALFQKRVK
TtCRSH3     423 CKNQAFCLI DKNGDG RI SIEE LTEVMEDLGAEGEDAMALMHLL DANSDG SL SFDE FESFQRQVG
HvCRSH1     477 CKNQAFCLI DKNGDG RI SIEE LTEVMEDLGAEGEDAMALMHLL DANSDG SL SFDE FESFQRQVE
TaCRSH2     482 CKNQAFCLI DKNGDG RI SIEE LTEVMEDLGAEGEDAMALMHLL DANSDG SL SFDE FESFQRQVE
TdCRSH2     482 CKNQAFCLI DKNGDG RI SIEE LTEVMEDLGAEGEDAMALMHLL DANSDG SL SFDE FESFQRQVE
TaCRSH3     482 CKNQAFCLI DKNGDG RI SIEE LTEVMEDLGAEGEDAMALMHLL DANSDG SL SFDE FESFQRQVG
TtCRSH2     482 CKNQAFCLI DKNGDG RI SIEE LTEVMEDLGAEGEDAMALMHLL DANSDG SL SFDE FESFQRQVG
AtaCRSH1    481 CKNQAFCLI DKNGDG RI SIEE LTEVMEDLGAEGEDAMALMHLL DANSDG SL SFDE FESFQRQVE
TdCRSH1     483 CKNQAFCLI DKNGDG RI SIEE LTEVMEDLGAEGEDAMALMHLL DANSDG SL SFDE FESFQRQVE
TaCRSH1     481 CKNQAFCLI DKNGDG RI SIEE LTEVMEDLGAEGEDAMALMHLL DANSDG SL SFDE FESFQRQVE
TtCRSH1     481 CKNQAFCLI DKNGDG RI SIEE LTEVMEDLGAEGEDAMALMHLL DANSDG SL SFDE FESFQRQVG
EcuCRSH     534 SKNRAFYLL DKNGDG RI SIEE LTEVMEDLGAGVKDATELMHLL DANSDG SL SSEE FESFQRQVE
EcCRSH1     471 FKNRAFCLL DKNGDG RI SIEE LTEVMEDLGAGGKDATELMHLL DANSDG SL SSEE FESFQRQVE
EcCRSH2     471 FKNRAFCLL DKNGDG RI SIEE LTEVMEDLGAGGKDATELMHLL DANSDG SL SSEE FESFQRQVE
MlCRSH2     382 CKNPAFCQL DKNGDG RI SIEE LTEVMEDLGAGGKDAPELMHLL DANSDG SL SSDE FESFQRQVE
MlCRSH1     475 CKNPAFCQL DKNGDG RI SIEE LTEVMEDLGAGGEDATELMHLL DANSDG SL SSDE FESFQRQIE
SbCRSH1     479 CKNPAFCQL DKNGDG RI SIEE LTEVMEDLGAGGEDATELMHLL DANSDG SL SSDE FESFQRQIE
SbCRSH2     476 CKNPAFCQL DKNGDG RI SIEE LTEVMEDLGAGGEDATELMHLL DANSDG SL SSDE FESFQRQIE
SbCRSH3     347 CKNPAFCQL DKNGDG RI SIEE LTEVMEDLGAGGEDATELMHLL DANSDG SL SSDE FESFQRQIE
PvCRSH1     463 SNNPAFCQL DKNGDG RI SIEE LTEVMEDLGAGGEDATELMHLL DANSDG SL SSDE FESFQRQIE
SvCRSH1     469 CKNPAFRQL DKNGDG RI SIEE LTEVMEDLGAGGEDATELMHLL DANSDG SL SSDE FESFQRQIE
DoCRSH1     471 CKNPAFCQL DKNGDG RI SIEE LTEVMEDLGAGGEDATELMRLL DANSDG SL SSDE FDSFQRQIE
DeCRSH1     471 CKNPAFRQL DKNGDG RI SIEE LTEVMEDLGAGGKDATELMHLL DANSDG SL SSDE FESFQRQIE
DeCRSH2     493 CKNPAFRQL DKNGDG RI SIEE LTEVMEDLGAGGKDATELMHLL DANSDG SL SSDE FESFQRQIE
DeCRSH3     493 CKNPAFRQL DKNGDG RI SIEE LTEVMEDLGAGGKDATELMHLL DANSDG SL SSDE FESFQRQIE
DeCRSH4     395 CKNPAFRQL DKNGDG RI SIEE LTEVMEDLGAGGKDATELMHLL DANSDG SL SSDE FESFQRQIE
AcCRSH1     488 CRNRVFCLL DKNGDG RI SIEE LTEVMEELGAGGEDAKELMHLL DENSDG SL SSEE FDLFQRQVQ
AcCRSH2     533 CRNRVFCLL DKNGDG RI SIEE LTEVMEELGAGGEDAKELMHLL DENSDG SL SSEE FDLFQRQVQ
CnCRSH1     485 PRNRVFHLL DKNGDG RI SIEE LKEVMEELGAGGEDAWELMQLL DSNSDG SL SSEE FDLFQRQVE
PdCRSH1     488 HRNLVFHLL DKNGDG RI SIEE LAEVMEELGAGGEDAQELMQLL DSNSDG SL SSEE FDLFQRQVE
PdCRSH2     485 HRNRVFHLL DKNGDG RI SIEE LAEVMEELGAGGEDAQELMQLL DSNSDG SL SSEE FDLFQRQVE
                  : .*  : ****** ** *:** * *:**:***  .**  **:** * *.** ** * :* *  **:: :
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Fig. 2. (*continued*).

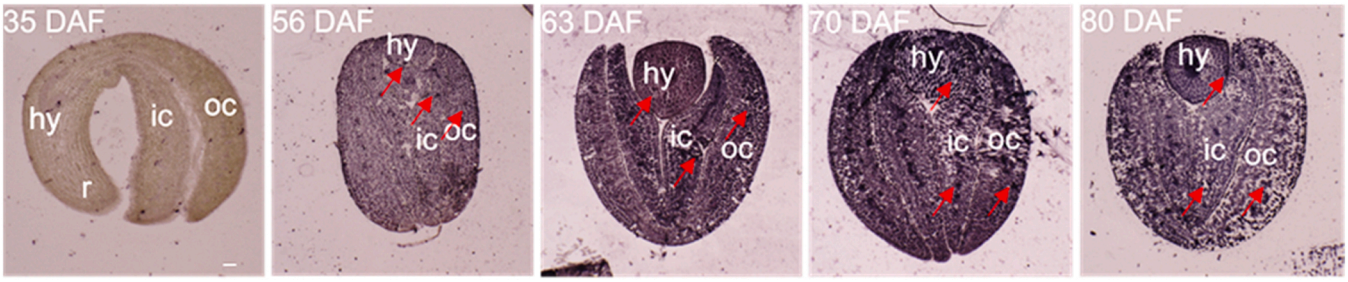


Fig. 3. The localization of *BnCRSH* transcripts in seeds of *B. napus* collected at 35, 56, 63, 70, and 80 days after flowering (DAF). Abbreviations: hy – hypocotyl, ic – inner cotyledons, oc – outer cotyledons, r – radicle. Scale bar – 100 μm. Red arrows show signals of hybridization.

production but also to increase the valuable traits of seeds.

2. Materials and methods

2.1. Plant growth conditions

Plants of *B. napus* spring cultivar 'Karo' were grown in garden soil in the growth chamber, at the constant temperature of 24 °C, at 16 h light and 8 h dark cycles, with the light intensity of 250 μmol m^{-2} s^{-1}. The flowering time ranged from 127 to 136 days after sowing. Each flower was tagged at the time of opening for determination of the days after flowering (DAF). Seeds were collected at five different developmental stages, i.e., 35, 56, 63, 70, and 80 days after flowering, frozen in liquid nitrogen, and stored at − 80 °C.

2.2. Flow cytometry

For flow cytometric analysis, embryo axes were dissected from seeds collected at five developmental stages. Samples were prepared as previously described by Rewers and Śliwińska (2012) using Galbraith's buffer (45 mM magnesium chloride, 30 mM sodium citrate, 20 mM MOPS (3-(N-morpholino) propane sulfonic acid, 0.1% Triton X-100; pH 7.0) (Galbraith et al., 1983); supplemented with 2 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI). Before analysis, samples were incubated for 10 min on ice. For each sample, fluorescence was measured in at least 1400 nuclei, using a CyFlow Ploidy Analyser flow cytometer (Sysmex--Partec GmbH, Gorlitz, Germany). Analyses were performed on five biological replicates using logarithmic amplification. Histograms were collected as FCS files and evaluated using FCS Express v.5, De Novo Software (Sysmex-Partec GmbH, Gorlitz, Germany). The proportion of nuclei with different DNA contents, mean C-value, and the (Σ > 2C)/2C ratio was established. The (Σ > 2C)/2C ratio is the ratio between the number of nuclei with DNA content higher than 2C (4C and 8C) to the number of nuclei with 2C DNA content. In this study, nuclei possessing at least 8C DNA content were classified as endopolyploid, since it is not possible to distinguish by flow cytometry the 4 C nuclei in cells that have just entered the endoreduplication cycle (being in the G1 phase of the first endocycle) from those in cells in the G2 phase of the mitotic cycle.

2.3. In silico analysis of plants CRSH

Plant CRSH amino acid sequences were obtained from the NCBI protein database and then analyzed using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Clustal Omega (http://www.clustal.org/omega/) was used for multiple sequence alignments. In the putative amino acid sequences of CRSH proteins the amino acids that form EF-hand motifs were identified based on sequence alignments described previously (Grabarek, 2006; Tozawa et al., 2007). The phylogenetic analysis was carried out in Clustal Omega using the neighbor-joining method and visualized by iTOL (https://itol.embl.de/).

2.4. Analysis of BnCRSH transcript level during seed development

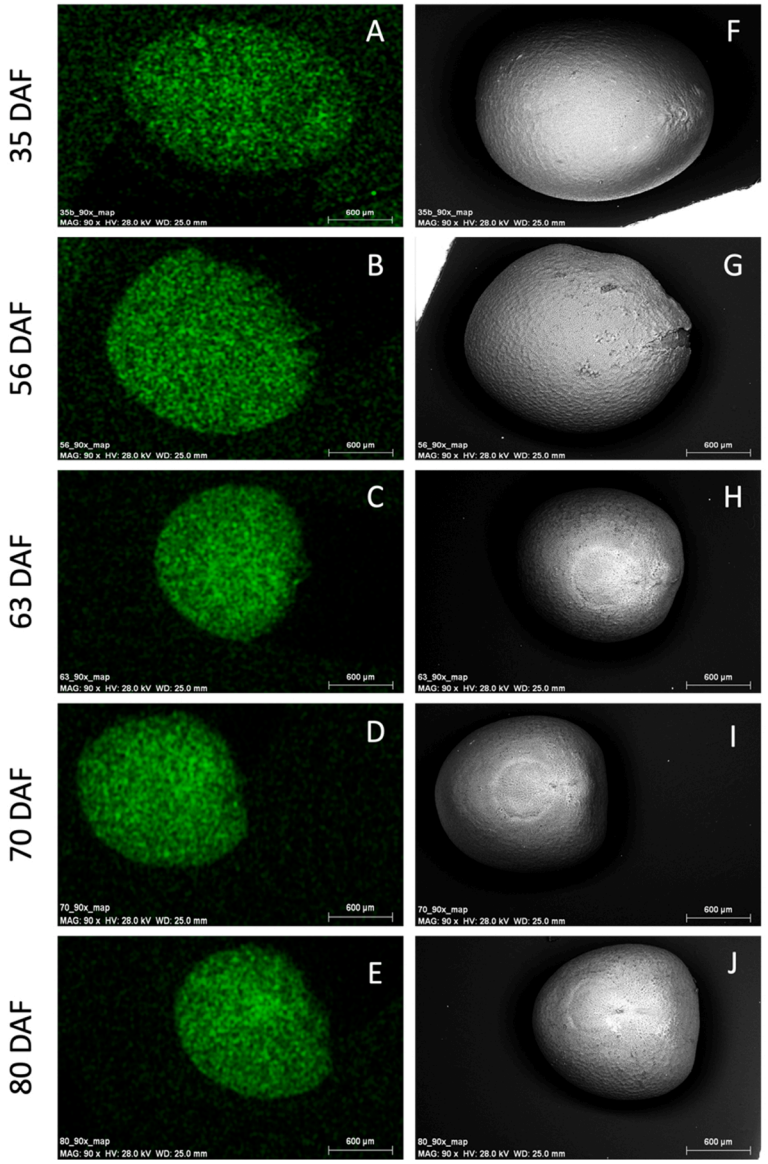
Total RNAs were isolated from *B. napus* seeds (35, 56, 63, 70, and 80 DAF) using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The integrity and quantity of RNA were analyzed by spectrophotometric measurement and gel electrophoresis in 1% agarose gel in 1x TAE (Tris-Acetate-EDTA) buffer stained with ethidium bromide. To remove genomic DNA contamination RNA was treated with 1 U of DNase I (Thermo Fisher Scientific, Waltham, MA, USA). The DNase I was heat-inactivated in the presence of 5 mM EDTA. The cDNA was synthesized from 1.5 μg of total RNA using 0.25 μg oligo $(dT)_{20}$ primer and 0.2 μg of random hexamers with NG dART RT Kit (EURx, Gdańsk, Poland) according to the manufacturer's protocol. The reaction was performed at 25 °C for 10 min, followed by 50 min at 50 °C.

The RT-qPCR reaction mixture included 4 μL of 2-fold diluted cDNA, gene-specific primers at a final concentration of 0.5 μM each, and 5 μL of LightCycler 480 SYBR Green I Master (Roche, Penzberg, Germany) in a total volume of 10 μL. Primers for *BnCRSH* (XM_013829418.2) gene are as follows: forward 5′- ACGTTCTCGGTCTCCGTGTC-3′ and reverse 5′-CGCTTTCGGCTTAGCGATGT-3′. Ubiquitin-conjugating enzyme 9 (*BnUBC9*, XM_013816206.2) was used as a reference gene, and primers were as follows: forward 5′-GCATCTGCCTCGACATCTTGA-3′ and reverse 5′-GACAGCAGCACCTTGGAAATG-3′. The reaction was performed in triplicate (technical replicates) in LightCycler 480 Instrument II (Roche, Penzberg, Germany). The thermal cycling conditions were as follows: initial denaturation 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 59 °C for 20 s, and 72 °C for 20 s. The SYBR Green I fluorescence signal was recorded at the end of the extension step in each cycle. The specificity of the assay was confirmed by the melt curve analysis (55–95 °C at a ramp rate of 0.11 °C/s). The fold-change in transcript level was calculated using LightCycler 480 Software release 1.5.1.62 (Roche, Penzberg, Germany).

2.5. In situ localization of BnCRSH transcripts

B. napus seeds (35, 56, 63, 70, and 80 DAF), dissected from the pods and submerged in a solution containing 4% paraformaldehyde and 0.1% glutaraldehyde, were vacuum infiltrated at 28 psi for 1 min. Following five 10-min washes in phosphate-buffered saline (PBS; pH 7.0), samples were fixed overnight at 4 °C in PBS buffer. Seeds were washed for 15 min in PBS and subjected to 2.3 M sucrose gradient infiltration performed overnight at 4 °C. The gradient consisted of 10%, 15%, and 30% 2.3 M sucrose, respectively, in a 0.1 M PBS buffer. For cryosectioning, seeds were transferred to a Hyrax C25 PLMC cryostat (Zeiss, Warsaw, Poland), cooled down to − 25 °C, and embedded in O.C.T. embedding matrix (Cell Path, Newton, Wales, UK). The frozen seeds were cut into 18- or 20-μm thick cross-sections transferred on SuperFrost® Plus microscope slides (Thermo Fisher Scientific, Braunschweig, Germany), and stored for 3–4 days at − 20 °C.

The probe for the in situ localization of *BnCRSH* transcript was prepared by PCR using *BnCRSH* partial cDNA cloned into pJET1.2 vector



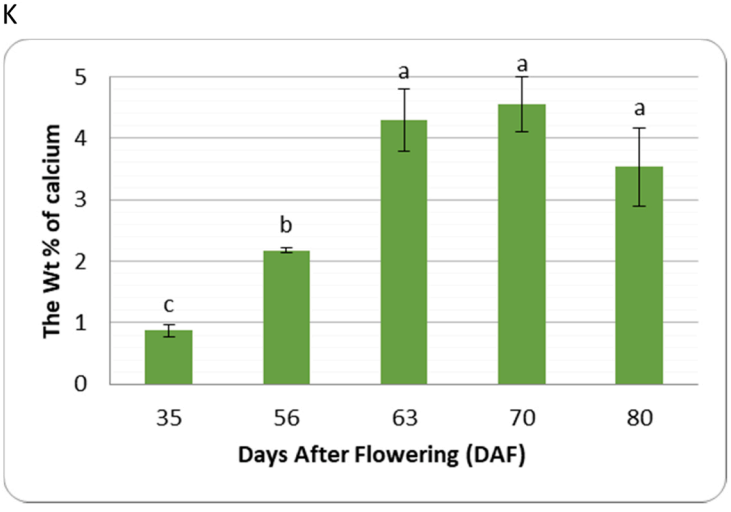


Fig. 4. The analysis of calcium content in seeds of *B. napus* collected at 35, 56, 63, 70, and 80 days after flowering (DAF) using SEM-EDX. (A, B, C, D, and E**)** represent the SEM with the EDX map of calcium in seeds of *B. napus* L. at 35, 56, 63, 70, and 80 DAF (green color represents calcium ions). (F, G, H, I, and J) represent the SEM without the EDX map images of (A, B, C, D, and E), respectively; (K) The weight percent (wt %) of the calcium in seeds collected 35, 56, 63, 70, and 80 DAF. Bars represent means ± SD. The statistically significant differences (labeled with different letters) were analyzed using one-way ANOVA test followed by Tukey's honest significance test ($p < 0.01$).

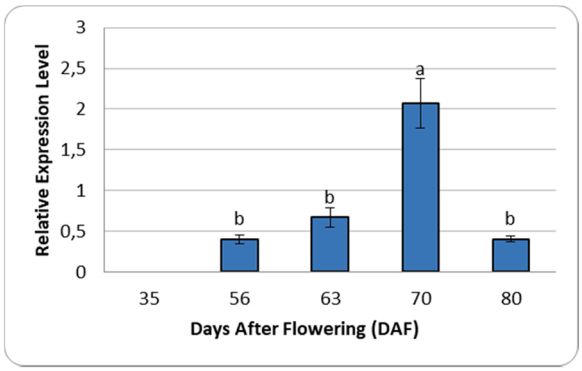


Fig. 5. The expression analysis of *BnCRSH* in seeds of *B. napus* collected at 35, 56, 63, 70, and 80 days after flowering (DAF). The chart shows the relative transcript level of *BnCRSH* normalized to the expression of the reference gene (*BnUBC9*). Bars represent means ± SD. The statistically significant differences (labeled with different letters) were analyzed using one-way ANOVA test followed by Tukey's honest significance test ($p < 0.01$).

as a template. A PCR reaction mixture contained 1.25 U of Opti*Taq* DNA polymerase (EURx, Gdańsk, Poland), 2 μL of DIG DNA Labelling Mix (Roche Molecular Biochemicals, Basel, Switzerland), and gene-specific primers at a final concentration of 0.2 μM in a total reaction volume of 20 μL. The PCR primers used for the synthesis of the *BnCRSH* probe were as follows: forward 5′- ACGTTCTCGGTCTCCGTGTC-3′ and reverse 5′-CGCTTTCGGCTTAGCGATGT-3′. To evaluate the probe labeling efficiency, the control PCR reaction was performed i.e., in the PCR reaction mixture DIG DNA Labelling Mix was replaced with 2 μL of 2 mM dNTP. The specificity and efficiency of the labeling reaction were determined by gel electrophoresis in 1.5% agarose gel in 1x TAE buffer stained with ethidium bromide. The probe was purified using PCR/DNA Clean-Up Purification Kit (EURx, Gdańsk, Poland) according to the manufacturer's protocol.

