



Differential activity of *CYTOKININ OXIDASE/DEHYDROGENASE3* (*CKX3*) and *CKX5* genes in regulating yield components in *Brassica napus* L.

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Abstract

Cytokinin is a plant hormone that regulates several yield-related traits in plants. Previously, it was demonstrated that in tetraploid oilseed rape (*Brassica napus* L.), mutation of all four cytokinin-degrading *BnCKX3* and both *BnCKX5* genes resulted in increased cytokinin concentration, larger and more active inflorescence meristems, and a higher number of ovules per gynoeceum. This resulted in the formation of more flowers and pods on the main stem, thereby increasing seed yield from the main stem of the plants. Here, we investigated the relative contributions of distinct combinations of *BnCKX3* and *BnCKX5* genes of the A and C genomes to these yield components. Our analysis revealed an unexpectedly strong role for *BnCKX5* in regulating these traits and identified distinct supportive *BnCKX3* gene mutant combinations. These findings facilitate the selection of relevant alleles for breeding. Furthermore, seeds from *BnCKX* gene mutant plants showed oil content and concentrations of unsaturated fatty acids similar to those of the wild type. Taken together, this study provides further insight into the role of cytokinin and *BnCKX* genes in regulating yield components in oilseed rape and provides novel information on functionally relevant alleles.

Key message

Genetic analysis demonstrates the importance of *BnCKX5* genes and specific *BnCKX3* alleles of the oilseed rape A and C genomes in regulating inflorescence meristem activity and ovule number.

Keywords *Brassica napus* L. · Cytokinin · Cytokinin oxidase/dehydrogenase · Inflorescence meristem · Oilseed rape · Ovules · Seed yield · Yield components

Introduction

Increasing seed yield is an important breeding goal for many crop plants. Seed yield is a complex trait controlled by numerous genes (Ding et al. 2012; Siles et al. 2021). To achieve a steady increase in yield, both source and sink

capacity must be improved (Körner 2015; Sonnewald and Fernie 2018). Studies of plants with enhanced sink strength in shoots or roots strongly support the view that plants are often not source-limited and that enhanced sink strength may be reached by altering organ growth or number without negative tradeoffs (Ashikari et al. 2005; Werner et al. 2010; Bartrina et al. 2011, 2017; Ramireddy et al. 2018; Schwarz et al. 2020).

In seed-bearing plants, enhanced sink strength and increased seed yield can be achieved by increasing the size and activity of reproductive meristems to form more flowers and more ovules, which together cause the formation of more seeds. A further option is to increase metabolic sink strength, leading to the formation of more storage compounds. One known factor to regulate the size and activity of reproductive tissues, as well as metabolic sink strength in both mono- and dicotyledonous plants, is the hormone

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cytokinin (CK) (Jameson and Song 2016, 2020; Kieber and Schaller 2018).

The CK content or signaling can be modified locally in a targeted fashion to alter plant growth (Werner et al. 2010, 2021; Ramireddy et al. 2018; Zeng et al. 2022). One important tool to alter the cytokinin content in plants to modify yield-related traits are genes encoding cytokinin oxidases/dehydrogenases (CKX), which are enzymes catalyzing the breakdown of CK (Schmülling et al. 2003; Werner et al. 2006). The first evidence for the role of CKX genes in regulating yield came from the discovery that the *Grain number1* (*Gn1*) locus, which causes an increase in grain number in rice, encodes OsCKX2 (Ashikari et al. 2005).

CKX genes also play a role in regulating yield components in dicots, as was shown for *Arabidopsis thaliana* (Bartrina et al. 2011) and *Brassica napus* L. (oilseed rape) (Schwarz et al. 2020). In *Arabidopsis*, it was found that the combined mutation of the *AtCKX3* gene, which is the *Arabidopsis* orthologue of the *OsCKX2* gene, but whose mutation alone was ineffective, and *AtCKX5*, alters several yield-related traits, namely flower number, silique number, distance of ovules, and seed density in siliques. The sum of all changes caused a significant increase in seed yield of more than 50% (Bartrina et al. 2011). Subsequently, it was shown that mutation of the four *BnCKX3* and two *BnCKX5* genes of the tetraploid *B. napus*, which is the closest relative of *Arabidopsis* among the crop plants and globally the third most important source of vegetable oil (Weselake et al. 2024), enhances several of these yield components as well (Schwarz et al. 2020), consistent with changes caused by exogenous treatment with CK (Zuñiga-Mayo et al. 2018).

