



## Chalazal seed coat development in *Brassica napus*



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### ABSTRACT

The chalazal seed coat (CZSC) is a maternal subregion adjacent to the funiculus which serves as the first point of entry into the developing seed. This subregion is of particular interest in *Brassica napus* (canola) because of its location within the seed and its putative contribution to seed filling processes. In this study, the CZSC of canola was characterized at an anatomical and molecular level to (i) describe the cellular and subcellular features of the CZSC throughout seed development, (ii) reveal cellular features of the CZSC that relate to transport processes, (iii) study gene activity of transporters and transcriptional regulators in the CZSC subregion over developmental time, and (iv) briefly investigate the contribution of the A and C constituent genomes to *B. napus* CZSC gene activity.

We found that the CZSC contains terminating ends of xylem and phloem as well as a mosaic of endomembrane and plasmodesmatal connections, suggesting that this subregion is likely involved in the transport of material and information from the maternal tissues of the plant to other regions of the seed. Laser microdissection coupled with quantitative RT-PCR identified the relative abundance of sugar, water, auxin and amino acid transporter homologs inherited from the constituent genomes of this complex polyploid. We also studied the expression of three transcription factors that were shown to co-express with these biological processes providing a preliminary framework for the regulatory networks responsible for seed filling in canola and discuss the relationship of the CZSC to other regions and subregions of the seed and its role in seed development.

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### 1. Introduction

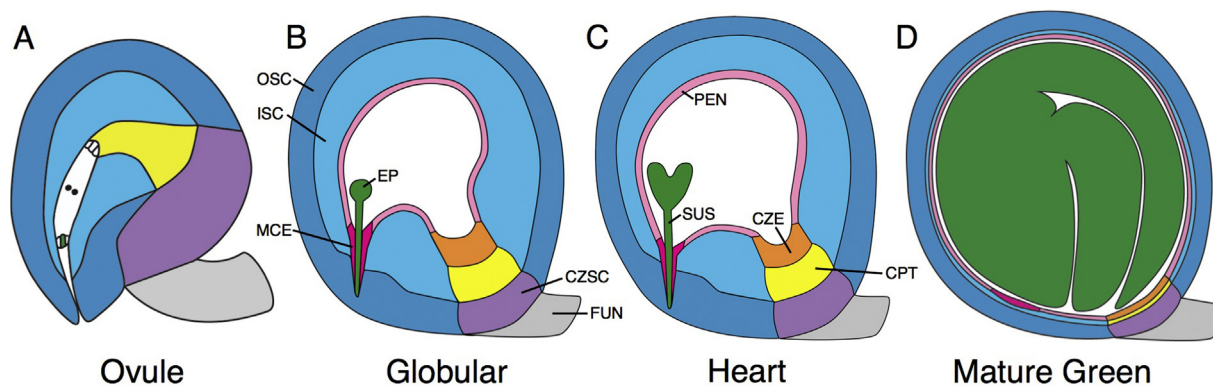
Seed development in oilseeds of the Brassicaceae begins with a double fertilization event that results in the production of a diploid zygote and triploid endosperm. Throughout development, the growing embryo is supported by the endosperm [1,2] and both are enclosed and protected by the seed coat. In addition to serving a protective function, the seed coat functions to release nutrients and transmit signals from the maternal tissues to the embryo, which are necessary for proper embryo development [3,4]. The seed coat is divided into two subregions: the distal seed coat (SC) and the chalazal seed coat (CZSC) (Fig. 1A–D). The SC, the portion of the seed that envelops the interior seed regions, confers a supportive and protective function, and is essential for dormancy and germination [4]. The SC has been extensively studied on both an anatomical [5,6] and molecular level [3,7–9] in *Arabidopsis*, and has also been well-characterized in economically important crop

plants including legumes [10], *Glycine max* [11], barley [12,13], rice [14] and *Brassica* species [15]. The less-studied CZSC is adjacent to the funiculus (Fig. 1A–D), which serves to channel nutrients and water to the developing seed. Given its location within the seed, the paucity of available information regarding this structure is surprising. However, recent advances in *Arabidopsis* research have begun to uncover some of the developmental and genetic mechanisms underlying the development of this poorly characterized seed subregion by combining histology, laser microdissection, and RNA profiling technologies [9,16].