All cryosections of seeds were treated with proteinase K (1 μg/mL) in 100 mM Tris-HCl pH 8.0, 50 mM EDTA at 37 °C before in situ hybridization. The samples were hybridized in a solution containing 50% (v/v) deionized formamide, 10% dextran sulfate, 50% Denhardt's solution, and in situ salt (2 M NaCl, 50 mM EDTA, 100 mM Tris-HCl pH 8.0, 50 mM NaH_2PO_4, 50 mM Na_2HPO_4) with the probe (1:4) in 50% formamide overnight at 65 °C. After hybridization, the slides were washed in prewarmed 0.2 × SSC buffer (30 mM NaCl, 3 mM sodium citrate; pH 7.0) for 2 h. The hybridized probes were detected using an alkaline phosphatase-conjugated anti-DIG antibody and NBT/BCIP solution as substrates (Roche Molecular Biochemicals, Basel, Switzerland). Stained slides were analyzed by using a 3D VHX-7000 Digital Microscope (KEYENCE, Mechelen, Belgium). The following negative controls were included: digestion of RNA with RNase A (50 μg/mL in NTE buffer at 37 °C for 30 min), digestion of DNA with DNase (3 U/μL at 37 °C for 30 min), hybridization without the probe, and detection of the probe without the anti-DIG antibody.

2.6. Analysis of calcium levels in B. napus seeds

The calcium content in *B. napus* seeds (35, 56, 63, 70, and 80 DAF) was analyzed with a Scanning Electron Microscope (SEM), LEO Electron Microscopy Ltd., 1430 VP (Cambridge, UK) equipped with detectors of backscattered electron (BSE), cathodoluminescence (CL), and an energy dispersive X-ray spectrometer (EDX) Quantax with an XFlash 4010 detector (Bruker AXS microanalysis GmbH, Berlin, Germany). Calcium content analysis was carried out at acceleration voltage, HV: 28.0 kV, live time 40 s, working distance, WD: 25.0 mm, and 100 × magnification.

2.7. Statistical analysis

Statistical differences were assessed using one-way ANOVA ($p < 0.05$) followed by Tukey's honest significance test ($p < 0.01$) or Duncan's test ($p < 0.05$). Statistical analyses were performed using R version 4.1.1 and packages DescTools and ggplot2 (r-project.org).

3. Results

3.1. Flow cytometry

Flow cytometric analysis showed the presence of nuclei with 2C and 4C DNA content in all investigated developmental stages (Table 1). Additionally, endopolyploid nuclei (8C DNA content) were observed in seeds collected 35 DAF; however, during further stages of seed development 8C nuclei were no longer present. From 56 DAF the percentage of 4C nuclei gradually decreased and reached the lowest value of 2.6% in seeds collected at 70 and 80 DAF. DNA replication intensity (cell cycle activity), described by (Σ>2C)/2C ratio was at the highest level in seeds

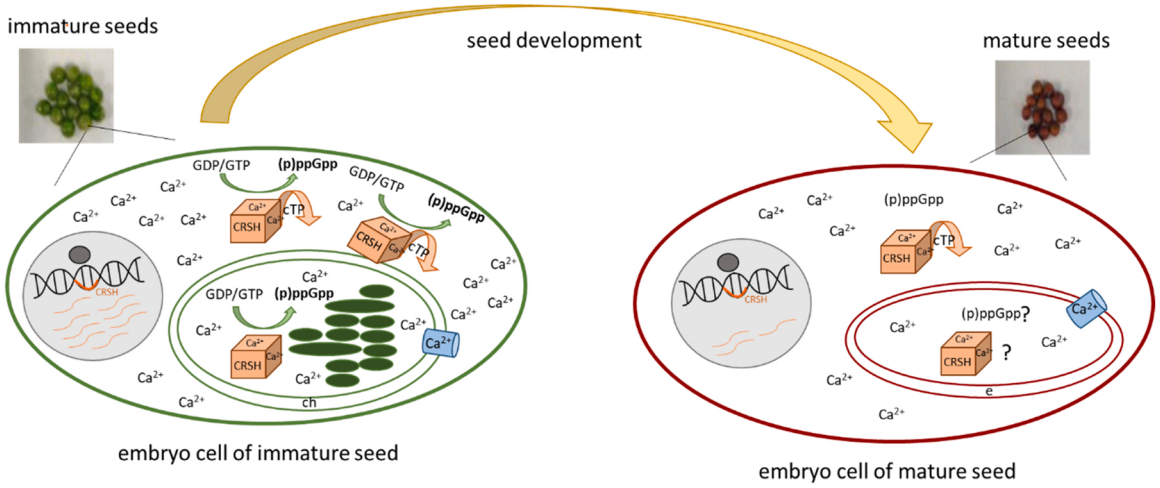


Fig. 6. The overview of the proposed model of functioning of Ca^{2+} -dependent stringent response during *B. napus* seed development. In immature seeds i.e., 35, 56, 63, and 70 days after flowering (DAF) high level of calcium and high level of *BnCRSH* expression led to the high level of (p)ppGpp both in the cytoplasm and the chloroplast. In mature seeds (80 DAF) the level of Ca^{2+} and of *BnCRSH* transcripts is lower than during seed development (35, 56, 63, and 70 DAF) and thus probably also the level of (p)ppGpp is lower. It is not clear whether CRSH proteins can be transported to eoplasts. Abbreviations: cTP – chloroplast transit peptide, ch – chloroplast, e – eoplast, GTP- guanosine-5'-triphosphate, GDP- guanosine-5'-diphosphate.

at 35 DAF, and next constantly decreased to the lowest level at 70 and 80 DAF (Table 1).

3.2. In silico analysis of plant CRSHs

The analysis of the evolutionary relationship among plant CRSHs based on analysis of 84 full-length amino acid sequences from 63 different plants species was performed (Fig. 1). The phylogenetic analysis showed the presence of two separate CRSH groups i.e., monocots CRSH (Fig. 1, highlighted in blue) and dicots CRSH (Fig. 1, highlighted in green and yellow). In the dicot species, CRSHs of the *Brassicaceae* family are grouped into a separate branch of the phylogenetic tree. CRSH from *Tarenaya hassleriana* (Chodat) Iltis (spider flower) that belongs to the *Brassicaceae* sister family, the *Cleomaceae*, is localized onto the same branch of the tree.

To compare the structure of EF-hand motifs among plant CRSHs, amino acid sequences of this Ca^{2+}-binding domain of CRSH proteins from 62 plant species were aligned (Fig. 2). EF-hand motif is localized on C-termini of CRSH proteins and is highly evolutionary conserved among analyzed plant species. The evolutionary conservation of this calcium-binding motif indicates that Ca^{2+}-dependent (p)ppGpp signaling plays an important physiological role.

3.3. In situ localization of BnCRSH transcripts during seed development

To investigate the possible role of the Ca^{2+}-dependent stringent response in seed development, the in situ localization of *CRSH* transcripts was performed in *B. napus* seeds collected 35, 56, 63, 70, and 80 days after flowering (Fig. 3). Hybridization signal was not observed in seeds collected 35 days after flowering. After 56, 63, 70, and 80 DAF, the transcripts of *BnCRSH* were detected in hypocotyl, inner and outer cotyledons. The localization of *BnCRSH* mRNA remained unchanged during development but the intensity of the hybridization signal increased at later stages of seed development. A hybridization signal was not observed in any of the control experiments (Supplementary Fig. 1).

3.4. Level of Ca^{2+} in developing B. napus seeds

The content and distribution of calcium ions in developing canola seeds (35, 56, 63, 70, and 80 DAF) were determined using dispersive energy spectroscopy coupled with the SEM instrument (Fig. 4). The obtained results showed that the weight percent (wt%) of calcium increased gradually during canola seed development and reached the maximum level in seeds collected at 70 DAF. In seeds collected 35 days after flowering, the calcium level was relatively low while the seed was unripe (green) and hydrated. A significant increase in the calcium content compared to 35 DAF was observed in seeds collected 56 DAF i.e., the level of calcium increased almost 2.5-fold. Between 56 DAF and 63 DAF the amount of calcium in seeds doubled. There was a 5-fold increment of calcium wt% in seeds collected 70 days after flowering compared to 35 DAF. Interestingly, the calcium level slightly decreased in mature seeds i.e., collected 80 DAF however the difference between 70 DAF and 80 DAF was not statistically significant (Fig. 4).

3.5. BnCRSH transcript level in developing B. napus seeds

The transcript level of *BnCRSH* was examined in canola seeds collected 35, 56, 63, 70, and 80 DAF (Fig. 5). During seed development the expression of *BnCRSH* gradually increased and reached the highest level in seeds collected 70 days after flowering, 4-fold higher than in seeds collected 56 DAF. The amount of *BnCRSH* transcript was under the detection limit in seeds collected 35 DAF. The expression of *BnCRSH* in mature seeds (80 DAF) significantly decreased in comparison to seeds collected 70 DAF i.e., the transcript level of *BnCRSH* in seeds collected after 80 days after flowering was almost 4.5-fold lower than in seeds collected 70 days after flowering (Fig. 5).

4. Discussion

Over the last 20 years, it has become apparent that the plant mechanism homologous to the bacterial stringent response is not just a leftover from the endosymbiotic ancestor of plastids. In plants, stringent response plays a crucial role in the regulation of chloroplast gene expression, photosynthesis, growth, nutrient remobilization, immunity, and adaptation to environmental changes. This study analyzed the potential role of Ca^{2+}-dependent stringent response in *B. napus* seed development.

The rate of DNA synthesis during seed development could be utilized as a predictor of seed germination ability. Flow cytometry is currently an effective, rapid, and accurate method for the measurement of nuclear DNA content and cell cycle activity. By isolating nuclei from the embryo and/or endosperm tissues, this method can be utilized for analysis of cell cycle/endoreduplication in seeds (Śliwińska, 2006). The use of flow cytometry to characterize cell cycle progression during seed development, maturation, and germination is a convenient tool for the analysis of seed quality (Śliwińska, 2009). Furthermore, some nuclei in endosperm tissues undergo endoreduplication i.e., DNA replication without subsequent cell division. The exact role of this process remains elusive however it may be related to increased cell size, higher gene expression, and accelerated growth. Endoreduplication occurs frequently in higher plants including green pea (*Pisum sativum* L.), corn, potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.), rice (*Oryza sativa* L.), and cucumber (*Cucumis sativus* L.) (Joubes and Chevalier, 2000). At the beginning of embryo development cells actively divide, but mitotic activity decreases during maturation. Our flow cytometry results showed that the cell cycle activity constantly decreased during canola seed development and reached the lowest level 70 days after flowering. Flow cytometry can be utilized to define an appropriate harvest time (Śliwińska, 2009) and cell cycle arrest can be a marker of seed maturity (Rewers and Śliwińska, 2012).

Calcium is a well-known signaling molecule involved in multiple processes of plant growth and development. Relatively less is known about the role of calcium during the development of seeds. Recent studies have shown that Ca^{2+} ions are located around nuclei in developing endosperm in seeds of plants belonging to the genus *Agave*. It was suggested that calcium plays a key role in the division of the primary nucleus of endosperm and in nuclei migration during further endosperm development (Barranco-Guzmán et al., 2019). In mature rice seeds calcium is localized mostly in the hull and scutellum and during germination Ca^{2+} ions move to rachilla and leaf primordia (Lu et al., 2013). Here we analyzed the distribution, abundance, and dynamics of calcium by SEM-EDX analysis. Calcium ions were localized in the seed coat and in the embryonic axis in developing canola seeds and the localization was unchanged during seed development. We showed that the level of calcium increased during seed maturation, and the highest level of calcium was observed in seeds collected 70 days after flowering. The level of Ca^{2+} ions in the cytoplasm can change substantially and the magnitude, duration, and location of those Ca^{2+} spikes or waves form a unique signal decoded by Ca^{2+} sensors and effectors (Tian et al., 2020). The EF-hand motif is the most common Ca^{2+}-binding domain and is present in proteins involved in the modulation and detection of calcium signals (Kawasaki and Kretsinger, 2017). The presence of the EF-hand motif in plant CRSH proteins makes them the most interesting group of RSH since the presence of this motif is unprecedented in other plants and bacterial alarmones synthetases and hydrolases (Ito et al., 2017). It is even more interesting when we notice that only two other chloroplast proteins possess EF-hand domains i.e., S-adenosylmethionine transporter1-like and type-II NAD(P)H dehydrogenase NDA2 (Field, 2018). In vitro analysis of three rice CRSH proteins showed that (p)ppGpp was synthesized only in the presence of Ca^{2+} in the concentration of at least 100 μM. Further, it was confirmed that both EF-motifs are necessary for synthase activity (Tozawa et al., 2007). Also, for AtCRSH it was shown that the synthesis of (p)ppGpp occurs only in the presence of

calcium ions (Masuda et al., 2008a). The comparison of the amino acid sequences of the EF-hand motif of plant CRSH showed that the sequence of the EF-hand domain is highly conserved among both monocotyledonous and dicotyledonous plant species. The total number of genes encoding CRSH proteins varies among plant species. There are three *CRSH* genes in rice and in radish (*Raphanus sativus* L.) whereas there is only one *CRSH* gene in *A. thaliana* and *Z. mays*. The multiple *CRSH* genes likely arose because of duplication of the ancestral gene during evolution. Interestingly, *B. napus* has a single *CRSH* gene despite being an allotetraploid plant. Ito et al. (2017) suggested that Ca^{2+}-dependent (p)ppGpp signaling may have arisen during evolution concomitantly with Ca^{2+} signaling in chloroplasts of land plants to adapt to adverse conditions, such as environmental stress, pathogen attack, and physical injury.

Our RT-qPCR results showed that the level of *BnCRSH* transcripts significantly increases between 35 and 70 DAF, and drastically decreases in mature seeds (80 DAF). Relatively little is known about the expression pattern of any plant *RSH* during seed development. In *A. thaliana CRSH* is expressed in flowers in mature pistils, green petals, and immature sepals (Masuda et al., 2008a) however not in mature petals (Mizusawa et al., 2008). An overview of expression patterns of *CRSH* genes in various plant species (Supplementary Fig. 2) is available via the ePlant tool at the Bio-Analytic Resource for Plant Biology (bar.utoronto.ca). The transcript level of *AtCRSH (*AT3G17470) is low and stable during seed maturation. In *G. max*, the *CRSH* gene (*GLYMA_13G119600*) is highly expressed in flowers whereas in developing seeds the expression is rather low. The mRNA level of *GmCRSH*, similar to the transcript level of *BnCRSH*, increases during seed maturation and then decreases in mature seeds. The transcript level of rice *OsCRSH1* (*Loc_Os05g06890*) is very low both in developing and mature seeds. Analysis of *BnCRSH* (BnaA03G0349600ZS) expression using the eFP Browser at http://yanglab.hzau.edu.cn/BnTIR showed that during seed development the expression is stable during early phases of seed development and then gradually decreased and reached an undetectable level in mature canola seeds (Supplementary Fig. 2).

This is the first study showing the localization of the transcript of any plant RSH in developing seeds. In *B. napus* and other oil plants, during seed development there are two events of plastid differentiation. First, proplastids inherited from parents differentiate into functional chloroplasts and seeds turn green. Chloroplast produced O_2 and ATP that are necessary for seed filling. Further, during the desiccation phase chloroplasts de-differentiate into non-photosynthetic eoplasts present in mature canola seeds (Liebers et al., 2017). Based on the observation that the relative expression level of *BnCRSH* and the level of Ca^{2+} reached their maximum 70 days after flowering and then significantly decreased in mature seeds it might be speculated that this is due to re-differentiation of chloroplasts into eoplasts occurring at the end of seed development. Our results might suggest that stringent response is limited to photosynthetic chloroplasts. There is no data in the literature showing that stringent response functions also in non-photosynthetic plastids however this possibility cannot be completely ruled out (Masuda et al., 2008b). It should be also noticed that different chloroplast transit peptides mediated the translocation of reporter proteins not only to chloroplasts but also to other types of plastids including root leucoplasts (Eseverri et al., 2020).

In *A. thaliana* CRSH is important for fertilization and silique formation. In flowers of *AtCRSH* knockdown lines reduced level of pollination was observed. Moreover, those mutant plants produced smaller siliques and over 300 times fewer seeds than wild type plants (Masuda et al., 2008a). Knockdown mutation of *AtCRSH* led also to the production of lighter seeds probably due to the impairment of nutrient remobilization from senescent leaves (Sugliani et al., 2016). Interestingly, knockout mutation of *CRSH* in *A. thaliana* did not lead to an abnormal phenotype of flowers (Ono et al., 2021) which shows that further analyses are needed to determine the physiological role of calcium-dependent (p)ppGpp synthase in flowering and seed production. During seed development, developmental signals lead to increases in the levels of the secondary messenger Ca^{2+} in immature seeds. We hypothesize that during the maturation of canola seeds, the increasing level of Ca^{2+} leads to increased activity of CRSH and accumulation of (p)ppGpp (Fig. 6). Elevated level of alarmones leads to inhibition of the expression of not only plastidial but also nuclear genes (Boniecka et al., 2017) which is necessary for the fine-tuning of the metabolism of developing seeds. During the desiccation phase, the level of *BnCRSH* expression and the level of calcium ions decreased therefore in mature seeds the level of alarmones is possibly lower than in developing seeds. It is also possible that stringent response functions in eoplast and other types of plastids (Fig. 6).

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CRediT authorship contribution statement

G.B.D. conceived and designed the research. S.T., A.M-A., and G.B.D. wrote the main manuscript. K.G. and S.T. analyzed of in situ localization. M.R., I.J., and S.T. did the flow cytometry analysis. A. Sz-Cz., S.T., and G.B.D. did the analysis of calcium content. A.M-A. and S.T did the gene expression analysis. S.T. did the statistical analysis. All the authors read and approved the manuscript.

Declaration of Competing Interest

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.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2023.116439.

References

Abdelkefi, H., Sugliani, M., Ke, H., Harchouni, S., Soubigou-Taconnat, L., Citerne, S., Mouille, G., Fakhfakh, H., Robaglia, C., Field, B., 2018. Guanosine tetraphosphate modulates salicylic acid signalling and the resistance of *Arabidopsis thaliana* to Turnip mosaic virus. Mol. Plant Pathol. 19, 634–646. https://doi.org/10.1111/mpp.12548.

Anil, V.S., Sankara Rao, K., 2001. Calcium-mediated signal transduction in plants: a perspective on the role of Ca^{2+} and CDPKs during early plant development. J. Plant Physiol. 158, 1237–1256. https://doi.org/10.1078/0176-1617-00550.