A comparison of the mutant phenotypes shows that CKX3 and CKX5 collectively control similar yield components to a different extent in *Arabidopsis* and oilseed rape (Bartrina et al. 2011; Schwarz et al. 2020). In oilseed rape, the size and activity of the inflorescence meristem of the *Bnckx3,5* mutants are increased, and more flowers and pods are formed. *Bnckx3,5* mutants have larger gynoeceia, and the distance of ovules is reduced, although to a lesser extent in oilseed rape than in *Arabidopsis*. In contrast to *Arabidopsis*, where the formation of larger siliques containing more densely packed seeds contributes significantly to the increase in seed yield, in *B. napus*, a portion of the higher number of ovules per pod does not develop into seeds. Therefore, the seed number per pod is not increased, thus limiting the increase in seed yield in *B. napus*, which was in the range of 20–32% on the main stem of *Bnckx3,5* mutants (Schwarz et al. 2020). The oil content of seeds from these lines was not reported, although it is a crucial factor for productivity, and high oil content is a major breeding goal for oilseed rape. Oil from oilseed rape contains a healthy lipid profile with a high proportion of mono- and polyunsaturated

fatty acids (Weselake et al. 2024). Also, the content of glucosinolates, which are secondary metabolites protecting the plants from insect pests and pathogens, is as yet unknown in seeds of *Bnckx3,5* mutants. Because high glucosinolate concentrations can be harmful for humans and livestock, breeding aims to produce varieties with a low glucosinolate content (Miao et al. 2021).

B. napus is an allotetraploid plant originating from natural hybridization between *B. rapa* and *B. oleracea*, which contribute the A and C subgenomes (Chalhoub et al. 2014; Gu et al. 2024). Preceding genome triplications in the diploid ancestors and chromosomal rearrangements have led to a complex genome structure with different gene numbers. For example, there are 23 *BnCKX* genes belonging to seven *BnCKX* gene families (Liu et al. 2013; Schwarz et al. 2020). Among these, there are four *BnCKX3* and two *BnCKX5* genes. The redundancy due to the complex evolutionary genome history and polyploidy makes it challenging to study gene function and its potential relevance to improve agronomically relevant traits.

With the combined mutation of four *BnCKX3* and two *BnCKX5* genes, the maximum number of potentially relevant *BnCKX3/5* genes from both subgenomes had been mutated. To follow this number of genes in practical breeding is challenging, even if it is assisted by molecular markers. It would therefore be desirable to lower the number of mutated *BnCKX* genes required to obtain an enhancement of these yield components. It could indeed be that not all the mutated genes are required to obtain the observed effect. For example, a partial subfunctionalization or neofunctionalization may have occurred for the different *BnCKX3* genes, as is indicated by their differential expression pattern (Schwarz et al. 2020). Therefore, the regulation of different yield components may be exerted (partly) by different *BnCKX3* alleles of the A and C genomes. It could also be that a complete loss of gene function is not optimal, as some CKX activity might be necessary during certain developmental stages of reproductive tissues (Di Marzo et al. 2020). Furthermore, the change of CK concentration achieved by reducing its breakdown is relevant as supraoptimal concentrations of CK may have negative effects (Ferreira and Kieber 2005). Taken together, the mutation of all *BnCKX3,5* genes may not result in optimal changes, at least not for all yield traits. Finally, given their differential expression and contribution of homoeologous genes to phenotypic traits, it would be interesting to explore which *BnCKX3* and *BnCKX5* gene copies of the A or C genomes contribute most to the phenotypic changes in the *Bnckx3,5* mutant (Chalhoub et al. 2014; Wu et al. 2018).