The first comprehensive molecular analysis of the *Arabidopsis* CZSC revealed that populations of mRNAs accumulated specifically in the CZSC and not in other seed regions. These data provide strong evidence that the CZSC is a transcriptionally distinct subregion of the maternal seed coat and is controlled by dynamic sets of actively transcribed genes [16]. Subsequently, Khan et al., [9] analyzed the *Arabidopsis* CZSC transcriptome and its underlying transcriptional circuitry at a higher resolution by employing vigorous bioinformatic analyses. Using a systems biology approach, the CZSC transcriptome was compared to the SC transcriptome in both space and time, revealing profound differences in the

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**Fig. 1.** Line drawings of *Brassica napus* seeds showing changes in tissue morphology at the (A) ovule, (B) globular, (C) heart, and (D) mature green stage of seed development. Embryo (EP, green), distal seed coat [outer (OSC, dark blue); inner (ISC, light blue)], chalazal seed coat (CZSC, purple), chalazal proliferating tissue (CPT, yellow), chalazal endosperm (CZE, orange), peripheral endosperm (PEN, pink), micropylar endosperm (MCE, fuschia), and funiculus (FUN, gray). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

molecular machinery controlling adjacent seed coat subregions at the transcriptional level. It was found that transcripts involved in the transport of sugar, water, amino acids, lipids, and hormonal regulation are expressed in the CZSC at specific stages of seed development that coincide with the timing of integral processes associated with embryonic growth, and oil and protein accumulation. In addition, funiculus vasculature was shown to terminate in the CZSC, suggesting the CZSC is an unloading zone in *Arabidopsis* [9]. Finally, the identification of genes involved in transport processes and the putative transcriptional regulators controlling the activity of these processes were also identified [9]. This research provided the basis for similar studies on more economically important crop plants that are close relatives to *Arabidopsis*, such as *Brassica napus*.

The developing seed imports materials including organic nutrients, sucrose, amino acids, and potassium to support growth, especially early in embryo and endosperm development. These nutrients are imported from the maternal plant via the vascular tissues found in the central region of the funiculus, to the seed coat, which further distributes the nutrients to other parts of the seed [17–19]. Maternal tissues of the developing seed must release and then target these nutrients into the apoplast by apoplastic transporters, as a symplastic barrier is present between maternal and filial seed tissues [17]. In *Arabidopsis*, mRNA encoding sucrose efflux co-transporters (SWEET4 and SWEET10) are expressed in the CZSC. Similarly, mRNAs encoding amino acid co-transporters (AAP2 and SIAR1), as well as water transporters (TIP1;1), and auxin efflux transporters (PIN1 and PIN3) were also identified within the CZSC [9]. The presence of these apoplastic transporters within the CZSC of *Arabidopsis* suggests this maternal seed subregion is involved in the release of nutrients to the filial seed tissues. Investigating the presence and transcriptional regulation of these transporters within the CZSC of canola could provide information regarding the role the CZSC plays in seed filling and development.

*B. napus* is an allopolyploid, resulting from the hybridization of *Brassica rapa* (A genome) and *Brassica oleracea* (C genome) with subsequent genome duplication. This gives *B. napus* the capacity to possess up to six copies of each gene, three inherited from *B. rapa* and three from *B. oleracea*. The expression of the A and C gene homologs are not always equal. For example, Chalhoub et al. [20] found that 3.7% of gene pairs had higher expression of the A homologue compared to the C homologue and 3.3% of gene pairs had lower expression of the A homologue compared to the C homologue in leaves while the opposite was found in roots [20]. Thus, the progenitor genomes may contribute unevenly in different organs of the plant in complex polyploids like *B. napus*. Additionally, the con-

stituent genomes could individually contribute to specific traits. For example, the C genome has been suggested to contribute to important agronomic traits such as oil content, seed yield, and biotic and abiotic stress tolerance because of the higher nucleotide diversity [21]. Additionally, the A genome has been shown to play a strong role in plant disease resistance [22]. Thus, investigating the contribution of the two constituent genomes to CZSC function and development could have implications in plant breeding and seed improvement research.

The present study explores the CZSC across seed development in the economically important species, *B. napus*. We provide a detailed anatomical analysis using light and transmission electron microscopy to reveal the complex cellular composition and organization of the CZSC relative to the timing of important seed processes such as seed filling and embryo development. We further studied the expression of genes associated with sugar, amino acid, water, and auxin transport and provide further evidence for the co-expression of transcriptional regulators possibly controlling the expression of the genes over time and the contribution of each gene copy from this large and genetically complex polyploid. Finally, we discuss the role of the CZSC in relation to other subregions of the seed and how the CZSC can contribute to advances in seed improvement research.

## 2. Materials and methods

### 2.1. Plant material and growing conditions

*B. napus* (cv. topaz) “canola” plants were grown in a peat-based medium (Sunshine Mix #1) at 22 °C with a relative humidity of 50–70% and long-day light conditions [16 h light (100–150  $\mu\text{E}/\text{m}^2/\text{s}$ ), 8 h dark]. To mitigate possible time-of-day effects on development and gene activity, flowers were pollinated and siliques were harvested between 3:00 PM and 5:00 PM. To provide a thorough evaluation of the changes in cellular composition, organization, and gene expression in the CZSC in the mature ovule and throughout seed development, siliques from four major developmental stages were collected (Fig. 1A–D): the mature ovule, prior to pollination; the globular stage, 7 DAP (days after pollination); the heart stage, 10 DAP; and the mature green stage, 28 DAP.

### 2.2. Light microscopy

Light microscopy was used to characterize the anatomy and cellular organization of the CZSC just prior to fertilization and throughout seed development. At least 20 different seeds were

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