Anil, V.S., Harmon, A.C., Rao, K.S., 2000. Spatio-temporal accumulation and activity of calcium-dependent protein kinases during embryogenesis, seed development, and germination in sandalwood. Plant Physiol. 122, 1035–1043. https://doi.org/10.1104/PP.122.4.1035.

Atkinson, G.C., Tenson, T., Hauryliuk, V., 2011. The RelA/SpoT Homolog (RSH) superfamily: Distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. PLoS One 6, e23479. https://doi.org/10.1371/journal.pone.0023479.

Barranco-Guzmán, A.M., González-Gutiérrez, A.G., Rout, N.P., Verdín, J., Rodríguez-Garay, B., 2019. Cytosolic calcium localization and dynamics during early endosperm development in the genus *Agave* (Asparagales, Asparagaceae). Protoplasma 256, 1079–1092. https://doi.org/10.1007/S00709-019-01366-2.

Berdychowska, J., Boniecka, J., Dąbrowska, G.B., 2019. The stringent response and its involvement in the actions of bacterial cells to stress. Adv. Microbiol 58, 127–142. https://doi.org/10.21307/pm-2019.58.2.127.

Bewley, J.D., Bradford, K.J., Hilhorst, H.W.M., Nonogaki, H., 2013. In: Agrawal, G., Rakwal, R. (Eds.), Seeds: Physiology of development, germination and dormancy. Springer, New York, NY, pp. 1–392. https://doi.org/10.1007/978-1-4614-4693-4.

Boniecka, J., Prusińska, J., Dąbrowska, G.B., Goc, A., 2017. Within and beyond the stringent response-RSH and (p)ppGpp in plants. Planta 246, 817–842. https://doi.org/10.1007/s00425-017-2780-y.

Burton, M.G., Lauer, M.J., McDonald, M.B., 2000. Calcium effects on soybean seed production, elemental concentration, and seed quality. Crop Sci. 40, 476–482. https://doi.org/10.2135/cropsci2000.402476x.

Cashel, M., Gallant, J., 1969. Two compounds implicated in the function of the RC gene of *Escherichia coli*. Nature 224, 177–178. https://doi.org/10.1038/221838a0.

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I.A.P., Tang, H., Wang, Xiyin, Chiquet, J., Belcram, H., Tong, C., Samans, B., Corréa, M., Da Silva, C., Just, J., Falentin, C., Koh, C.S., Le Clainche, I., Bernard, M., Bento, P., Noel, B., Labadie, K., Alberti, A., Charles, M., Arnaud, D., Guo, H., Daviaud, C., Alamery, S., Jabbari, K., Zhao, M., Edger, P.P., Chelaifa, H., Tack, D., Lassalle, G., Mestiri, I., Schnel, N., Le Paslier, M. C., Fan, G., Renault, V., Bayer, P.E., Golicz, A.A., Manoli, S., Lee, T.H., Thi, V.H.D., Chalabi, S., Hu, Q., Fan, C., Tollenaere, R., Lu, Y., Battail, C., Shen, J., Sidebottom, C. H.D., Wang, Xinfa, Canaguier, A., Chauveau, A., Bérard, A., Deniot, G., Guan, M., Liu, Z., Sun, F., Lim, Y.P., Lyons, E., Town, C.D., Bancroft, I., Wang, Xiaowu, Meng, J., Ma, J., Pires, J.C., King, G.J., Brunel, D., Delourme, R., Renard, M., Aury, J. M., Adams, K.L., Batley, J., Snowdon, R.J., Tost, J., Edwards, D., Zhou, Y., Hua, W., Sharpe, A.G., Paterson, A.H., Guan, C., Wincker, P., 2014. Early allopolyploid evolution in the post-neolithic *Brassica napus* oilseed genome. In: Science, 345, pp. 950–953. https://doi.org/10.1126/science.1253435.

Chen, H., Yang, Q., Chen, K., Zhao, S., Zhang, C., Pan, R., Cai, T., Deng, Y., Wang, X., Chen, Y., Chu, W., Xie, W., Zhuang, W., 2019. Integrated microRNA and transcriptome profiling reveals a miRNA-mediated regulatory network of embryo abortion under calcium deficiency in peanut (*Arachis hypogaea* L.). BMC Genom. 20, 392. https://doi.org/10.1186/s12864-019-5770-6.

Costa, A., Navazio, L., Szabo, I., 2018. The contribution of organelles to plant intracellular calcium signalling. J. Exp. Bot. 69, 4175–4193. https://doi.org/10.1093/JXB/ERY185.

Dąbrowska, G., Prusińska, J., Goc, A., 2006. The stringent response - the mechanism of bacterial adaptive response to stress conditions. Adv. Biochem. 52, 87–93.

Dąbrowska, G., Prusińska, J., Goc, A., 2006a. Plant mechanism of an adaptive stress response homologous to bacterial stringent response. Adv. Biochem. 52, 94–100.

Dąbrowska, G.B., Turkan, S., Tylman-Mojżeszek, W., Mierek-Adamska, A., 2021. In silico study of the RSH (RelA/SpoT Homologs) gene family and expression analysis in response to PGPR bacteria and salinity in *Brassica napus*. Int. J. Mol. Sci. 22, 10666. https://doi.org/10.3390/IJMS221910666.

Day, I.S., Reddy, V.S., Shad Ali, G., Reddy, A., 2002. Analysis of EF-hand-containing proteins in *Arabidopsis*. research0056.1-0056.24 Genome Biol. 3. https://doi.org/10.1186/GB-2002-3-10-RESEARCH0056.

Dumas, C., Gaude, T., 2006. Fertilization in plants: is calcium a key player? Semin. Cell Dev. Biol. 17, 244–253. https://doi.org/10.1016/j.semcdb.2006.02.005.

Edwards, J., Hertel, K., 2011. Canola growth & development. Department of Primary Industries.

Eseverri, Á., López-Torrejón, G., Jiang, X., Burén, S., Rubio, L.M., Caro, E., 2020. Use of synthetic biology tools to optimize the production of active nitrogenase Fe protein in chloroplasts of tobacco leaf cells. Plant Biotechnol. J. 18, 1882–1896. https://doi.org/10.1111/PBI.13347.

Field, B., 2018. Green magic: regulation of the chloroplast stress response by (p)ppGpp in plants and algae. J. Exp. Bot. 69, 2797–2807. https://doi.org/10.1093/jxb/erx485.

Galbraith, D.W., Harkins, K.R., Maddox, J.M., Ayres, N.M., Sharma, D.P., Firoozabady, E., 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220, 1049–1051. https://doi.org/10.1126/SCIENCE.220.4601.1049.

Grabarek, Z., 2006. Structural basis for diversity of the EF-hand calcium-binding proteins. J. Mol. Biol. 359, 509–525. https://doi.org/10.1016/J.JMB.2006.03.066.

Hepler, P.K., 2005. Calcium: a central regulator of plant growth and development. Plant Cell. https://doi.org/10.1105/tpc.105.032508.

Honoki, R., Ono, S., Oikawa, A., Saito, K., Masuda, S., 2018. Significance of accumulation of the alarmone (p)ppGpp in chloroplasts for controlling photosynthesis and metabolite balance during nitrogen starvation in *Arabidopsis*. Photosynth. Res. 135, 299–308. https://doi.org/10.1007/s11120-017-0402-y.

Ito, D., Ihara, Y., Nishihara, H., Masuda, S., 2017. Phylogenetic analysis of proteins involved in the stringent response in plant cells. J. Plant Res 130, 625–634. https://doi.org/10.1007/s10265-017-0922-8.

Joubes, J., Chevalier, C., 2000. Endoreduplication in higher plants. Plant Mol. Biol. 43, 735–745. https://doi.org/10.1023/A:1006446417196.

Kawasaki, H., Kretsinger, R.H., 2017. Structural and functional diversity of EF-hand proteins: Evolutionary perspectives. Protein Sci. 26, 1898–1920. https://doi.org/10.1002/PRO.3233.

Liebers, M., Grübler, B., Chevalier, F., Lerbs-Mache, S., Merendino, L., Blanvillain, R., Pfannschmidt, T., 2017. Regulatory shifts in plastid transcription play a key role in morphological conversions of plastids during plant development. Front. Plant Sci. 8, 23. https://doi.org/10.3389/FPLS.2017.00023/BIBTEX.

Lu, L., Tian, S., Liao, H., Zhang, J., Yang, X., Labavitch, J.M., Chen, W., 2013. Analysis of metal element distributions in rice (*Oryza sativa* L.) seeds and relocation during germination based on X-Ray fluorescence imaging of Zn, Fe, K, Ca, and Mn. PLoS One 8, e57360. https://doi.org/10.1371/JOURNAL.PONE.0057360.

Masuda, S., Mizusawa, K., Narisawa, T., Tozawa, Y., Ohta, H., Takamiya, K.I., 2008a. The bacterial stringent response, conserved in chloroplasts, controls plant fertilization. Plant Cell Physiol. 49, 135–141. https://doi.org/10.1093/pcp/pcm177.

Masuda, S., Tozawa, Y., Ohta, H., 2008b. Possible targets of "magic spots" in plant signalling. Plant Signal. Behav. 3, 1021–1023. https://doi.org/10.4161/psb.6766.

Mizusawa, K., Masuda, S., Ohta, H., 2008. Expression profiling of four RelA/SpoT-like proteins, homologues of bacterial stringent factors, in *Arabidopsis thaliana*. Planta 228, 553–562. https://doi.org/10.1007/s00425-008-0758-5.

Nakada-Freitas, P.G., Santos, J.T., dos, Hidalgo, G.F., Anjos, L.V.S., dos, Souza, E.P., de, Martins, I.R., Cardoso, A.I.I., Bardiviesso, E.M., Lanna, N., de, B.L., Catão, H.C.R.M., Heinrichs, R., 2021. Calcium in the production and quality of cauliflower seeds. Res. Soc. Dev. 10, e44710212763 https://doi.org/10.33448/rsd-v10i2.12763.

Navazio, L., Formentin, E., Cendron, L., Szabò, I., 2020. Chloroplast calcium signaling in the spotlight. Front. Plant Sci. 11, 186. https://doi.org/10.3389/fpls.2020.00186.

Nomura, H., Shiina, T., 2014. Calcium signaling in plant endosymbiotic organelles: mechanism and role in physiology. Mol. Plant 7, 1094–1104. https://doi.org/10.1093/MP/SSU020.

Ono, S., Suzuki, S., Ito, D., Tagawa, S., Shiina, T., Masuda, S., 2021. Plastidial (p)ppGpp synthesis by the Ca^{2+}-dependent RelA–SpoT homolog regulates the adaptation of chloroplast gene expression to darkness in *Arabidopsis*. Plant Cell Physiol. 61, 2077–2086. https://doi.org/10.1093/PCP/PCAA124.

Prusińska, J.M., Boniecka, J., Dąbrowska, G.B., Goc, A., 2019. Identification and characterization of the *Ipomoea nil* RelA/SpoT Homologs (*InRSHs*) and potential directions of their transcriptional regulation. Plant Sci. 284, 161–176. https://doi.org/10.1016/j.plantsci.2019.01.026.

Reddy, V.S., Reddy, A.S.N., 2004. Proteomics of calcium-signaling components in plants. Phytochemistry 65, 1745–1776. https://doi.org/10.1016/j.phytochem.2004.04.033.

Rewers, M., Śliwińska, E., 2012. Endoreduplication intensity as a marker of seed developmental stage in the *Fabaceae*. Cytom. Part A 81A, 1067–1075. https://doi.org/10.1002/cyto.a.22202.

Romand, S., Abdelkefi, H., Lecampion, C., Belaroussi, M., Dussenne, M., Ksas, B., Citerne, S., Caius, J., D'alessandro, S., Fakhfakh, H., Caffarri, S., Havaux, M., Field, B., 2022. A guanosine tetraphosphate (ppGpp) mediated brake on photosynthesis is required for acclimation to nitrogen limitation in *Arabidopsis*. eLlife 11, e75041. https://doi.org/10.7554/ELIFE.75041.

Śliwińska, E., 2006. Nuclear DNA content analysis of plant seeds by flow cytometry. Curr. Protoc. Cytom. Chapter 7, Unit 7.29. https://doi.org/10.1002/0471142956.CY0729S35.

Śliwińska, E., 2009. Nuclear DNA replication and seed quality. Seed Sci. Res. 19, 15–25. https://doi.org/10.1017/S0960258508186275.

Sreenivasulu, N., Wobus, U., 2013. Seed-development programs: a systems biology–based comparison between dicots and monocots. Annu. Rev. Plant Biol. 64, 189–217. https://doi.org/10.1146/ANNUREV-ARPLANT-050312-120215.

Sugliani, M., Abdelkefi, H., Ke, H., Bouveret, E., Robaglia, C., Caffarri, S., Field, B., 2016. An ancient bacterial signaling pathway regulates chloroplast function to influence growth and development in *Arabidopsis*. Plant Cell 28, 661–679. https://doi.org/10.1105/tpc.16.00045.

Takahashi, K., Kasai, K., Ochi, K., 2004. Identification of the bacterial alarmone guanosine 5′-diphosphate 3′-diphosphate (ppGpp) in plants. Proc. Natl. Acad. Sci. U. S. A 101, 4320–4324. https://doi.org/10.1073/pnas.0308555101.

Tian, W., Wang, C., Gao, Q., Li, L., Luan, S., 2020. Calcium spikes, waves and oscillations in plant development and biotic interactions. Nat. Plants 6, 750–759. https://doi.org/10.1038/s41477-020-0667-6.

Tozawa, Y., Nozawa, A., Kanno, T., Narisawa, T., Masuda, S., Kasai, K., Nanamiya, H., 2007. Calcium-activated (p)ppGpp synthetase in chloroplasts of land plants. J. Biol. Chem. 282, 35536–35545. https://doi.org/10.1074/jbc.M703820200.

van der Biezen, E.A., 2000. Arabidopsis RelA/SpoT homologs implicate (p)ppGpp in plant signaling. Proc. Natl. Acad. Sci. 97, 3747–3752. https://doi.org/10.1073/pnas.060392397.

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511. https://doi.org/10.1093/AOB/MCG164.

Woodfield, H.K., Sturtevant, D., Borisjuk, L., Munz, E., Guschina, I.A., Chapman, K., Harwood, J.L., 2017. Spatial and temporal mapping of key lipid species in *Brassica napus* seeds. Plant Physiol. 173, 1998–2009. https://doi.org/10.1104/PP.16.01705.

Zeng, H., Zhang, Y., Zhang, X., Pi, E., Zhu, Y., 2017. Analysis of EF-hand proteins in soybean genome suggests their potential roles in environmental and nutritional stress signaling. Front. Plant Sci. 8, 877. https://doi.org/10.3389/fpls.2017.00877.

New seed coating containing *Trichoderma viride* with anti-pathogenic properties

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ABSTRACT

Background: To ensure food security in the face of climate change and the growing world population, multi-pronged measures should be taken. One promising approach uses plant growth-promoting fungi (PGPF), such as *Trichoderma*, to reduce the usage of agrochemicals and increase plant yield, stress tolerance, and nutritional value. However, large-scale applications of PGPF have been hampered by several constraints, and, consequently, usage on a large scale is still limited. Seed coating, a process that consists of covering seeds with low quantities of exogenous materials, is gaining attention as an efficient and feasible delivery system for PGPF.
Methods: We have designed a new seed coating composed of chitin, methylcellulose, and *Trichoderma viride* spores and assessed its effect on canola (*Brassica napus* L.) growth and development. For this purpose, we analyzed the antifungal activity of *T. viride* against common canola pathogenic fungi (*Botrytis cinerea*, *Fusarium culmorum*, and *Colletotrichum* sp.). Moreover, the effect of seed coating on germination ratio and seedling growth was evaluated. To verify the effect of seed coating on plant metabolism, we determined superoxide dismutase (SOD) activity and expression of the stress-related *RSH* (*RelA*/*SpoT* homologs).
Results: Our results showed that the *T. viride* strains used for seed coating significantly restricted the growth of all three pathogens, especially *F. culmorum*, for which the growth was inhibited by over 40%. Additionally, the new seed coating did not negatively affect the ability of the seeds to complete germination, increased seedling growth, and did not induce the plant stress response. To summarize, we have successfully developed a cost-effective and environmentally responsible seed coating, which will also be easy to exploit on an industrial scale.

Subjects Agricultural Science, Microbiology, Molecular Biology, Mycology, Plant Science
Keywords Seed coating, Germination, *Brassica napus* L., Plant stringent response, RelA/SpoT homologs, Plant growth-promoting fungi (PGPF), *Trichoderma*

INTRODUCTION

In 2021, as many as 828 million people around the world suffered from hunger, and, due to the slow economic recovery from the COVID-19 pandemic, it is predicted that nearly 670 million people will still be facing hunger in 2030 (*Food and Agriculture Organization (FAO), 2022*). Currently, to achieve a high yield, significant inputs of crop protection

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chemicals and fertilizers are required. Besides adverse effects on human health, these preparations pollute water, soil, and air and diminish the biological diversity in ecosystems. Therefore, there is an urgent need to develop new agricultural methods to minimize agrochemical use while ensuring production sustainability, food security, environmental responsibility, and cost-effectiveness. The application of plant growth-promoting fungi (PGPF) has been considered a promising approach to replace agrochemicals. Successful application of PGPF in the field is challenging because of the low level of survival, low efficiency of colonization of soil, and short shelf-life of inoculum (*Rojas-Sánchez et al., 2022*). Moreover, it is widely observed that bioformulations beneficial in laboratory conditions do not enhance plant growth and development in field studies (*Mawar, Manjunatha & Kumar, 2021*). The most common methods of application of PGPF are direct foliar, seed, root, and soil inoculation, which might give rise to problems when used on a large scale (*Lopes et al., 2021*). Recently, seed coating has been proposed as an efficient and cost-effective technology for delivering beneficial microorganisms (*Rocha et al., 2019b*; *Ma, 2019*). Seed dressing, film coating, and pelleting are the three major methods of seed coating application and are selected depending on the used chemicals, the form of seed, and selected microbes (*Pedrini et al., 2017*; *Rocha et al., 2019b*). In seed coating, three main types of materials are used: (i) binders, *i.e.*, usually a liquid with adhesive properties, (ii) fillers, *i.e.*, inner material that increases seed size, (iii) active compounds, *e.g.*, microorganisms, micronutrients, and plant growth promoters (*Pedrini et al., 2017*). The coating is performed to enhance seed morphology, to improve handle traits, including seed size and weight, and to deliver active substances (*Pedrini et al., 2017*; *Rocha et al., 2019a*).