To explore the functional relevance of gene dosage and specific combinations of *BnCKX3* and *BnCKX5* mutant alleles in regulating yield components, we have analyzed a

variety of *Bnckx3,5* mutant plants. In each of these, half of the genes of *BnCKX3* and *BnCKX5* are mutated in different combinations in the background of the full set of mutated alleles of the respective other gene. Thus, these eight different genotypes harbour only one mutant allele of the two *BnCKX5* genes from the A and C genomes, or different combinations of two of the four different *BnCKX3* genes of the A and C genomes, always in a background of a fully mutated set of *BnCKX5* or *BnCKX3* genes. The results show that mutation of both *BnCKX5* alleles is necessary to obtain full expressivity of the mutant phenotypes. In contrast, two of the four *Bnckx3* mutant alleles, preferentially those of the A genome, are sufficient to cause the formation of significantly more flowers and ovules. The results identify the functionally important alleles to enhance yield components in oilseed rape with a lower number of mutant alleles than known previously, thus facilitating their use in breeding.

Material and methods

Plant material

An elite spring oilseed rape breeding line of *Brassica napus* L. was used as the wild type (WT) (Schwarz et al. 2020). Mutants carrying point mutations in the four *BnCKX3* and two *BnCKX5* genes were originally identified by TILLING (McCallum et al. 2000) in a mutant population generated by treatment with ethyl methanesulfonate (EMS) (Lammerlyn et al. 2017). These individual mutants were backcrossed

four times to wild type before their combination by crossing to generate the quadruple *Bnckx3* mutant (*a1/a2/c1/c2*), the double *Bnckx5* mutant (*a1/c1*), and the sextuple *Bnckx3,5* mutant (*a1/a2/c1/c2; a1/c1*) (Schwarz et al. 2020).

Plants containing different combinations of *Bnckx3* and *Bnckx5* mutant alleles (genotypes G1, G2, and G7 to G12 according to Table 1) were obtained by crossing *Bnckx3,5* to the wild type. The F1 generation was backcrossed to *Bnckx3,5*. 250 BC1 plants were genotyped using competitive allele-specific PCR (KASP) (Majeed et al. 2019) to identify the 64 genotypes containing all possible combinations of homozygote or heterozygote *Bnckx3* and *Bnckx5* mutant alleles from the A and C genomes. In the next generation, obtained by selfing, ~1300 BC1S1 plants of the selected genotypes G1, G2, and G7 to G12, shown in Table 1, were again genotyped to eliminate plants containing heterozygous alleles. From the selected plants, the BC1S2 was obtained and used for all further experiments. Genotyping was carried out by BASF (Gent, Belgium) (BC1) and by VHLGenetics (Wageningen, Netherlands) (BC1S1).

Plant growth conditions

Plants were grown in the greenhouse in pots with a diameter of 13 cm filled with 1.5 L of soil (Stender D400 from Stender AG, Schermbeck, Germany) and fertilised with Osmocote START at 19–22 °C under long-day conditions (16 h light/8 h dark) and watered every day. Before planting in soil, seeds were surface-sterilized with 0.12% H₂O₂ + 0.01% Triton X-100 for 10 min, washed several times with water,

Table 1 Different *Bnckx3,5* mutant genotypes of *B.napus*. *B.napus* genotypes analyzed in this study harboring different combinations of mutated *BnCKX3* and *BnCKX5* genes of the A and C genomes are shown. Genes written in capitals and marked in light grey are wild-type, whereas small letters marked in darkgrey indicate mutant alleles