Relatively little is known about the possible adverse effects of seed coating on seed metabolism. Reactive oxygen species (ROS) are oxygen-containing radicals commonly considered harmful at high levels that may lead to DNA, protein, and lipid damage. However, when maintained at a moderate level, they play a pivotal role in regulating many processes, including seed germination (*Bailly, 2019*). During seed imbibition, environmental cues are perceived and transduced with the participation of ROS. In permissive conditions, elevated ROS levels trigger germination (*Oracz & Karpiński, 2016*). The task of the cellular antioxidant system is to fine-tune the ROS level to match the current signaling needs. One of the primary antioxidant enzymes is superoxide dismutase (SOD) which catalyzes the conversion of superoxide radical ($O_2 \cdot^-$) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). Since SOD is the first line of defense against enhanced ROS production, the level of SOD activity is often used as a marker of oxidative stress (*Sarker & Oba, 2018*; *Luo et al., 2019*). Recently, much attention has been drawn to the stringent response-related plant *RSH* (*RelA*/*SpoT* homolog) genes that encode synthases/hydrolases of (pp)pGpp (guanosine tri-, tetra-, and pentaphosphate) called "alarmones", which serve as stress-signaling molecules. The stringent response was observed for the first time over a half-century ago in *Escherichia coli* (*Cashel & Gallant, 1969*), and plant *RSH* genes were first identified in *Arabidopsis thaliana* (L.) Heynh. (*van der Biezen et al., 2000*) and later in other plant species, including *Ipomoea nil* (L.) Roth (syn. *Pharbitis nil* (L.) Choisy) (*Dąbrowska, Prusińska & Goc, 2006*). Plant RSH proteins

have been proven to play a role in stress response to salinity (*Takahashi, Kasai & Ochi, 2004*; *Prusińska et al., 2019*; *Dąbrowska et al., 2021b*), drought (*Takahashi, Kasai & Ochi, 2004*; *Prusińska et al., 2019*), darkness (*Ono et al., 2021*), wounding, UV, heat shock, and pathogens (*Takahashi, Kasai & Ochi, 2004*). *Abdelkefi et al. (2018)* showed that, in *A. thaliana*, the accumulation of alarmones directly correlates with the susceptibility to *turnip mosaic virus* (TuMV) infection. Plant RSHs also play a role in reutilizing micro- and macro-elements from aging organs to seeds (*Boniecka et al., 2017*), photoperiod (*Prusińska et al., 2019*), and the interaction with plant growth-promoting rhizobacteria (*Szymańska et al., 2019*; *Dąbrowska et al., 2021b*).

Canola (*Brassica napus* L.) is, after soybean, the second most important plant oil source used not only for food but also for biofuel production. Canola is sensitive to various environmental stresses, and significant yearly yield loss therefore occurs (*Elferjani & Soolanayakanahally, 2018*). In this study, we aimed to develop a new, biodegradable coating for canola seeds to stimulate germination—one of the most critical stages in plant development that affects all further stages of plant growth and, consequently, yield. Although seed coating as a method of PGPF delivery has been gaining popularity during the last decade, there are still gaps in existing knowledge that prevent this approach being used on an industrial scale. A suitable formulation is challenging to select since the physical and chemical properties of different types of seed coating ingredients may differ depending on other ingredients. Some ingredients promoting the growth of microorganisms might negatively affect plant germination and growth and vice versa. Moreover, seed coating might reduce the shelf life of seeds (*Rocha et al., 2019b*). Since different microbes might react differently with coating materials, more research on different coating formulas is still needed. We created a seed coating consisting of *Trichoderma viride* Pers. spores, chitin as a filler, and methylcellulose as a binder. Fungi belonging to the genus *Trichoderma* have great potential to be used in agriculture because of their ability to stimulate plant growth and development, including in adverse environmental conditions (*Lorito & Woo, 2015*; *Contreras-Cornejo et al., 2016*; *Al-Ani, 2018*; *Macías-Rodríguez et al., 2020*) *via* various mechanisms including modifying the rhizosphere, modulating root architecture, increasing the availability of nutrients, and producing specific growth and development-promoting compounds (*Guzmán-Guzmán et al., 2019*; *Mastan et al., 2021*; *Antoszewski, Mierek-Adamska & Dąbrowska, 2022*). In addition, *T. viride* can grow on polymeric materials and possibly can degrade them (*Dąbrowska et al., 2021a*). We chose spores of *T. viride* as the active compound of the seed coating because our recent study showed the positive effect of this species on the development of canola (*Znajewska, Dąbrowska & Narbutt, 2018*). In addition, fungi belonging to *Trichoderma* might control populations of plant pathogens (*Rodríguez et al., 2021*) *via* the secretion of hydrolytic enzymes (*Pérez et al., 2002*). Therefore, we hypothesized that the tested *T. viride* strains possess anti-fungal activity and verified *T. viride* activity against plant pathogen fungi, *i.e.*, *Botrytis cinerea* Pers., *Fusarium culmorum* (Wm.G. Sm.) Sacc., and *Colletotrichum* sp. Those fungi are the most significant plant pathogens worldwide, causing diseases in a wide range of hosts, including cereals, legumes, vegetables, and fruit trees (*Kthiri et al., 2020*) and additionally posing serious health risks (*Juergensen & Madsen, 2009*; *Shivaprakash*

et al., 2011). They are present in food and feed made from contaminated cereal crops (*Błaszczyk et al., 2017*). Large-scale disease control techniques are implemented using chemical fungicides, which are expensive, harmful to living organisms, and environmentally irresponsible. Spraying with a spore suspension of *Trichoderma* fungi has a well-documented protective anti-fungal effect, *e.g.*, on soybean (*John et al., 2010*), chickpea (*Pradhan et al., 2022*) or cocoa (*Seng et al., 2014*). Recently, *Trichoderma*-enriched seed coatings have been developed for many plant seeds (*Cortés-Rojas et al., 2021*), and the coating allows beneficial microorganisms to colonize the roots at an early stage of growth (*Tavares et al., 2013*; *Ben-Jabeur et al., 2019*). Secondly, we checked the effect of seed coating on canola seed germination and seedling growth. We aimed to show that chitin could be used as a filler instead of commonly used chitosan. Although chitin has lower solubility than chitosan, chitosan is less stable, more hydrophilic, and more sensitive to changes in pH. Moreover, chitosan can form complexes with metals, including microelements, thereby reducing their bioavailability for plants and fungi. Chitosan also has stronger antimicrobial activity than chitin, which is a disadvantage for microbe-containing seed coatings (*Rinaudo, 2006*; *Aranaz et al., 2009*). From the industrial point of view, the coating materials should be low-cost, and chitin is significantly less expensive than chitosan. Lastly, we hypothesized that the newly developed seed coating would be inert to seed metabolism, and thus we assessed the expression of the *RSH* genes and the activity of SOD as markers of stress in plants.

MATERIALS AND METHODS

Microorganisms

The saprophytic fungi used in this study were obtained from the culture collection of the Department of Microbiology (GenBank NCBI accession numbers: OL221594.1–*T. viride* strain I (TvI) and OL221590.1–*T. viride* strain II (TvII), and from the culture collection of the Department of Environmental Microbiology and Biotechnology (The Bank of Pathogens, Institute of Plant Protection in Poznań; Faculty of Forestry and Wood Technology Poznań University of Life Sciences; *B. cinerea* - 873, *Colletotrichum* sp.- 1202, and *F. culmorum*- 2333), Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University in Toruń. The culture of fungi stored in agar slopes at 4 °C was transferred to a solid PDA (Potato Dextrose Agar, Difco, US) medium and incubated at 23 °C for 7 days. Fungal spores were collected with a sterile cotton-tipped applicator and suspended in sterile water.

In vitro antagonistic activity of *T. viride* strains against plant pathogens

In vitro antagonist activity of *T. viride* against plant pathogens (*B. cinerea*, *Colletotrichum* sp., and *F. culmorum*) was evaluated using the dual culture technique according to *Li et al. (2016)* and *Kunova et al. (2016)*. Mycelial discs of 5 mm diameter of 1-week-old *T. viride* and 1-week-old plant pathogens were placed on the opposite sides of Petri dishes (maintaining a distance of 6 cm between discs) containing Czapek-Dox agar (2.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \times 7\ H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \times 7\ H_2O$, 20 g glucose, 14 g agar, 1 g yeast extract dissolved in 1,000 mL distilled water). Control plates contained only

Table 1 Experimental variants tested in this study.

Variant	Binding agent	Filler	Active compound	Seed weight (mg)
Uncoated seed	–	–	–	5.142 ± 0.291b
M	+	–	–	5.863 ± 0.169a
C	–	+	–	5.301 ± 0.300b
M+C	+	+	–	5.902 ± 0.117a
TvI	–	–	+	5.220 ± 0.290b
TvII	–	–	+	5.219 ± 0.285b
M+C+TvI	+	+	+	5.903 ± 0.358a
M+C+TvII	+	+	+	5.905 ± 0.197a

Note:
The full seed coating (M+C+TvI, M+C+TvII) consists of *Trichoderma viride* spores (active compound), methylcellulose (binding agent), and chitin (filler). Uncoated seeds, seeds coated with methylcellulose (M), chitin (C), methylcellulose and chitin (M+C), and *T. viride* spores (TvI/TvII) served as control. The mass of one seed for each experimental variant was determined based on the weight of 100 random seeds. Values are the mean ± SD (n = 4). Different letters indicate significant differences between variants (ANOVA with Tukey's *post-hoc* test, $p < 0.05$).

mycelial discs of *B. cinerea*, *Colletotrichum* sp., and *F. culmorum*. The plates were incubated at 25 °C for 6 days in the dark, and the diameter of mycelia was measured. The pathogen growth inhibition was calculated according to the following formula:

$$\text{Inhibition}(\%) = [(C - T)/C] \times 100 \quad (1)$$

where C is the radial growth of plant pathogen (mm) when grown without *T. viride* (control), and T is the radial growth of plant pathogen (mm) in the presence of *T. viride* strains.

Seed coating

Seeds of *B. napus* 'Karo' were obtained from Plant Breeding Strzelce Ltd., Co. (Strzelce, Poland; IHAR-PIB Group). 'Karo' is a spring open-pollinated variety of canola, registered in Poland in 2016. This cultivar produces a high yield, seeds contain a high level of oil, and the plant is resistant to fungal pathogens. The seeds were surface sterilized with a mixture of 30% hydrogen peroxide and 96% ethanol (1:1, v:v) for 3 min and then rinsed at least six times with sterile distilled water. The complete coating mixture (C+M+TvI, C+M+TvII) consisted of a solution of 0.5% chitin dissolved in methylcellulose (final concentration of 2.5%) and *T. viride* spore suspension at a final concentration of 10^5 spores mL^{-1}.
For control, the following variants were used: (i) 0.5% chitin (C) that was dissolved in 1% acetic acid (the pH of the solution was adjusted to 6.0 using 1% NaOH), (ii) 2.5% methylcellulose (M), (iii) the mixture of methylcellulose (final concentration 2.5%) and chitin (final concentration 0.5%) (M+C), (iv) *T. viride* spores at a final concentration of 10^5 spores mL^{-1} in distilled sterile water (TvI/TvII). In each variant, 100 mg of canola seeds were incubated with 5 mL of mixture with shaking at 180 rpm for 15 min at room temperature. The summary of experimental variants and the means mass of seed tested in this study are presented in Table 1.

Impact of seed coating on *B. napus* germination and seedling growth

For the germination test, 25 seeds were placed in glass Petri dish (90 mm) with filter paper moistened with distilled water (conductivity < 0.08 μS cm^{-1}). Subsequently, the dishes were placed in a growth chamber, temperature regulated to 25 °C (at 16 h light and 8 h dark cycles), with supplemental lighting to maintain a light intensity, PAR = 100 μmol photons m^{-2} s^{-1}. The seed germination was monitored for 7 days. To assess the rate of germination, three different germination parameters were calculated. FGP and MGT were calculated following the formula by *Ranal & Santana (2006)*, while IGV was calculated using the formula reported by *Khan & Ungar (1984)*:

$$\text{Final germination percentage, FGP} = 100(\text{N}/\text{S}) \tag{2}$$

where N is the number of total seeds completed germination at the end of the experiment and S is the number of initial seeds used;
Index of germination velocity (modified Timson's index),

$$\text{IGV} = \Sigma \text{G}/\text{t} \tag{3}$$

where G is the percentage of seed germination at 1-day intervals, and t is the total germination period;
Mean germination time,

$$\text{MGT} = (\text{N1T1} + \text{N2T2} + \ldots + \text{NxTx})/(\text{N1} + \text{N2} + \ldots + \text{Nx}) \tag{4}$$

where N is the germination count on any counting period, and T is the time point in days until the last day (x).

To assess the growth, we measured the lengths of roots and shoots and the fresh and dry biomass of 100 seven-day-old seedlings. The plants were carefully separated into shoots and roots using a razor blade, and the lengths of roots and shoots were measured using a calibrated ruler. To assess the dry mass, samples were dried and weighed using an MA 50 moisture analyzer (Radwag, Radom, Poland).

Determination of SOD activity

For SOD activity analysis plants were grown as described above. Protein extracts were prepared according to *Rusaczonek et al. (2015)*. Briefly, 6-day-old seedlings were ground in liquid nitrogen, and then ~50 mg of the grounded tissue was suspended in 1 mL of ice-cold protein extraction buffer (100 mM tricine, 3 mM $MgSO_4$, 3 mM EGTA, 1 mM DTT, pH 7.5). Following the incubation on ice for 15 min, samples were centrifuged (4 °C, 20 min, 13,000 rcf). The obtained supernatant was used to determine total soluble protein concentration using PierceTM Coomassie (Bradford) Protein Assay Kit (Merck, Darmstadt, Germany) according to the manufacturer's protocol, with bovine serum albumin as a protein standard. SOD activity in the protein extract was measured according to *Beauchamp & Fridovich (1971)*, adapted by *Rusaczonek et al. (2015)*. Briefly, 100 μL of the working solution (0.1 M phosphate buffer pH 7.5, 2.4 μM riboflavin, 840 μM NBT, 150 mM methionine, 12 mM Na_2EDTA mixed in the ratio: 8:1:1:1:1 (v/v/v/v)) was added to 2 μL of the protein extract in a 96-well transparent microplate in three technical

Table 2 Primers used for *Brassica napus* L. *RSH* genes expression analysis.

Target gene	Gene ID	Name of oligomer	Nucleotide sequence (5′ → 3′)
BnRSH1	106399012	Sense antisense	5′- GGAGGTTCAGATCAGAACGG - 3′ 5′- CCATTCACCTTCGCTGCTAC - 3′
BnRSH2	111206471	Sense antisense	5′- GCAAGATGTTGAAGAATCTAACG - 3′ 5′- GCACAGACATCTTGTCATTTTCG - 3′
BnRSH3	106431664	Sense antisense	5′- CCGAAACTTTCCGATTTCAA - 3′ 5′- TCGTAGTCAACGCACGAGTC - 3′
BnCRSH	106439579	Sense antisense	5′- ACGTTCTCGGTCTCCGTGTC - 3′ 5′- CGCTTTCGGCTTAGCGATGT - 3′
BnUBC9	106376144	Sense antisense	5′- GCATCTGCCTCGACATCTTGA - 3′ 5′- GACAGCAGCACCTTGGAAATG - 3′

replicates for each of three biological replicates. The reaction was set in two identical plates, one illuminated with 400 µE warm white LED light for 16 min, and the other (blank) was kept in darkness. Then absorbance at 560 nm was measured. One unit of SOD activity was defined as the amount of enzyme required for 50% inhibition of NBT reduction in 1 min in the assay conditions.

Expression analysis of *BnRSH* genes

Real-time PCR (qPCR) assays were performed to evaluate the effects of seed coating on *BnRSH1, BnRSH2*, *BnRSH3*, and *BnCRSH* expression. Total RNA was isolated from 6-day-old seedlings using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The integrity and quantity of RNA were analyzed by spectrophotometric measurement and electrophoresis in 1% agarose gel in 1x TAE buffer stained with ethidium bromide. To remove genomic DNA contamination, RNA was treated with 1 U of DNase I (Thermo Fisher Scientific, Waltham, MA, US). The DNase I was heat-inactivated in the presence of 5 mM EDTA. The cDNA was synthesized from 1.5 µg of total RNA using both 250 ng oligo $(dT)_{20}$ primer and 200 ng random hexamers with NG dART RT Kit (EURx, Gdańsk, Poland). The reaction was performed at 25 °C for 10 min, followed by 50 min at 50 °C.

The qPCR reaction mixture included 4 µL of 2-fold diluted cDNA, gene-specific primers at a final concentration of 0.5 µM each, and 5 µL of LightCycler 480 SYBR Green I Master (Roche, Penzberg, Germany) in a total volume of 10 µL. Primers for *BnRSH* are listed in Table 2. The reaction was performed in three technical replicates for three biological replicates in LightCycler 480 Instrument II (Roche, Penzberg, Germany). PCR conditions were as follows: 95 °C for 5 min for initial denaturation, and 40 cycles of 95 °C for 10 s, 59 °C for 20 s, and 72 °C for 20 s. The fluorescence signal was recorded at the end of each cycle. To verify the specificity of the PCR reaction melt curve analysis was used (55 °C to 95 °C at a ramp rate of 0.11 °C/s). The relative gene expression was calculated using LightCycler 480 Software version 1.5.1.62 (Roche, Penzberg, Germany).

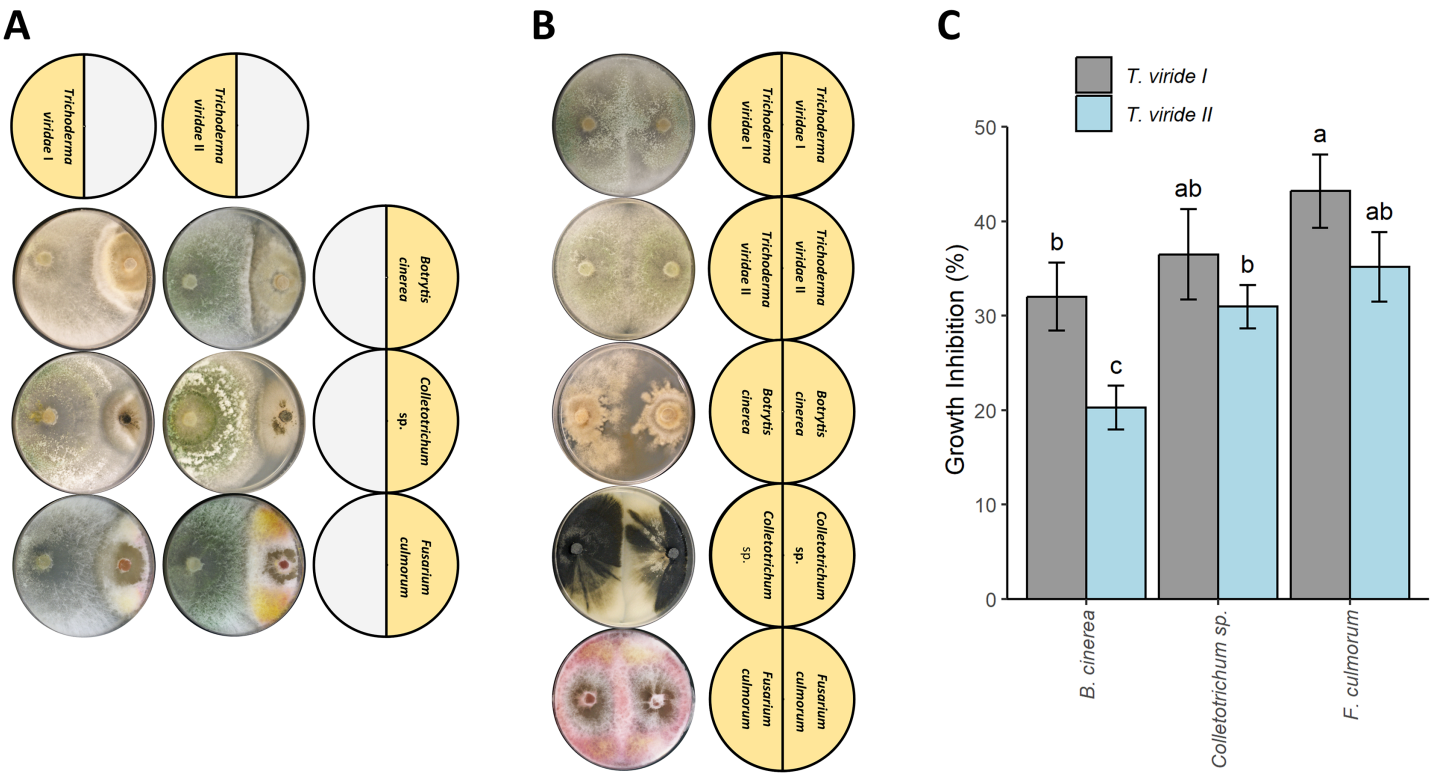

Figure 1 Effect of *Trichoderma viride* isolates (Tv I–II) on the growth of plant pathogens *Botritis cinerea*, *Colletotrichum* sp., and *Fusarium culmorum*. (A) Co-culture experiment showing the antagonism of *T. viride* I and II (left side of Petri dish) against plant pathogens: *B. cinerea, Colletotrichum* sp., and *F. culmorum* (right side of Petri dish). (B) Control showing the growth of tested *T. viride* strains and pathogens. (C) Percentage of inhibition of *T. viride* isolates (Tv I–II) against the plant pathogens *B. cinerea*, *Colletotrichum* sp., and *F. culmorum*.

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Statistical analyses

The homogeneity of variances and normality of the data was tested with the Bartlett and Shapiro–Wilk tests, respectively. To test the significance of the differences in means, the ANOVA test was applied, followed by Tukey's *post-hoc* test when data met the normality assumption and the Mann–Whitney *post-hoc* test when data did not meet the normality assumption. Data were visualized using the 'ggplot2' R package.

RESULTS

The effect of *T. viride* on the growth of pathogenic fungi

Both tested *T. viride* isolates (*T. viride* I and *T. viride* II) significantly inhibited the growth of all plant pathogens analyzed in this study *i.e.*, *B. cinerea*, *Colletotrichum* sp., and *F. culmorum* (Figs. 1A and 1C). *T. viride* I showed a percentage of inhibition in the range of 33.03–43.21% whereas *T. viride* II showed a percentage of inhibition in the range of 20.27–35.19% (Fig. 1C). *T. viride* I suppressed the growth of all analyzed pathogens more than *T. viride* II. The strongest inhibition was observed for *T. viride* I and *F. culmorum*, *i.e.*, 43.21% of inhibition, while the weakest inhibitory effect was observed for *T. viride* II and *B. cinerea*, *i.e.*, 20.27%. In fact, both tested *T. viride* strains the least effectively inhibited the growth of *B. cinerea* (Fig. 1C). Interestingly, the discoloration of *F. culmorum* mycelia

Table 3 Final germination percentage (FGP), index of germination velocity (IGV), and mean germination time (MGT) of *Brassica napus* L. seeds depending on type of seed coating.

Treatments	FGP (%)	IGV (a.u.)	MGT (day)
Control	85 ± 13.78a	51.6 ± 7.69a	4.76 ± 0.06a
M+C+TvI	98 ± 4.08a	62.8 ± 6.7 a	4.65 ± 0.12a
M+C+TvII	98 ± 4.08a	64.2 ± 5.86a	4.64 ± 0.12a
Methylcellulose	91 ± 9.83a	53.0 ± 10.86a	4.75 ± 0.12a
Chitin	86 ± 8.16a	52.1 ± 8.93a	4.73 ± 0.08a
Methylcellulose + Chitin	90 ± 10.95a	52.8 ± 9.73a	4.71 ± 0.09a
TvI	95 ± 5.48a	60.9 ± 2.81a	4.63 ± 0.05a
TvII	93 ± 8.16a	62.1 ± 4.12a	4.63 ± 0.04a

Note:
Values are mean ± SD (n = 4). Different letters indicate significant differences between groups (ANOVA with Tukey's *post-hoc* test, $p < 0.05$). Uncoated—control seeds, M+C+TvI—methylcellulose-chitin-*Trichoderma viride* I treated seeds, M+C+TvII—methylcellulose-chitin-*T. viride* II treated seeds, M—methylcellulose treated seeds, C—chitin treated seeds, M+C—methylcellulose–chitin treated seeds, TvI —*T. viride* I treated seeds, and TvII —*T. viride* II treated seeds.

upon contact with *T. viride* was observed, *i.e.*, pink *F. culmorum* mycelia in control (Fig. 1B) changed into white/yellow when grown together with *T. viride* (Fig. 1A). The two-way ANOVA revealed no statistically significant interaction between the effects of *T. viride* strains and pathogens (F-value = 1.147, p-value = 0.350043). However, simple main effects analyses showed that both *T. viride* strains and pathogens did have a statistically significant effect on inhibition (for *Trichoderma*: F-value = 24.967, p-value = 0.000311, for pathogens: F-value = 20.082, p-value = 0.000148).

The effect of seed coating on seed germination and seedling growth

To verify the beneficial influence of the developed *T. viride*-containing seed coating on plant development and growth, we performed the germination test (Table 3) and measured the growth dynamics of seedlings (Fig. 2). Final germination percentage (FGP), which reflects only the final ratio of seeds that completed germination, was the highest for seeds with the complete coating (*i.e.*, methylcellulose + chitin + *T. viride* spores), *i.e.*, 98%. In contrast, the lowest value of FGP was observed for control seeds, *i.e.*, 85% (Table 3). However, those differences were not statistically significant. FGP does not provide information about the speed or uniformity of germination; therefore, the index of germination velocity (IGV) and the mean germination time (MGT) were calculated. IGV, which indicates the rapidity of germination, was the highest for seeds with complete seed coating (*i.e.*, methylcellulose + chitin + *T. viride* spores) and the lowest for control seeds. Interestingly, the IGV value for seeds with complete coating was higher in comparison to seeds inoculated only with *T. viride* spores, which showed that other ingredients of seed coating, *i.e.*, methylcellulose and chitin, did not negatively affect the germination-promoting ability of *T. viride* (Table 3). MGT reflects the time needed for a group of seeds to complete germination and focuses on the day when most of the germination was completed. Faster germination was observed in seeds with complete coating and seeds inoculated with *T. viride* spores, whereas, for other experimental variants, the MGT was the same as for control seeds (Table 3). To assess the effect of seed

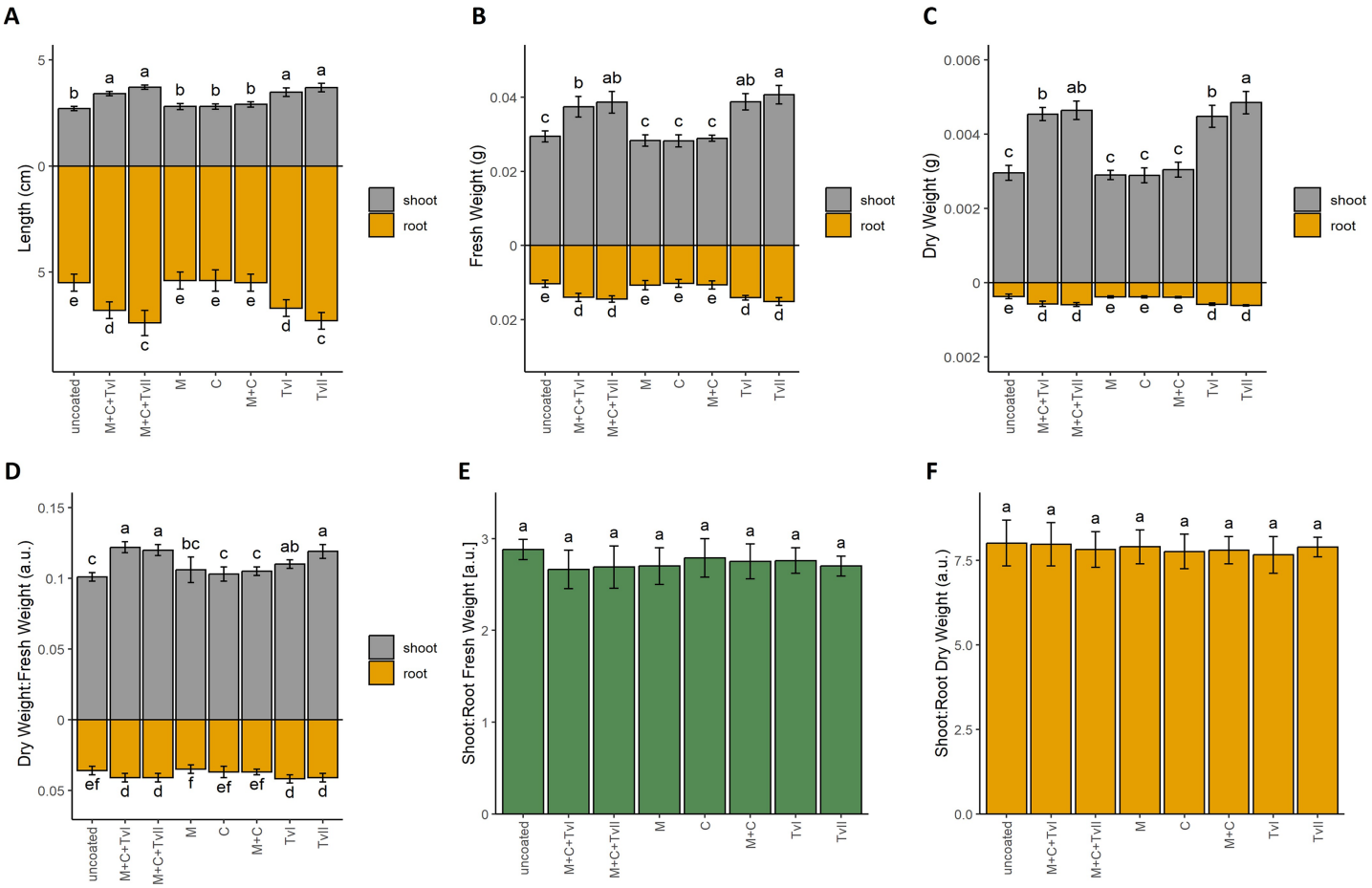

Figure 2 Length (A), fresh weight (B), dry weight (C), and dry weight:fresh weight ratio (D) of shoot and roots, and fresh weight (E) and dry weight (F) shoot:root ratio of *Brassica napus* L. seedlings depending on type of seed coating. Values are mean ± SD (n = 4). Different letters indicate significant differences between groups (ANOVA with Tukey's *post-hoc* test and Mann–Whitney *post-hoc* test, $p < 0.05$). Uncoated—control seeds, M+C+TvI—methylcellulose-chitin-*Trichoderma viride* I treated seeds, M+C+TvII—methylcellulose-chitin-*T. viride* II treated seeds, M—methylcellulose treated seeds, C—chitin treated seeds, M+C—methylcellulose–chitin treated seeds, TvI—*T. viride* I treated seeds, and TvII—*T. viride* II treated seeds.
Full-size DOI: 10.7717/peerj.15392/fig-2

coating on further seedling growth, we measured the length of roots and hypocotyls of 6-day-old seedlings (Fig. 2A). The seedlings that grew from seeds coated with a complete coating (*i.e.*, methylcellulose + chitin + *T. viride* spores) displayed longer shoots and roots than those from uncoated seeds. Seedlings that grew from seeds inoculated with spores of *T. viride* had also significantly longer shoots and roots compared to seedlings that grew from uncoated seeds (Fig. 2A), which showed that chitin and methylcellulose did not affect the ability of *T. viride* to promote the growth of canola seedlings.

Fresh (Fig. 2B) and dry (Fig. 2C) weight, as well as dry matter content (Fig. 2D), of 6-day-old canola seedlings were higher for seedlings that grew from seeds with the complete coating (*i.e.*, methylcellulose + chitin + *T. viride* spores) and from seeds inoculated with *T. viride* spores when compared to uncoated seeds and other control variants (*i.e.*, chitin, methylcellulose, and chitin + methylcellulose). The fungus' plant growth promoting ability was thus unaffected by the filler and binder in seed coating.

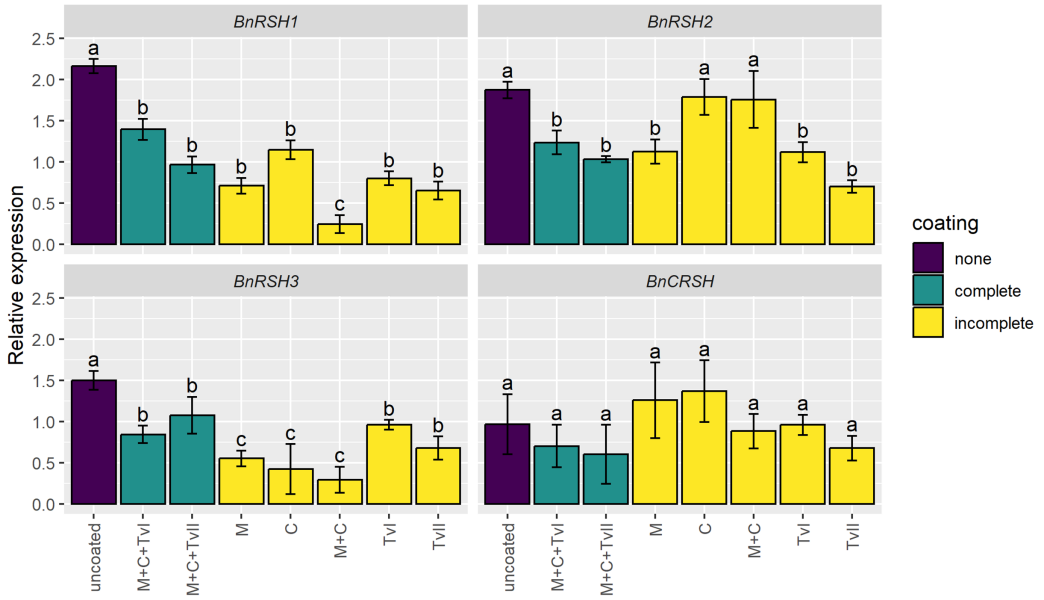

Figure 3 Relative expression level of *BnRSH* genes in 6-day-old *Brassica napus* L. seedlings germinated from uncoated (control) or coated seeds. Values are mean ± SD (n = 4). Different letters indicate significant differences between groups (ANOVA with Tukey's *post-hoc* test, $p < 0.05$). Uncoated—control seeds, M+C+TvI—methylcellulose-chitin-*Trichoderma viride* I treated seeds, M+C+TvII—methylcellulose-chitin-*T. viride* II treated seeds, M—methylcellulose treated seeds, C—chitin treated seeds, M+C—methylcellulose–chitin treated seeds, TvI—*T. viride* I treated seeds, and TvII—*T. viride* II treated seeds. Full-size DOI: 10.7717/peerj.15392/fig-3

In each case, the highest biomass was observed for seedlings inoculated with spores of *T. viride* II (*i.e.*, 1.5-fold higher for fresh root weight, 1.7-fold higher for fresh shoot weight, 1.4-fold higher for dry root weight, and 1.6-fold higher for dry shoot weight than seedlings that grew from uncoated seeds). The biomasses of seedlings that grew from seeds with a complete coating containing spores of *T. viride* II, chitin, and methylcellulose were only slightly lower than the previously mentioned (*i.e.*, 1.4-fold higher for fresh root weight, 1.6-fold higher for fresh shoot weight, 1.3-fold higher for dry root weight, and 1.6-fold higher for dry shoot weight than seedlings that grew from uncoated seeds). The comparison of shoot:root ratio (Figs. 2E and 2F) showed that seedlings that grew from tested seed coatings had the same proportion of shoots and roots. These results suggest that seed coating similarly promoted the growth of both roots and shoots in all tested variants.

The effect of seed coating on *BnRSH* gene expression

To assess whether the seed coating induces stress-related genes, the expression of genes encoding synthases and/or hydrolases of alarmones, *i.e.*, *BnRSH*s, was evaluated in 6-day-old seedlings grown from coated and uncoated seeds. The expression of no *BnRSH* genes was induced by seed coating (Fig. 3). The expression of *BnCRSH* was not affected by seed coating, whereas the expression of *BnRSH1–3* was down-regulated in seedlings grown from coated seeds compared to seedlings that grew from uncoated seeds. The transcript levels of *BnRSH1* and *BnRSH3* were significantly reduced in seedlings grown from all coated seeds, but the lowest level of expression (*i.e.*, almost nine-times lower than in

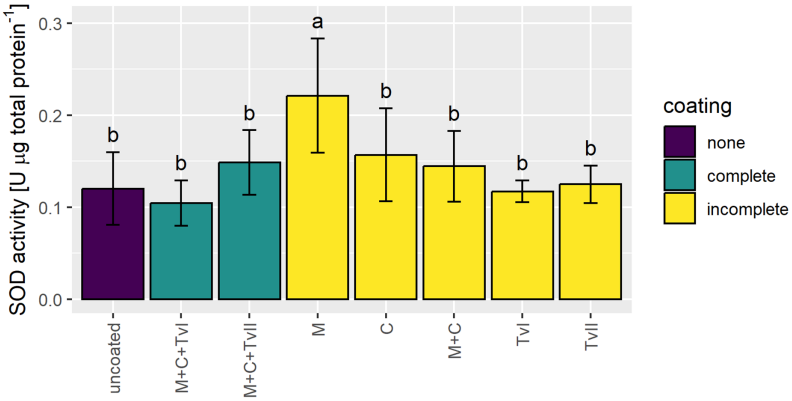

Figure 4 Activity of SOD in 6-day-old *Brassica napus* L. seedlings germinated from uncoated (control) and coated seeds. Values are mean ± SD (n = 4). Different letters indicate significant differences between groups (ANOVA with Tukey's *post-hoc* test, $p < 0.05$). Uncoated—control seeds, M+C+TvI—methylcellulose-chitin-*Trichoderma viride* I treated seeds, M+C+TvII—methylcellulose-chitin-*T. viride* II treated seeds, M—methylcellulose treated seeds, C—chitin treated seeds, M+C—methylcellulose–chitin treated seeds, TvI—*T. viride* I treated seeds, and TvII —*T. viride* II treated seeds. Full-size DOI: 10.7717/peerj.15392/fig-4

control seedlings) was observed for *BnRSH1* in seedlings grown from seeds coated with the mixture of chitin and methylcellulose (Fig. 3).

The effect of seed coating on SOD activity

Superoxide dismutase is a ubiquitous metalloenzyme that comprises the first level of protection against reactive oxygen species (ROS) and is widely used as a biochemical marker for abiotic stress tolerance in plants (*Szechyńska-Hebda et al., 2007*; *Berwal & Ram, 2019*). Therefore, to verify whether the seed coating developed in this study induced stress in plants, the activity of SOD was measured in 6-day-old seedlings grown from uncoated and coated seeds. The SOD activity significantly increased only in seedlings grown from seeds coated with methylcellulose and was not affected in seedlings grown from other variants of coated seeds in comparison to seedlings grown from uncoated seeds (Fig. 4).