Line	<i>BnCKX3</i>				<i>BnCKX5</i>	
	<i>CKX3_A1</i>	<i>CKX3_A2</i>	<i>CKX3_C1</i>	<i>CKX3_C2</i>	<i>CKX5_A1</i>	<i>CKX5_C1</i>
WT	A1	A2	C1	C2	A1	C1
<i>ckx3</i>	a1	a2	c1	c2	A1	C1
<i>ckx5</i>	A1	A2	C1	C2	a1	c1
<i>ckx3,5</i>	a1	a2	c1	c2	a1	c1
G1	a1	a2	c1	c2	A1	c1
G2	a1	a2	c1	c2	a1	C1
G7	a1	a2	C1	C2	a1	c1
G8	A1	A2	c1	c2	a1	c1
G9	a1	A2	c1	C2	a1	c1
G10	A1	a2	C1	c2	a1	c1
G11	a1	A2	C1	c2	a1	c1
G12	A1	a2	c1	C2	a1	c1

and germinated in vitro on wet filter paper under long-day conditions. After one week, seedlings were transferred to soil and grown in 6 cm pots for three weeks, then in 9 cm pots for an additional 1–2 weeks, and finally transferred into 13 cm pots before the reproductive phase started.

Plants grown in the field were germinated under standard greenhouse conditions and transferred after four weeks to a field trial area located at FU Berlin, Germany. Plants were grown in 12 plots of 1.2 m × 1.2 m containing one plant of each of the 12 genotypes planted in a mixed arrangement. Seeds from these plants were used to determine the content of oil and protein in seeds, the percentage of oleic acid (18:1) and linolenic acid (18:3), as well as of glucosinolate content.

Measurement of morphological yield components

To determine the inflorescence meristem activity, the number of flowers formed between day 2 and day 23 after the beginning of flowering was counted. The beginning of flowering was defined as the opening of the first flower. The number of developed pods was counted at the end of the reproductive growth period. The analysis was mostly limited to the main stem as it shows the most stable growth behaviour and is the principal source of seed yield (Diepenbrock 2000; Siles et al. 2021).

To determine the number of ovules per gynoecium, the number of developed seeds per pod, and empty positions were counted at the end of pod development using a stereomicroscope. Seeds from both halves of the pod were counted in three pods obtained from the main stem of 5–6 plants.

Analysis of seed oil content

Seed quality parameters were determined by NIRS using the FOSS monochromator model 6500 (NIRSystem Inc., Silver Spring, MD, USA). The spectra of 2 g of seeds in small ring cups were recorded from 400 to 2498 nm at 2-nm intervals. WinISI software (Version 4.12.0.15440, FOSS NIR Systems Inc., USA) was used to analyze the spectra of the harvested seeds. Calibration 'raps2024.eqa' provided by VDLUFA Qualitätssicherung NIRS GmbH (<http://www.vdlufa-nirs.de>) was used to determine the seed content of oil (%), protein (%) and glucosinolate (μmol/g seed). Seed oil, protein, and glucosinolate contents were determined on a 91% seed dry matter basis (Holzenkamp and Möllers 2024). The calibration 'raps2024.eqa' also allowed for determining the oleic acid and linolenic acid content of the seed oil as described (Velasco and Becker 1998; Velasco et al. 1998, 1999).

Accession numbers

The accession numbers of the *B. napus* *CKX3* genes are BnaA10g28940D (*CKX3_A1*), BnaA02g08420D (*CKX3_A2*), BnaC09g33450D (*CKX3_C1*), and BnaCnng41060D (*CKX3_C2*). The accession numbers of the *B. napus* *CKX5* genes are BnaAnng09190D (*CKX5_A1*) and BnaC06g36220D (*CKX5_C1*).

Statistical analysis

Data were statistically evaluated using GraphPad Prism, version 8 (GraphPad Software, La Jolla, CA, United States). Statistical tests used are indicated in the figure and table legends. A p-value < 0.05 was considered to indicate a significant difference.

Results

Generation of different *Bnckx3,5* mutant genotypes

Mutation of either all four *BnCKX3* genes or both *BnCKX5* genes alone did not have any or only slight phenotypic consequences on yield-related traits, while the mutation of all six genes caused distinct and strong phenotypic changes (Schwarz et al. 2020). To study the functional relevance of *BnCKX3* and *BnCKX5* genes from the A and C genomes and their combinations, we constructed lines harbouring different numbers and combinations of mutated genes. To obtain these lines, *Bnckx3,5* was first crossed to the wild type. The resulting F1 generation was backcrossed to *Bnckx3,5*, and the BC1 plants were genotyped to identify the 64 possible different *Bnckx3 Bnckx5* mutant combinations. Selected genotypes were propagated, self-fertilized, and the resulting BC1S1 again genotyped to eliminate plants containing heterozygous alleles. BC1S2 plants homozygous for the selected individual mutant alleles were used for all further experiments.