DISCUSSION

Over the last decades, several biotechnological and agricultural advances have been made in developing new stress-resistant varieties, varieties producing higher yields, and bioinoculants improving plant growth (*Gao, 2021*). However, there is still room for improvement in the agricultural sector. Since many countries have banned genetically modified crops from the market, one of the most promising techniques for yield improvement is artificial seed covers created to aid plant development (*Rihan et al., 2017*). Nowadays, they are made mostly from synthetic materials that are expensive and non-degradable (*Pirzada et al., 2020*; *Britt, 2021*; *Zia et al., 2021*; *Sohail et al., 2022*). As seeds complete germination and young seedlings break through the soil and expand upwards, the plastic capsule suppresses the vegetation underneath (*Bosker et al., 2019*).

The new seed coating has the potential to improve plant immunity to fungal pathogens

In this study, we have demonstrated the ability of analyzed strains of *T. viride* to suppress the growth of the three plant pathogens (Fig. 1). Similar levels of antagonistic potential between *T. viride* and *F. culmorum* were also observed in other studies (*Modrzewska et al., 2022*). We report here the first quantitative analyses concerning *B. cinerea* and *Colletotrichum* sp. For *B. cinerea*, similar (~30%) inhibition was observed when co-cultured with spore suspension *Trichoderma harzianum* Rifai (*Geng et al., 2022*). For *T. viride* and *Colletotrichum* sp. inhibition, the data published so far is descriptive and image-based only (*Bankole & Adebanjo, 1996*).

A great body of evidence suggests *Trichoderma* treatment contributes to the defense of plants against pathogens far beyond germination. Biopriming of tomato seeds with *T. asperellum* increased the accumulation of total phenol and antioxidant enzyme activities in plants, consequently, and induced resistance against Fusarium Wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hansen (*Singh et al., 2020*). Isolates of *T. harzianum* also promoted growth and systemic protection against downy mildew in a highly susceptible sunflower cultivar (Morden) (*Nagaraju et al., 2012*). Biopriming of durum wheat (*Triticum durum* L.) seeds with *Trichoderma* strains enhanced the systemic resistance against *Fusarium* crown rot caused by *Fusarium culmorum* while also promoting growth (*Kthiri et al., 2020*). Treatment of cowpea seeds with *T. viride* increased the immunity against *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore; however, the treatment had to be repeated on a 2-week basis (*Bankole & Adebanjo, 1996*). On this basis, we assume that the *in-vitro* inhibition observed in our study will translate into a similar effect *in planta*, significantly contributing to the improvement of its resistance to the studied pathogens.

It was recognized that the inhibitory effect of *T. viride* and the pathogens probably involves the activation of various mechanisms. First of all, *Trichoderma* spp. have been regarded as necrotrophic mycoparasites, and this lifestyle is supported by the enzymes that break down chitin (*Ihrmark et al., 2010*), reviewed in (*Mukherjee et al., 2022*). Other mechanisms may involve the degradation of pathogen cell walls, production of antibiotics, and competition for nutrients (*Sivan & Chet, 1989*; *Sarrocco et al., 2009*) and ecological niches (*Chet & Inbar, 1994*; *Vinale et al., 2008*; *Vos et al., 2015*; *Waghunde, Shelake & Sabalpara, 2016*; *Oszust, Cybulska & Frąc, 2020*). Recently, *T. harzianum* S. INAT was found to induce the systemic resistance of durum wheat against foot crown rot disease caused by *Fusarium* (*Kthiri et al., 2020*). Significantly, several *Trichoderma* isolates were found to be capable of detoxifying zearalenone, a mycotoxin produced by some *Fusarium* species that contaminate grains (*Tian et al., 2018*), which suggests an additional potential benefit of the new seed coating. However, this needs to be verified in the future.

The new materials for seed coating promote plant growth

Our results showed that both tested strains of *T. viride* inoculated separately and as a component of seed coating did not impair the ability of the seeds to complete germination (Table 3), and both strains significantly enhanced seedling development (Fig. 2).

The impact of seed treatment with spores of various *Trichoderma* species on germination and growth seems to be plant-species-dependent. Some studies indicate no effect of these fungi on germination (*Lustosa et al., 2020*). However, most of the data show the positive effect of germination to mature plants. In tomato (*Solanum lycopersicum* L.), biopriming with *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg increased seed germination and plant height (*Singh et al., 2020*). In maize (*Zea mays* L.), single inoculation improved the ability of seeds to complete germination and vigor, field emergence, as well as plant height and seed mass (*Nayaka et al., 2010*). In wheat, seed biopriming with *T. harzianum* induced significant effects, specifically increased leaf area, ear length, ear weight, test weight, and grain yield, while reducing chemical fertilization (*Meena et al., 2017*). Similarly, pea seed biopriming with the *T. asperellum* strain BHUT8 effectively increased the length of the shoot and root, number of leaves, and fresh weight of the shoot and root compared to the control (*Singh et al., 2016*). Higher seed ability to complete germination, seedling establishment, and shoot length were observed by *Piri et al. (2019)* following the bio-priming of cumin seeds with *T. harzianum*. In soybean (*Glycine max* L.), seed biopriming with *T. viride* strain BHU-2953 increased root length and phosphorus (P) uptake and thus reduced demand for P-fertilizer (*Paul & Rakshit, 2021*). In sunflower, *T. harzianum* biopriming led not only to higher germination rate, vigor index, and height but also accelerated maturation (*Nagaraju et al., 2012*). In rice, seed biopriming with *Trichoderma* strains improved seed vigor, germination, chlorophyll content, and plant growth and additionally enhanced straw degradation capacity (*Swain et al., 2021*). Therefore, it is apparent that *Trichoderma* biopriming has the potential to benefit crop growth and development beyond germination, and this phenomenon would be seen in mature bio-primed rapeseed plants.

Canola seedlings showed significantly longer shoots and roots in all variants containing *T. viride* (Fig. 2A). Interestingly, *Trichoderma atroviride* P. Karst. was proven to decelerate *A. thaliana* root elongation by rhizosphere acidification (*Pelagio-Flores et al., 2017*). The observed effect seems to be specific to *Trichoderma*–plant species duets, and in *T. viride*–canola, this pathway is probably not induced. This is supported by the study of *Nieto-Jacobo et al. (2017)*, who showed the opposite effects of two *Trichoderma* species on *A. thaliana*. Moreover, the effect of *T. asperellum* LU1370 depended on growth conditions: in the soil, *A. thaliana* exhibited dwarfism, whereas on agar, its growth accelerated (*Nieto-Jacobo et al., 2017*). Also, fresh and dry weights of both roots and shoots were significantly higher in all variants containing *T. viride* spores (Figs. 2B and 2C). *Trichoderma* may induce local or systemic plant resistance through salicylic acid (SA), jasmonic acid (JA), and/or auxin pathways during interaction with plants (*Nawrocka & Małolepsza, 2013*). The mechanism of the observed growth promotion is probably *via* the auxin pathway, a key phytohormone that orchestrates plant growth and development. *Gravel, Antoun & Tweddell (2007)* found that IAA synthesis by a *Trichoderma* sp. was correlated with tomato growth. Since IAA may differently influence various tissues, one of the possible explanations for the phenomenon observed in our study is that, in shoots, both cell division and elongation are induced, and, in roots, only cell division is accelerated. This hypothesis needs to be verified by histological and immunohistochemical analyses.

Trichoderma spp. might also boost rapeseed growth through other mechanisms, including the production of volatile organic compounds (*Neik et al., 2020*) and various secondary metabolites (*Vinale et al., 2009*). Moreover, *Trichoderma* strains may be capable of colonizing the roots during early growth of seedlings and hence facilitate early development through enhancing nutrient absorbance (*Saba, 2012*; *Lutts et al., 2016*; *Ben-Jabeur et al., 2019*; *Kthiri et al., 2020*). In addition, these fungi might reduce the activity of harmful root microflora and deactivate toxins in the root area, resulting in improved root growth (*Roberti et al., 2008*).

The developed seed coating does not induce stress in plants

Based on available results, it was hypothesized that plant RSH proteins might play a role in many physiological processes, including germination and plant growth and development (*Dąbrowska, Prusińska & Goc, 2006*; *Boniecka et al., 2017*) The data about the expression of *RSH* genes during the early stages of seedling development are rather scarce. It was shown that during early seedling growth of *A. thaliana*, *RSH2* and *RSH3* are more strongly expressed than *RSH1* and *CRSH* (*Schmid et al., 2005*; *Mizusawa, Masuda & Ohta, 2008*; *Sugliani et al., 2016*). Interestingly, 8-day-old seedlings of *A. thaliana RSH3*-overexpression lines showed dwarf chloroplasts, metabolite reduction, and significantly inhibited plastid translation and transcription (*Maekawa et al., 2015*). Previously, we showed that, in the presence of plant growth-promoting rhizobacteria, the expression level of *BnRSH* genes in canola seedlings significantly raised (*Dąbrowska et al., 2021b*), so it was surprising that in this study *BnRSH1–3* and *BnCRSH* genes were unaffected or significantly downregulated in several experimental variants, including ones with *T. viride* (Fig. 3). Probably, the response to beneficial fungi is executed *via* different pathways or has different pacing than the response to beneficial bacteria. Importantly, we did not observe any abnormalities in the early stages of seedling development (File S2). In *I. nil* seedlings, *InRSH*s showed dynamic expression patterns during the early stages of seedling growth. During the 1-day sampling period, the expression level increase for *InRSH1*, decreased for *InRSH2*, and for *InCRSH* remained unchanged (*Prusińska et al., 2019*), showing that *RSH*s are differentially involved in seedling growth, not only plant-PGPR interaction.
Our previous *in silico* promoter analysis of the *BnRSH* genes revealed the presence of biotic stress response elements only within *BnRSH1* and *BnCRSH* promoters (*Dąbrowska et al., 2021b*). *BnCRSH* harbors AT-rich sequence elements for fungal elicitor-mediated activation, while *BnRSH1* contains both W-box (WRKY binding site) and TC-rich repeats elements involved in wounding and pathogens response (*Diaz-De-Leon & Lagrimini, 1993*; *Dąbrowska et al., 2021b*). WRKY, a plant-specific transcription factor family, plays vital roles in pathogen defense, abiotic stress, and phytohormone signaling (*Jones & Dangl, 2006*). It is also engaged in plant growth and development (*Chen & Yin, 2017*). Our results demonstrated that *T. viride* used in seed coating was not perceived by the plant as a pathogen and did not trigger an alarmones-dependent stress response pathway. Moreover, chitin and methylcellulose also did not induce the expression of *BnRSH* genes (Fig. 3). If plant immunity established *via Trichoderma* inoculation involves the alarmones-dependent pathway, we would expect that, in the presence of plant pathogens,

the expression profile of *BnRSHs* would change, probably with higher amplitude for *Trichoderma*-coated seeds. However, this hypothesis needs to be verified in the future.

Superoxide dismutase is one of the first-line antioxidant enzymes and is localized in various subcellular structures. Many stresses accelerate its activity (*e.g.*, salinity (*Houmani et al., 2016*), drought (*Saed-Moucheshi et al., 2021*), heat (*Ji et al., 2021*), and plant pathogens (*Gajera et al., 2016*; *Lightfoot, Mcgrann & Able, 2017*), and thus it is often chosen as an indicator of plant stress. Previous studies showed that fungi belonging to *Trichoderma* increased SOD activity in unstressed plants and plants under stress conditions. In tomato, SOD activity was elevated by the presence of *T. harizanum* in control conditions and the increase was even more profound upon osmotic stress treatment (*Mastouri, Björkman & Harman, 2012*). The presence of *Trichoderma longibrachiatum* Rifai induced SOD activity in wheat seedlings in the control conditions and higher SOD activity was observed in seedlings inoculated with fungi under saline treatment (*Zhang, Gan & Xu, 2016*). In groundnut, the activity of SOD was significantly induced by the presence of the pathogen *Aspergillus niger*, and the co-inoculation of plants with *A. niger* and *T. viride* led to a greater increase in SOD activity (*Gajera et al., 2016*). Interestingly, the seedlings displayed a slight but significant increase in SOD activity only when grown from seeds coated exclusively with methylcellulose and not in other variants—even those containing this substance (Fig. 4). Such a situation may be due to the effect of the crosstalk between methylcellulose, chitin, and *T. viride* within a complex regulatory network that leads to an unchanged SOD activity level. Although methylcellulose is an inhibitor of cellulase that prevents the cellulolytic activity of pathogenic fungi (*Cheng et al., 1991*), and it is a potential signal of pathogen invasion, the higher SOD activity is not necessarily a sign of oxidative stress defined as an imbalance between ROS production and scavenging. The increase in SOD activity and/or expression of genes encoding SOD was found at early seedling development stages in many plant species, including soybean (*Puntarulo et al., 1991*; *Gidrol et al., 1994*), goosefoot (*Bogdanović, Radotić & Mitrović, 2008*), and mung bean (*Singh, Chaudhuri & Kar, 2014*). ROS homeostasis does not mean that the level of ROS remains unchanged throughout the plant ontogenesis but, rather, that its level is adjusted to the current developmental and environmental context. For example, during the germination of tomato seeds, the level of superoxide anion dramatically rises, while the activity of SOD is maintained at a constant level (*Anand et al., 2019*). This event cannot be regarded as oxidative stress but rather as an oxidative burst because it is closely related to the plan of plant development. Moreover, promoters of genes encoding SOD enzymes harbor *cis*-elements involved in response to hormones and light (*Feng et al., 2016*; *Wang et al., 2017*; *Huo et al., 2022*). This implies that the expression of *SOD* genes might be regulated by developmental-related factors.
In *Arabidopsis*, loss-of-function mutation of chloroplast Cu/ZnSOD-encoding gene results in significant inhibition of plant growth and development and decreased chloroplast size, chlorophyll content, and photosynthetic activity compared with the wild-type plant (*Rizhsky, Liang & Mittler, 2003*). The *A. thaliana fsd1* knockout mutant, lacking functional FeSOD, extends fewer lateral roots than the WT strain (*Dvořák et al., 2021*).

As we already mentioned, *Trichoderma* was found to induce systemic resistance during biotic stress (*Kthiri et al., 2020*). Thus, we would expect significant differences in the activity of antioxidant enzymes between fully coated and uncoated seeds in the presence of a stressor rather than in plants grown in optimal conditions. This hypothesis is worth exploring, and our future experiments will focus on verifying it.

CONCLUSIONS

We conclude that the complete products that contain methylcellulose, chitin, and spores of *T. viride* strain I and II outperformed other variants in different ways. *T. viride* strain I had greater potential to inhibit the growth of plant pathogens, *i.e.*, *B. cinerea*, *F. culmorum*, and *Colletotrichum* sp., whereas *T. viride* strain II was slightly more effective in the promotion of plant growth. The key features of the seed coating obtained in this study are: (1) the ability to promote seedling growth and limit the growth of plant pathogens contained in a single product, and (2) seed coating is safe for the environment because it is fully biodegradable. The multidimensional positive effects of our seed coating make it of great interest for sustainable agriculture. The developed seed coating is currently being tested in field conditions.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Sena Turkan performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Agnieszka Mierek-Adamska analyzed the data, authored or reviewed drafts of the article, and approved the final draft.

- Milena Kulasek performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Wiktoria B Konieczna performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Grażyna B Dąbrowska conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The data is available at Zenodo: Sena Turkan, Agnieszka Mierek-Adamska, Milena Kulasek, Wiktoria Beata Konieczna, & Grażyna Barbara Dąbrowska. (2022). Seed coating for *Brassica napus* L. [Data set]. Zenodo. https://doi.org/10.5281/zenodo.7628268.

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REFERENCES

Abdelkefi H, Sugliani M, Ke H, Harchouni S, Soubigou-Taconnat L, Citerne S, Mouille G, Fakhfakh H, Robaglia C, Field B. 2018. Guanosine tetraphosphate modulates salicylic acid signalling and the resistance of *Arabidopsis thaliana* to *Turnip mosaic virus*. *Molecular Plant Pathology* **19(3)**:634–646 DOI 10.1111/mpp.12548.

Al-Ani LKT. 2018. Trichoderma from extreme environments: physiology, diversity, and antagonistic activity. In: *Extremophiles in Eurasian Ecosystems: Ecology, Diversity, and Applications*. 389–403.

Anand A, Kumari A, Thakur M, Koul A. 2019. Hydrogen peroxide signaling integrates with phytohormones during the germination of magnetoprimed tomato seeds. *Scientific Reports* **9(1)**:8814 DOI 10.1038/s41598-019-45102-5.

Antoszewski M, Mierek-Adamska A, Dąbrowska GB. 2022. The importance of microorganisms for sustainable agriculture—a review. *Metabolites* **12(11)**:1100 DOI 10.3390/metabo12111100.

Aranaz I, Mengibar M, Harris R, Panos I, Miralles B, Acosta N, Galed G, Heras A. 2009. Functional characterization of chitin and chitosan. *Current Chemical Biology* **3(2)**:203–230 DOI 10.2174/2212796810903020203.

Bailly C. 2019. The signalling role of ROS in the regulation of seed germination and dormancy. *Biochemical Journal* **476(20)**:3019–3032 DOI 10.1042/BCJ20190159.

Bankole SA, Adebanjo A. 1996. Biocontrol of brown blotch of cowpea caused by *Colletotrichum truncatum* with *Trichoderma viride*. *Crop Protection* **15(7)**:633–636 DOI 10.1016/0261-2194(96)00028-2.

Beauchamp C, Fridovich I. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry* **44(1)**:276–287 DOI 10.1016/0003-2697(71)90370-8.

Ben-Jabeur M, Vicente R, López-Cristoffanini C, Alesami N, Djébali N, Gracia-Romero A, Serret MD, López-Carbonell M, Araus JL, Hamada W. 2019. A novel aspect of essential oils: coating seeds with thyme essential oil induces drought resistance in wheat. *Plants* **8(10)**:371 DOI 10.3390/plants8100371.

Berwal M, Ram C. 2019. Superoxide dismutase: a stable biochemical marker for abiotic stress tolerance in higher plants. In: de Oliveira AB, ed. *Abiotic and Biotic Stress in Plants*. London, UK: IntechOpen.

Błaszczyk L, Basińska-Barczak A, Ćwiek-Kupczyńska H, Gromadzka K, Popiel D, Stępień Ł. 2017. Suppressive effect of *Trichoderma* spp. on toxigenic *Fusarium* species. *Polish Journal of Microbiology* **66(1)**:85–100 DOI 10.5604/17331331.1234996.

Bogdanović J, Radotić K, Mitrović A. 2008. Changes in activities of antioxidant enzymes during *Chenopodium murale* seed germination. *Biologia Plantarum* **52(2)**:396–400 DOI 10.1007/s10535-008-0083-7.

Boniecka J, Prusińska J, Dąbrowska GB, Goc A. 2017. Within and beyond the stringent response-RSH and (p)ppGpp in plants. *Planta* **246(5)**:817–842 DOI 10.1007/s00425-017-2780-y.

Bosker T, Bouwman LJ, Brun NR, Behrens P, Vijver MG. 2019. Microplastics accumulate on pores in seed capsule and delay germination and root growth of the terrestrial vascular plant *Lepidium sativum*. *Chemosphere* **226**:774–781 DOI 10.1016/j.chemosphere.2019.03.163.

Britt DW. 2021. Plug-and-play bioinspired seed coatings. *Nature Food* **2(7)**:456–457 DOI 10.1038/s43016-021-00325-6.

Cashel M, Gallant J. 1969. Two compounds implicated in the function of the *RC* gene of *Escherichia coli*. *Nature* **221**:838–841 DOI 10.1038/221838a0.

Chen J, Yin Y. 2017. WRKY transcription factors are involved in brassinosteroid signaling and mediate the crosstalk between plant growth and drought tolerance. *Plant Signaling and Behavior* **12(11)**:e1365212 DOI 10.1080/15592324.2017.1365212.

Cheng K, Kudo H, Duncan SH, Mesbah A, Stewart CS, Bernalier A, Fonty G, Costerton JW. 1991. Prevention of fungal colonization and digestion of cellulose by the addition of methylcellulose. *Canadian Journal of Microbiology* **37**:484–487 DOI 10.1139/m91-081.

Chet I, Inbar J. 1994. Biological control of fungal pathogens. *Applied Biochemistry and Biotechnology* **48(1)**:37–43 DOI 10.1007/BF02825358.

Contreras-Cornejo HA, Macías-Rodríguez L, del-Val E, Larsen J. 2016. Ecological functions of *Trichoderma* spp. and their secondary metabolites in the rhizosphere: interactions with plants. *FEMS Microbiology Ecology* **92(4)**:fiw036 DOI 10.1093/femsec/fiw036.

Cortés-Rojas D, Beltrán-Acosta C, Zapata-Narvaez Y, Chaparro M, Gómez M, Cruz-Barrera M. 2021. Seed coating as a delivery system for the endophyte *Trichoderma koningiopsis* Th003 in rice (*Oryza sativa*). *Applied Microbiology and Biotechnology* **105(5)**:1889–1904 DOI 10.1007/s00253-021-11146-9.

Dąbrowska GB, Garstecka Z, Olewnik-Kruszkowska E, Szczepańska G, Ostrowski M, Mierek-Adamska A. 2021a. Comparative study of structural changes of polylactide and poly (ethylene terephthalate) in the presence of *Trichoderma viride*. *International Journal of Molecular Sciences* **22(7)**:3491 DOI 10.3390/ijms22073491.

Dąbrowska G, Prusińska J, Goc A. 2006. Identification of the *RSH* gene cDNA (*RelA/SpoT homolog*) involved in *Pharbitis nil* response to stress condition. *Advances of Agricultural Sciences Problem Issues* **509**:333–341.

Dąbrowska GB, Turkan S, Tylman-Mojżeszek W, Mierek-Adamska A. 2021b. In silico study of the *RSH (RelA/SpoT homologs)* gene family and expression analysis in response to PGPR bacteria and salinity in *Brassica napus*. *International Journal of Molecular Sciences* **22(19)**:10666 DOI 10.3390/ijms221910666.

Diaz-De-Leon F, Lagrimini M. 1993. Nucleotide sequence of the tobacco (*Nicotiana tabacum*) anionic peroxidase gene. *Plant Physiology* **101(3)**:1117–1118 DOI 10.1104/pp.101.3.1117.

Dvořák P, Krasylenko Y, Zeiner A, Šamaj J, Takáč T. 2021. Signaling toward reactive oxygen species-scavenging enzymes in plants. *Frontiers in Plant Science* **11**:2645 DOI 10.3389/fpls.2020.618835.

Elferjani R, Soolanayakanahally R. 2018. Canola responses to drought, heat, and combined stress: shared and specific effects on carbon assimilation, seed yield, and oil composition. *Frontiers in Plant Science* **9**:1224 DOI 10.3389/fpls.2018.01224.

Feng K, Yu J, Cheng Y, Ruan M, Wang R, Ye Q, Zhou G, Li Z, Yao Z, Yang Y, Zheng Q, Wan H. 2016. The SOD gene family in tomato: identification, phylogenetic relationships, and expression patterns. *Frontiers in Plant Science* **7**:1279 DOI 10.3389/fpls.2016.01279.

Food and Agriculture Organization (FAO). 2022. *The state of food security and nutrition in the world 2022*. Rome, Italy: FAO DOI 10.4060/cc0639en.

Gajera HP, Katakpara ZA, Patel SV, Golakiya BA. 2016. Antioxidant defense response induced by *Trichoderma viride* against *Aspergillus niger* Van Tieghem causing collar rot in groundnut (*Arachis hypogaea* L.). *Microbial Pathogenesis* **91**:26–34 DOI 10.1016/j.micpath.2015.11.010.

Gao C. 2021. Genome engineering for crop improvement and future agriculture. *Cell* **184(6)**:1621–1635 DOI 10.1016/j.cell.2021.01.005.

Geng L, Fu Y, Peng X, Yang Z, Zhang M, Song Z, Guo N, Chen S, Chen J, Bai B, Liu A, Ahammed GJ. 2022. Biocontrol potential of *Trichoderma harzianum* against *Botrytis cinerea* in tomato plants. *Biological Control* **174(1)**:105019 DOI 10.1016/j.biocontrol.2022.105019.

Gidrol X, Lin WS, Degousee N, Yip SF, Kush A. 1994. Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. *European Journal of Biochemistry* **224(1)**:21–28 DOI 10.1111/j.1432-1033.1994.tb19990.x.

Gravel V, Antoun H, Tweddell RJ. 2007. Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida* or *Trichoderma atroviride*: possible role of indole acetic acid (IAA). *Soil Biology and Biochemistry* **39(8)**:1968–1977 DOI 10.1016/j.soilbio.2007.02.015.

Guzmán-Guzmán P, Porras-Troncoso MD, Olmedo-Monfil V, Herrera-Estrella A. 2019. *Trichoderma* species: versatile plant symbionts. *Phytopathology* **109(1)**:6–16 DOI 10.1094/PHYTO-07-18-0218-RVW.

Houmani H, Rodríguez-Ruiz M, Palma JM, Abdelly C, Corpas FJ. 2016. Modulation of superoxide dismutase (SOD) isozymes by organ development and high long-term salinity in the halophyte *Cakile maritima*. *Protoplasma* **253(3)**:885–894 DOI 10.1007/s00709-015-0850-1.

Huo C, He L, Yu T, Ji X, Li R, Zhu S, Zhang F, Xie H, Liu W. 2022. The superoxide dismutase gene family in *Nicotiana tabacum*: genome-wide identification, characterization, expression profiling and functional analysis in response to heavy metal stress. *Frontiers in Plant Science* **13**:263 DOI 10.3389/fpls.2022.904105.

Ihrmark K, Asmail N, Ubhayasekera W, Melin P, Stenlid J, Karlsson M. 2010. Comparative molecular evolution of *Trichoderma chitinases* in response to mycoparasitic interactions. *Evolutionary Bioinformatics* **6**:6–7 DOI 10.4137/ebo.s4198.

Ji HS, Bang SG, Ahn M-A, Kim G, Kim E, Eom SH, Hyun TK. 2021. Molecular cloning and functional characterization of heat stress-responsive superoxide dismutases in garlic (*Allium sativum* L.). *Antioxidants* **10(5)**:815 DOI 10.3390/antiox10050815.

John RP, Tyagi RD, Prévost D, Brar SK, Pouleur S, Surampalli RY. 2010. Mycoparasitic *Trichoderma viride* as a biocontrol agent against *Fusarium oxysporum* f. sp. adzuki and *Pythium*

arrhenomanes and as a growth promoter of soybean. *Crop Protection* **29(12)**:1452–1459 DOI 10.1016/j.cropro.2010.08.004.

Jones JDG, Dangl JL. 2006. The plant immune system. *Nature* **444(7117)**:323–329 DOI 10.1038/nature05286.

Juergensen C, Madsen AM. 2009. Exposure to the airborne mould *Botrytis* and its health effects. *Annals of agricultural and environmental medicine: AAEM* **16**:183–196.

Khan MA, Ungar IA. 1984. The effect of salinity and temperature on the germination of polymorphic seeds and growth of *Atriplex triangularis* Willd. *American Journal of Botany* **71(4)**:481–489 DOI 10.2307/2443323.

Kthiri Z, Jabeur MB, Machraoui M, Gargouri S, Hiba K, Hamada W. 2020. Coating seeds with *Trichoderma* strains promotes plant growth and enhance the systemic resistance against *Fusarium* crown rot in durum wheat. *Egyptian Journal of Biological Pest Control* **30(1)**:139 DOI 10.1186/s41938-020-00338-6.

Kunova A, Bonaldi M, Saracchi M, Pizzatti C, Chen X, Cortesi P. 2016. Selection of *Streptomyces* against soil borne fungal pathogens by a standardized dual culture assay and evaluation of their effects on seed germination and plant growth. *BMC Microbiology* **16(1)**:272 DOI 10.1186/s12866-016-0886-1.

Li Y, Sun R, Yu J, Saravanakumar K, Chen J. 2016. Antagonistic and biocontrol potential of *Trichoderma asperellum* ZJSX5003 against the maize stalk rot pathogen *Fusarium graminearum*. *Indian Journal of Microbiology* **56(3)**:318–327 DOI 10.1007/s12088-016-0581-9.

Lightfoot DJ, Mcgrann GRD, Able AJ. 2017. The role of a cytosolic superoxide dismutase in barley-pathogen interactions. *Molecular Plant Pathology* **18(3)**:323–335 DOI 10.1111/mpp.12399.

Lopes MJ, dos S, Dias-Filho MB, Gurgel ESC. 2021. Successful plant growth-promoting microbes: inoculation methods and abiotic factors. *Frontiers in Sustainable Food Systems* **5**:606454 DOI 10.3389/fsufs.2021.606454.

Lorito M, Woo S. 2015. *Trichoderma*: a multi-purpose tool for integrated pest management. In: *Principles of Plant-Microbe Interactions: Microbes for Sustainable Agriculture*. Berlin: Springer International Publishing, 345–353.

Luo D, Hou X, Zhang Y, Meng Y, Zhang H, Liu S, Wang X, Chen R. 2019. *CaDHN5*, a dehydrin gene from pepper, plays an important role in salt and osmotic stress responses. *International Journal of Molecular Sciences* **20(8)**:1989 DOI 10.3390/ijms20081989.

Lustosa DC, Araújo AJC, Campos BF, Vieira TA. 2020. *Trichoderma* spp. and its effects on seeds physiological quality and seedlings development of African mahogany. *Revista Brasileira de Ciências Agrárias—Brazilian Journal of Agricultural Sciences* **15(1)**:1–7 DOI 10.5039/agraria.v15i1a5843.

Lutts S, Benincasa P, Wojtyla L, Kubala S, Pace R, Lechowska K, Quinet M, Garnczarska M. 2016. Seed priming: new comprehensive approaches for an old empirical technique. In: Susana A, Alma B, eds. *New Challenges in Seed Biology*. London, UK: IntechOpen, 1–46.

Ma Y. 2019. Seed coating with beneficial microorganisms for precision agriculture. *Biotechnology Advances* **37(7)**:107423 DOI 10.1016/j.biotechadv.2019.107423.

Macías-Rodríguez L, Contreras-Cornejo HA, Adame-Garnica SG, del-Val E, Larsen J. 2020. The interactions of *Trichoderma* at multiple trophic levels: inter-kingdom communication. *Microbiological Research* **240**:126552 DOI 10.1016/j.micres.2020.126552.

Maekawa M, Honoki R, Ihara Y, Sato R, Oikawa A, Kanno Y, Ohta H, Seo M, Saito K, Masuda S. 2015. Impact of the plastidial stringent response in plant growth and stress responses. *Nature Plants* **1(12)**:1458 DOI 10.1038/nplants.2015.167.

Mastan A, Rane D, Dastager SG, Vivek Babu CS. 2021. Molecular insights of fungal endophyte co-inoculation with *Trichoderma viride* for the augmentation of forskolin biosynthesis in *Coleus forskohlii*. *Phytochemistry* **184**:112654 DOI 10.1016/j.phytochem.2021.112654.

Mastouri F, Björkman T, Harman GE. 2012. *Trichoderma harzianum* enhances antioxidant defense of tomato seedlings and resistance to water deficit. *Molecular Plant-Microbe Interactions®* **25(9)**:1264–1271 DOI 10.1094/MPMI-09-11-0240.

Mawar R, Manjunatha BL, Kumar S. 2021. Commercialization, diffusion and adoption of bioformulations for sustainable disease management in Indian arid agriculture: prospects and challenges. *Circular Economy and Sustainability* **1(4)**:1367–1385 DOI 10.1007/s43615-021-00089-y.

Meena SK, Rakshit A, Singh HB, Meena VS. 2017. Effect of nitrogen levels and seed bio-priming on root infection, growth and yield attributes of wheat in varied soil type. *Biocatalysis and Agricultural Biotechnology* **12(2)**:172–178 DOI 10.1016/j.bcab.2017.10.006.

Mizusawa K, Masuda S, Ohta H. 2008. Expression profiling of four RelA/SpoT-like proteins, homologues of bacterial stringent factors, in *Arabidopsis thaliana*. *Planta* **228(4)**:553–562 DOI 10.1007/s00425-008-0758-5.

Modrzewska M, Błaszczyk L, Stępień Ł, Urbaniak M, Waśkiewicz A, Yoshinari T, Bryła M. 2022. *Trichoderma* versus *Fusarium*—inhibition of pathogen growth and mycotoxin biosynthesis. *Molecules* **27(23)**:8146 DOI 10.3390/molecules27238146.

Mukherjee PK, Mendoza-Mendoza A, Zeilinger S, Horwitz BA. 2022. Mycoparasitism as a mechanism of *Trichoderma*-mediated suppression of plant diseases. *Fungal Biology Reviews* **39**:15–33 DOI 10.1016/j.fbr.2021.11.004.

Nagaraju A, Sudisha J, Murthy SM, Ito SI. 2012. Seed priming with *Trichoderma harzianum* isolates enhances plant growth and induces resistance against *Plasmopara halstedii*, an incitant of sunflower downy mildew disease. *Australasian Plant Pathology* **41(6)**:609–620 DOI 10.1007/s13313-012-0165-z.

Nawrocka J, Małolepsza U. 2013. Diversity in plant systemic resistance induced by *Trichoderma*. *Biological Control* **67(2)**:149–156 DOI 10.1016/j.biocontrol.2013.07.005.

Nayaka SC, Niranjana SR, Uday Shankar AC, Niranjan Raj S, Reddy MS, Prakash HS, Mortensen CN. 2010. Seed biopriming with novel strain of *Trichoderma harzianum* for the control of toxigenic *Fusarium verticillioides* and *fumonisins* in maize. *Archives of Phytopathology and Plant Protection* **43(3)**:264–282 DOI 10.1080/03235400701803879.

Neik TX, Amas J, Barbetti M, Edwards D, Batley J. 2020. Understanding host-pathogen interactions in *Brassica napus* in the omics era. *Plants* **9(10)**:1–31 DOI 10.3390/plants9101336.

Nieto-Jacobo MF, Steyaert JM, Salazar-Badillo FB, Nguyen DV, Rostás M, Braithwaite M, de Souza JT, Jimenez-Bremont JF, Ohkura M, Stewart A, Mendoza-Mendoza A. 2017. Environmental growth conditions of *Trichoderma* spp. affects indole acetic acid derivatives, volatile organic compounds, and plant growth promotion. *Frontiers in Plant Science* **8**:102 DOI 10.3389/fpls.2017.00102.

Ono S, Suzuki S, Ito D, Tagawa S, Shiina T, Masuda S. 2021. Plastidial (p)ppGpp synthesis by the Ca^{2+}-dependent RelA-SpoT homolog regulates the adaptation of chloroplast gene expression to darkness in *Arabidopsis*. *Plant & Cell Physiology* **61(12)**:2077–2086 DOI 10.1093/pcp/pcaa124.

Oracz K, Karpiński S. 2016. Phytohormones signaling pathways and ROS involvement in seed germination. *Frontiers in Plant Science* **7(e1005373)**:864 DOI 10.3389/fpls.2016.00864.

Oszust K, Cybulska J, Frąc M. 2020. How do *Trichoderma* genus fungi win a nutritional competition battle against soft fruit pathogens? A report on niche overlap nutritional potentiates. *International Journal of Molecular Sciences* **21(12)**:4235 DOI 10.3390/ijms21124235.

Paul S, Rakshit A. 2021. Effect of seed bio-priming with *Trichoderma viride* strain BHU-2953 for enhancing soil phosphorus solubilization and uptake in soybean (*Glycine max*). *Journal of Soil Science and Plant Nutrition* **21**:1041–1052 DOI 10.1007/s42729-021-00420-4/Published.

Pedrini S, Merritt DJ, Stevens J, Dixon K. 2017. Seed coating: science or marketing spin? *Trends in Plant Science* **22(2)**:106–116 DOI 10.1016/j.tplants.2016.11.002.

Pelagio-Flores R, Esparza-Reynoso S, Garnica-Vergara A, López-Bucio J, Herrera-Estrella A. 2017. *Trichoderma*-induced acidification is an early trigger for changes in *Arabidopsis* root growth and determines fungal phytostimulation. *Frontiers in Plant Science* **8**:822 DOI 10.3389/fpls.2017.00822.

Pérez LM, Besoaín X, Reyes M, Pardo G, Montealegre J. 2002. The expression of extracellular fungal cell wall hydrolytic enzymes in different *Trichoderma harzianum* isolates correlates with their ability to control *Pyrenochaeta lycopersici*. *Biological Research* **35**:401–410 DOI 10.4067/S0716-97602002000300014.

Piri R, Moradi A, Balouchi H, Salehi A. 2019. Improvement of cumin (*Cuminum cyminum*) seed performance under drought stress by seed coating and biopriming. *Scientia Horticulturae* **257**:108667 DOI 10.1016/j.scienta.2019.108667.

Pirzada T, de Farias BV, Mathew R, Guenther RH, Byrd MV, Sit TL, Pal L, Opperman CH, Khan SA. 2020. Recent advances in biodegradable matrices for active ingredient release in crop protection: towards attaining sustainability in agriculture. *Current Opinion in Colloid & Interface Science* **48**:121–136 DOI 10.1016/j.cocis.2020.05.002.

Pradhan PC, Mukhopadhyay A, Kumar R, Kundu A, Patanjali N, Dutta A, Kamil D, Bag TK, Aggarwal R, Bharadwaj C, Singh PK, Singh A. 2022. Performance appraisal of *Trichoderma viride* based novel tablet and powder formulations for management of *Fusarium* wilt disease in chickpea. *Frontiers in Plant Science* **13**:990392 DOI 10.3389/fpls.2022.990392.

Prusińska JM, Boniecka J, Dąbrowska GB, Goc A. 2019. Identification and characterization of the *Ipomoea nil RelA/SpoT Homologs* (*InRSHs*) and potential directions of their transcriptional regulation. *Plant Science* **284**:161–176 DOI 10.1016/j.plantsci.2019.01.026.

Puntarulo S, Galleano M, Sanchez RA, Boveris A. 1991. Superoxide anion and hydrogen peroxide metabolism in soybean embryonic axes during germination. *Biochimica et Biophysica Acta (BBA)—General Subjects* **1074(2)**:277–283 DOI 10.1016/0304-4165(91)90164-C.

Ranal MA, Santana DGde. 2006. How and why to measure the germination process? *Revista Brasileira de Botânica* **29(1)**:1–11 DOI 10.1590/S0100-84042006000100002.

Rihan HZ, Kareem F, El-Mahrouk ME, Fuller MP. 2017. Artificial seeds (Principle, aspects and applications). *Agronomy* **7(4)**:71 DOI 10.3390/agronomy7040071.