Table 1 shows the different *Bnckx3 Bnckx5* mutant allele combinations analyzed in this study. These lines harbour different mutant alleles of *BnCKX3* or *BnCKX5* in the background of the full set of mutated alleles of the respective other gene. Two of the lines (genotypes G1, G2) are homozygous for all four *Bnckx3* mutant alleles and contain, in addition, a single *Bnckx5_a1* or *Bnckx5_c1* mutant allele. Six other lines (G7 to G12) contain both *Bnckx5* mutant alleles and all six possible combinations of the additional two *Bnckx3* mutant alleles. Thus, each of the eight lines contains a full set of mutated *Bnckx3* or *Bnckx5* genes plus half the gene dosage of the respective other genes in different allele combinations. During the vegetative phase, growth

and development of all mutants, including the *Bnckx3,5* sextuple mutant, were comparable to wild type (see also Schwarz et al. 2020). The focus of this work has been on changes during the reproductive phase as outlined below.

Activity of the inflorescence meristem

First, we studied the activity of the inflorescence meristem by counting the number of flowers formed between days 2 and 23 after the beginning of flowering as the readout. In three independent greenhouse trials, WT plants formed during that time span about 80 to 90 flowers on the main stem, while *Bnckx3,5* mutants formed between 100 and 120 flowers, i.e., on average one third more than the WT (Figs. 1, S1; see also Schwarz et al. 2020). In comparison, plants harboring mutations in only the *BnCKX3* or *BnCKX5* genes showed only a slight increase of 6–7% in the number of flowers at the main stem compared with their wild-type segregants (Schwarz et al. 2020).

Mutation of either one of the two *BnCKX5* gene copies in a *Bnckx3* mutant background (G1, G2) almost completely abolished the enhanced flower formation found in *Bnckx3,5*, revealing the functional importance of *BnCKX5* for this trait. This was a reproducible result in all three independent trials (Figs. 1, S1).

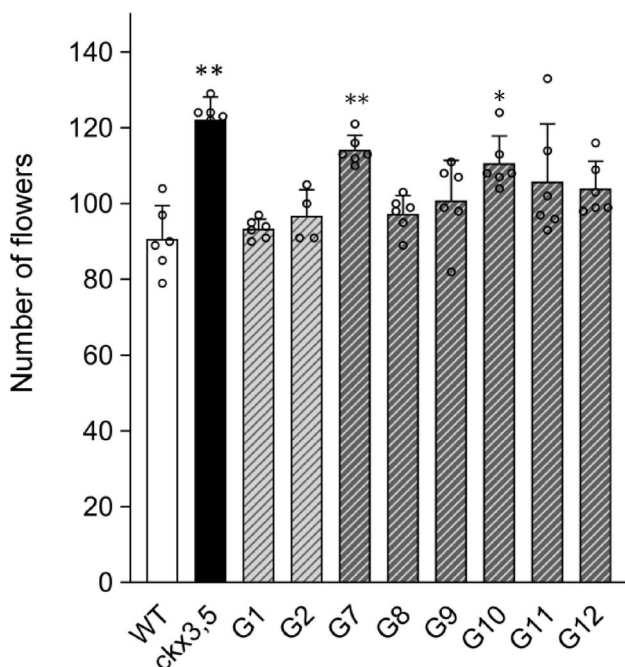


Fig. 1 Inflorescence meristem activity. The number of flowers formed between 2 and 23 days after the opening of the first flower at the main stem of plants grown in a greenhouse. Data shown are mean values $\pm$ SD, $n=6$. The statistical significance of differences compared to WT was calculated using one-way ANOVA (Kruskal–Wallis test). * $p \leq 0.05$, ** $p \leq 0.01$