Rinaudo M. 2006. Chitin and chitosan: properties and applications. *Progress in Polymer Science* **31(7)**:603–632 DOI 10.1016/j.progpolymsci.2006.06.001.

Rizhsky L, Liang H, Mittler R. 2003. The water-water cycle is essential for chloroplast protection in the absence of stress. *Journal of Biological Chemistry* **278(40)**:38921–38925 DOI 10.1074/jbc.M304987200.

Roberti R, Veronesi AR, Cesari A, Cascone A, di Berardino I, Bertini L, Caruso C. 2008. Induction of PR proteins and resistance by the biocontrol agent *Clonostachys rosea* in wheat

plants infected with *Fusarium culmorum*. *Plant Science* **175(3)**:339–347 DOI 10.1016/j.plantsci.2008.05.003.

Rocha I, Ma Y, Carvalho MF, Magalhães C, Janoušková M, Vosátka M, Freitas H, Oliveira RS. 2019a. Seed coating with inocula of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria for nutritional enhancement of maize under different fertilisation regimes. *Archives of Agronomy and Soil Science* **65(1)**:31–43 DOI 10.1080/03650340.2018.1479061.

Rocha I, Ma Y, Souza-Alonso P, Vosátka M, Freitas H, Oliveira RS. 2019b. Seed coating: a tool for delivering beneficial microbes to agricultural crops. *Frontiers in Plant Science* **10**:1357 DOI 10.3389/fpls.2019.01357.

Rodríguez MdCH, Evans HC, de Abreu LM, de Macedo DM, Ndacnou MK, Bekele KB, Barreto RW. 2021. New species and records of *Trichoderma* isolated as mycoparasites and endophytes from cultivated and wild coffee in Africa. *Scientific Reports* **11(1)**:e0160613 DOI 10.1038/s41598-021-84111-1.

Rojas-Sánchez B, Guzmán-Guzmán P, Morales-Cedeño LR, del Orozco-Mosqueda Ma C, Saucedo-Martínez BC, Sánchez-Yáñez JM, Fadiji AE, Babalola OO, Glick BR, Santoyo G. 2022. Bioencapsulation of microbial inoculants: mechanisms, formulation types and application techniques. *Applied Biosciences* **1(2)**:198–220 DOI 10.3390/applbiosci1020013.

Rusaczonek A, Czarnocka W, Kacprzak S, Witoń D, Ślesak I, Szechyńska-Hebda M, Gawroński P, Karpiński S. 2015. Role of phytochromes A and B in the regulation of cell death and acclimatory responses to UV stress in *Arabidopsis thaliana*. *Journal of Experimental Botany* **66(21)**:6679–6695 DOI 10.1093/jxb/erv375.

Saba H. 2012. *Trichoderma*—a promising plant growth stimulator and biocontrol agent. *Mycosphere* **3(4)**:524–531 DOI 10.5943/mycosphere/3/4/14.

Saed-Moucheshi A, Sohrabi F, Fasihfar E, Baniasadi F, Riasat M, Mozafari AA. 2021. Superoxide dismutase (SOD) as a selection criterion for triticale grain yield under drought stress: a comprehensive study on genomics and expression profiling, bioinformatics, heritability, and phenotypic variability. *BMC Plant Biology* **21(1)**:148 DOI 10.1186/s12870-021-02919-5.

Sarker U, Oba S. 2018. Catalase, superoxide dismutase and ascorbate-glutathione cycle enzymes confer drought tolerance of *Amaranthus tricolor*. *Scientific Reports* **8(1)**:16496 DOI 10.1038/s41598-018-34944-0.

Sarrocco S, Guidi L, Fambrini S, Degl'innocenti E, Vannacci G. 2009. Competition for cellulose exploitation between *Rhizoctonia solani* and two *Trichoderma* isolates in the decomposition of wheat straw. *Journal of Plant Pathology* **91**:331–338.

Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Schölkopf B, Weigel D, Lohmann JU. 2005. A gene expression map of *Arabidopsis thaliana* development. *Nature Genetics* **37(5)**:501–506 DOI 10.1038/ng1543.

Seng J, Herrera G, Vaughan CS, McCoy MB. 2014. Use of *Trichoderma* fungi in spray solutions to reduce *Moniliophthora roreri* infection of *Theobroma cacao* fruits in northeastern Costa Rica. *Revista de Biología Tropical* **62(3)**:899 DOI 10.15517/rbt.v62i3.14059.

Shivaprakash MR, Appannanavar SB, Dhaliwal M, Gupta A, Gupta S, Gupta A, Chakrabarti A. 2011. *Colletotrichum truncatum*: an unusual pathogen causing mycotic keratitis and endophthalmitis. *Journal of Clinical Microbiology* **49(8)**:2894–2898 DOI 10.1128/JCM.00151-11.

Singh KL, Chaudhuri A, Kar RK. 2014. Superoxide and its metabolism during germination and axis growth of *Vigna radiata* (L.) Wilczek seeds. *Plant Signaling & Behavior* **9(8)**:e29278 DOI 10.4161/psb.29278.

Singh P, Singh J, Ray S, Rajput RS, Vaishnav A, Singh RK, Singh HB. 2020. Seed biopriming with antagonistic microbes and ascorbic acid induce resistance in tomato against *Fusarium* wilt. *Microbiological Research* **237(2)**:126482 DOI 10.1016/j.micres.2020.126482.

Singh V, Upadhyay RS, Sarma BK, Singh HB. 2016. *Trichoderma asperellum* spore dose depended modulation of plant growth in vegetable crops. *Microbiological Research* **193**:74–86 DOI 10.1016/j.micres.2016.09.002.

Sivan A, Chet I. 1989. The possible role of competition between *Trichoderma harzianum* and *Fusarium oxysporum* on rhizosphere colonization. *Phytopathology* **79(2)**:198 DOI 10.1094/phyto-79-198.

Sohail M, Pirzada T, Opperman CH, Khan SA. 2022. Recent advances in seed coating technologies: transitioning toward sustainable agriculture. *Green Chemistry* **24(16)**:6052–6085 DOI 10.1039/D2GC02389J.

Sugliani M, Abdelkefi H, Ke H, Bouveret E, Robaglia C, Caffarri S, Field B. 2016. An ancient bacterial signaling pathway regulates chloroplast function to influence growth and development in *Arabidopsis*. *Plant Cell* **28(3)**:661–679 DOI 10.1105/tpc.16.00045.

Swain H, Adak T, Mukherjee AK, Sarangi S, Samal P, Khandual A, Jena R, Bhattacharyya P, Naik SK, Mehetre ST, Baite MS, Kumar MS, Zaidi NW. 2021. Seed biopriming with *Trichoderma* strains isolated from tree bark improves plant growth, antioxidative defense system in rice and enhance straw degradation capacity. *Frontiers in Microbiology* **12**:633881 DOI 10.3389/fmicb.2021.633881.

Szechyńska-Hebda M, Skrzypek E, Dąbrowska G, Biesaga-Kościelniak J, Filek M, Wędzony M. 2007. The role of oxidative stress induced by growth regulators in the regeneration process of wheat. *Acta Physiologiae Plantarum* **29(4)**:327–337 DOI 10.1007/s11738-007-0042-5.

Szymańska S, Dąbrowska GB, Tyburski J, Niedojadło K, Piernik A, Hrynkiewicz K. 2019. Boosting the *Brassica napus* L. tolerance to salinity by the halotolerant strain *Pseudomonas stutzeri* ISE12. *Environmental and Experimental Botany* **163(4)**:55–68 DOI 10.1016/j.envexpbot.2019.04.007.

Takahashi K, Kasai K, Ochi K. 2004. Identification of the bacterial alarmone guanosine 5′-diphosphate 3′-diphosphate (ppGpp) in plants. *Proceedings of the National Academy of Sciences of the United States of America* **101(12)**:4320–4324 DOI 10.1073/pnas.0308555101.

Tavares LC, de Araújo Rufino C, Brunes AP, Friedrich FF, Albuquerque Barros ACS, Villela FA. 2013. Physiological performance of wheat seeds coated with micronutrients. *Jounal of Seed Science* **35(1)**:28–34 DOI 10.1590/S2317-15372013000100004.

Tian Y, Tan Y, Yan Z, Liao Y, Chen J, de Boevre M, de Saeger S, Wu A. 2018. Antagonistic and detoxification potentials of *Trichoderma* isolates for control of zearalenone (ZEN) producing *Fusarium graminearum*. *Frontiers in Microbiology* **8**:2710 DOI 10.3389/fmicb.2017.02710.

van der Biezen EA, Sun J, Coleman MJ, Bibb MJ, Jones JDG. 2000. *Arabidopsis* RelA/SpoT homologs implicate (p)ppGpp in plant signaling. *Proceedings of the National Academy of Sciences of the United States of America* **97(7)**:3747–3752 DOI 10.1073/pnas.060392397.

Vinale F, Flematti G, Sivasithamparam K, Lorito M, Marra R, Skelton BW, Ghisalberti EL. 2009. Harzianic acid, an antifungal and plant growth promoting metabolite from *Trichoderma harzianum*. *Journal of Natural Products* **72(11)**:2032–2035 DOI 10.1021/np900548p.

Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Woo SL, Lorito M. 2008. *Trichoderma*-plant-pathogen interactions. *Soil Biology and Biochemistry* **40(1)**:1–10 DOI 10.1016/j.soilbio.2007.07.002.

Vos CMF, de Cremer K, Cammue BPA, de Coninck B. 2015. The toolbox of *Trichoderma* spp. in the biocontrol of *Botrytis cinerea* disease. *Molecular Plant Pathology* **16(4)**:400–412 DOI 10.1111/mpp.12189.

Waghunde R, Shelake RM, Sabalpara AN. 2016. *Trichoderma*: a significant fungus for agriculture and environment. *African Journal of Agricultural Research* **11(22)**:1952–1965 DOI 10.5897/ajar2015.10584.

Wang W, Zhang X, Deng F, Yuan R, Shen F. 2017. Genome-wide characterization and expression analyses of superoxide dismutase (SOD) genes in *Gossypium hirsutum*. *BMC Genomics* **18(1)**:376 DOI 10.1186/s12864-017-3768-5.

Zhang S, Gan Y, Xu B. 2016. Application of plant-growth-promoting fungi *Trichoderma longibrachiatum* T6 enhances tolerance of wheat to salt stress through Improvement of antioxidative defense system and gene expression. *Frontiers in Plant Science* **07(868)**:306 DOI 10.3389/fpls.2016.01405.

Zia KM, Akram N, Tabasum S, Noreen A, Akbar MU. 2021. Processing of bio-based polymers for industrial and medical applications. In: *Processing Technology for Bio-Based Polymers*. Amsterdam: Elsevier, 191–238.

Znajewska Z, Dąbrowska GB, Narbutt O. 2018. *Trichoderma viride* strains stimulating the growth and development of winter rapeseed (*Brassica napus* L.). *Progress in Plant Protection* **58**:264–269 DOI 10.14199/ppp-2018-036.

13.06.2023

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To whom it may concern,

I hereby declare that my contribution to the articles:

- In silico study of the *RSH* (*RelA/SpoT Homologs*) gene family and expression analysis in response to PGPR bacteria and salinity in *Brassica napus*. Dąbrowska GB, Turkan S, Tylman-Mojżeszek W, Mierek-Adamska A, Int. J. Mol. Sci. (2021) 22: 10666, was 50% (Conceptualization, review, editing, supervision, and funding acquisition).
- Localization and expression of *CRSH* transcript, level of calcium ions, and cell cycle activity during *Brassica napus* L. seed development. Turkan S, Mierek-Adamska A, Głowacka K, Szydłowska-Czerniak A, Rewers M, Jędrzejczyk I, Dąbrowska GB, Ind. Crops Prod. (2023) 195: 116439, was 5% (Designing the research, review and editing).
- New seed coating containing *Trichoderma viride* with anti-pathogenic properties. Turkan S, Mierek-Adamska A, Kulasek M, Konieczna WB, Dąbrowska GB. PeerJ. 2023 Jun 1;11:e15392. doi: 10.7717/peerj.15392, was 5% (Designing the experiments, data analysis, original draft preparation, review and editing).

Sincerely,

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and Veterinary Sciences

Dr Agnieszka Mierek-Adamska
Assistant Professor
Department of Genetics
Faculty of Biological and Veterinary Sciences
Nicolaus Copernicus University in Toruń

Toruń, 13.06.2023

To whom it may concern,

I hereby declare that my contribution to the articles:

- In silico study of the RSH (*RelA/SpoT* Homologs) gene family and expression analysis in response to PGPR bacteria and salinity in *Brassica napus*. Dąbrowska GB, Turkan S, Tylman-Mojżeszek W, Mierek-Adamska A, *Int. J. Mol. Sci.* (2021) 22: 10666, was 7.5% (data analysis, *in silico* analysis, original draft preparation, review and editing).

- Localization and expression of *CRSH* transcript, level of calcium ions, and cell cycle activity during *Brassica napus* L. seed development. Turkan S, Mierek-Adamska A, Głowacka K, Szydłowska-Czerniak A, Rewers M, Jędrzejczyk I, Dąbrowska GB *Ind. Crops Prod.* (2023) 195: 116439, was 5% (gene expression analysis, review and editing).

- New seed coating containing *Trichoderma viride* with anti-pathogenic properties. Turkan S, Mierek-Adamska A, Kulasek M, Konieczna WB, Dąbrowska GB, *PeerJ* (2023) 11: e15392, was 15% (data analysis, original draft preparation, review and editing).

Sincerely,
Agnieszka Mierek-Adamska

Toruń,13.06.2023

mgr Wioleta Tylman-Mojżeszek

Department of Genetics

Faculty of Biological and Veterinary Sciences

Nicolaus Copernicus University in Toruń

Declaration

I hereby declare my contribution to the article:

In silico study of the *RSH* (*RelA/SpoT Homologs*) gene family and expression analysis in response to PGPR bacteria and salinity in *Brassica napus*. Dąbrowska GB, Turkan S, Tylman-Mojżeszek W, Mierek-Adamska A, Int. J. Mol. Sci. (2021) 22: 10666, was 5% (formal analysis and investigation).

Yours sincerely,

Wioleta Tylman-Mojżeszek

Dr Katarzyna Głowacka
Department of Plant Physiology, Genetics and Biotechnology
University of Warmia and Mazury in Olsztyn
Faculty of Biology and Biotechnology
Oczapowskiego 1A
10-718 Olsztyn, Poland

Olsztyn, 2023-06-13

Declaration

I hereby declare that my contribution to the article:

Sena Turkan, Agnieszka Mierek-Adamska, Katarzyna Głowacka, Aleksandra Szydłowska-Czerniak, Monika Rewers, Iwona Jędrzejczyk, Grażyna B. Dąbrowska. (2023) Localization and expression of CRSH transcript, level of calcium ions, and cell cycle activity during *Brassica napus* L. seed development. Industrial Crops and Products. 195, 116439. https://doi.org/10.1016/j.indcrop.2023.116439 was 5% (*in situ* localization analysis).

Sincerely,

Katarzyna Głowacka

NICOLAUS COPERNICUS
UNIVERSITY
IN TORUŃ
Faculty of Chemistry

RESEARCH
UNIVERSITY
EXCELLENCE INITIATIVE

Toruń, 12.06.2023

Prof. dr hab. Aleksandra Szydłowska-Czerniak
Department of Analytical Chemistry and Applied Spectroscopy
Analytical and Food Chemistry Research Group
7 Gagarin Street, 87-100 Toruń

Declaration

I hereby declare my contribution to the article:
Sena Turkan, Agnieszka Mierek-Adamska, Katarzyna Głowacka, Aleksandra Szydłowska-Czerniak, Monika Rewers, Iwona Jędrzejczyk, Grażyna B. Dąbrowska. Localization and expression of *CRSH* transcript, level of calcium ions, and cell cycle activity during *Brassica napus* L. seed development. *Industrial Crops & Products* 195 (2023) 116439, https://doi.org/10.1016/j.indcrop.2023.116439 was 5% and consisted of calcium content analysis.

Yours sincerely,

PODPIS ZAUFANY
ALEKSANDRA BARBARA
SZYDŁOWSKA-CZERNIAK
12.06.2023 19:00:14 [GMT+2]
Dokument podpisany elektronicznie
podpisem zaufanym

Prof. dr. hab. Aleksandra Szydłowska-Czerniak

Bydgoszcz 13.06.2023

Dr. Monika Rewers

Department of Agricultural Biotechnology

Bydgoszcz University of Science and Technology

Kaliskiego Ave. 7, 85-796 Bydgoszcz

Declaration

I hereby declare my contribution to the article:

Sena Turkan, Agnieszka Mierek-Adamska, Katarzyna Głowacka, Aleksandra Szydłowska Czerniak, Monika Rewers, Iwona Jędrzejczyk, Grażyna B. Dąbrowska. Localization and expression of CRSH transcript, level of calcium ions, and cell cycle activity during *Brassica napus* L. seed development. Industrial Crops & Products 195 (2023) 116439, https://doi.org/10.1016/j.indcrop.2023.116439, was 5% (flow cytometry analysis).

Yours sincerely,

Monika Rewers

13.06.2023

Assoc. prof. Iwona Jędrzejczyk

Department of Agricultural Biotechnology

Bydgoszcz University of Science and Technology

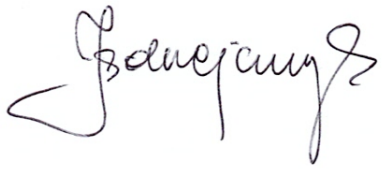
Kaliskiego Ave. 7, 85-796 Bydgoszcz

Declaration

I hereby declare my contribution to the article:

Sena Turkan, Agnieszka Mierek-Adamska, Katarzyna Głowacka, Aleksandra Szydłowska Czerniak, Monika Rewers, Iwona Jędrzejczyk, Grażyna B. Dąbrowska. Localization and expression of CRSH transcript, level of calcium ions, and cell cycle activity during *Brassica napus* L. seed development. Industrial Crops & Products 195 (2023) 116439, https://doi.org/10.1016/j.indcrop.2023.116439, was 5% (flow cytometry analysis).

Yours sincerely,



Dr Milena Kulasek
Assistant Professor
Department of Genetics
Faculty of Biological and Veterinary Sciences
Nicolaus Copernicus University in Toruń

Toruń, 13.06.2023

To whom it may concern,

I hereby declare my contribution to the article:

> New seed coating containing *Trichoderma viride* with anti-pathogenic properties. Turkan S, Mierek-Adamska A, Kulasek M, Konieczna WB, Dąbrowska GB, *PeerJ* (2023) 11: e15392

was 10% (I performed the antioxidant enzyme (SOD) experiment, analyzed the data, prepared figures and tables, authored and reviewed drafts of the article, and approved the final draft).

Sincerely,
Milena Kulasek

13.06.2023

Mgr Wiktoria Konieczna

Department of Genetics

Faculty of Biological and Veterinary Sciences

Nicolaus Copernicus University in Toruń

Declaration

I hereby declare my contribution to the article:

Sena Turkan, Agnieszka Mierek-Adamska, Milena Kulasek, Wiktoria B Konieczna, Grażyna B Dąbrowska. New seed coating containing Trichoderma viride with anti-pathogenic properties. PeerJ. 2023 Jun 1;11:e15392. doi:10.7717/peerj.15392 was 5% and consisted of performing the experiments and reviewing the draft of the article.

Yours sincerely,

Wiktoria Konieczna