In the background of the *Bnckx5* mutant, the contribution of distinct *Bnckx3* mutant alleles became apparent. Among the *Bnckx3* mutant gene combinations, the *Bnckx3a1a2* mutant combination (G7) showed in all three trials a significant increase (24% to 35%) in flower number compared to WT (Fig. 1, S1). The increase was in all three trials close to or even similar to the one obtained with the *Bnckx3,5* mutant. In contrast, the rate of flower formation in plants carrying mutations in both *BnCKX3* gene copies from the C genome (G8) was in all three trials not statistically significantly different from WT. Mutants carrying combinations of mutated *BnCKX3* from both genomes (G9 to G12) mostly also showed a tendency to a higher frequency of flower formation than WT. The increase in the rate of flower formation of up to 29% in comparison to WT was statistically significant in one of the three trials for each of the genotypes G9, G10, and G11 (Fig. 1, S1). Taken together, this comparison highlights the functional importance of the *BnCKX3* gene copies from the A genome as compared to those of the C genome. The rate of flower formation in *Bnckx3a1a2ckx5* (G7) mutants carrying four mutant alleles could be as high as the rate of flower formation in the sextuple *Bnckx3,5* mutant.

Ovule number

The number of ovules formed per gynoecium, calculated as the total number of aborted and developed seeds, was another yield-related trait that was increased in *Bnckx3,5* mutants. The increase in ovule number was due to a reduced distance between individual ovules that had formed on the placenta and longer gynoecia (Schwarz et al. 2020). In four independent trials, *Bnckx3,5* mutants showed an increase in the ovule number per gynoecium between 16 and 32% (Figs. 2, S2), which is consistent with previous data (Schwarz et al. 2020). Of the *Bnckx3* and *Bnckx5* mutants, which were not analysed by Schwarz et al. (2020), only *Bnckx5* showed an increased ovule number of 21% (Fig. 2a), which was almost in the range of the increase of 31% shown by *Bnckx3,5*. In this trial, *Bnckx5* mutants had, on average, 44 ovules per gynoecium, while WT had 36, and *Bnckx3,5* had 48. In contrast, the ovule number in gynoecia of the *Bnckx3* mutant was not significantly different from WT (Fig. 2a).

Mutation of only one of the two *BnCKX5* gene copies in a *Bnckx3* mutant background reduced the number of ovules per gynoecium to WT level, highlighting the relevance of *BnCKX5* to control the ovule number in the placenta tissue (Figs. 2b, S2). All mutants carrying two mutated *BnCKX3* gene copies in the *Bnckx5* mutant background (G7 to G12) showed an increase in ovule number per gynoecium, which is consistent with an important role for *BnCKX5*. The highest increase was shown by the genotypes G7 and G10, which

Fig. 2 Number of ovules per gynoecium. **a** The number of ovules in wild type (WT) and the mutant lines *Bnckx3*, *Bnckx5*, and *Bnckx3,5*. **b** The number of ovules in genotypes with different combinations of *Bnckx3* and *Bnckx5* mutant alleles. Plants were grown in a greenhouse. Data shown are mean values $\pm$ SD, $n = 15$ – 18 . The statistical significance of differences compared to WT was calculated using one-way ANOVA (Tukey's test) in (A) and one-way ANOVA (Kruskal–Wallis test) in (B). * $p \leq 0.05$, ** $p \leq 0.01$

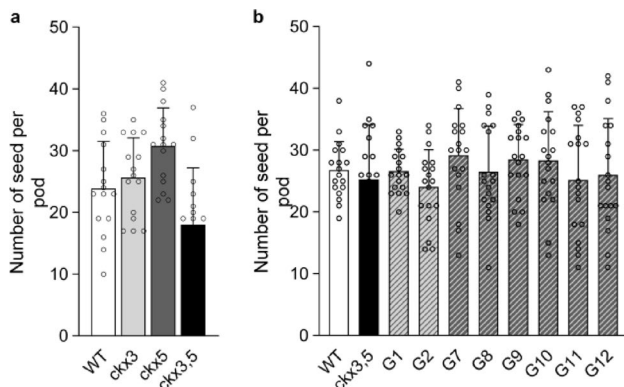
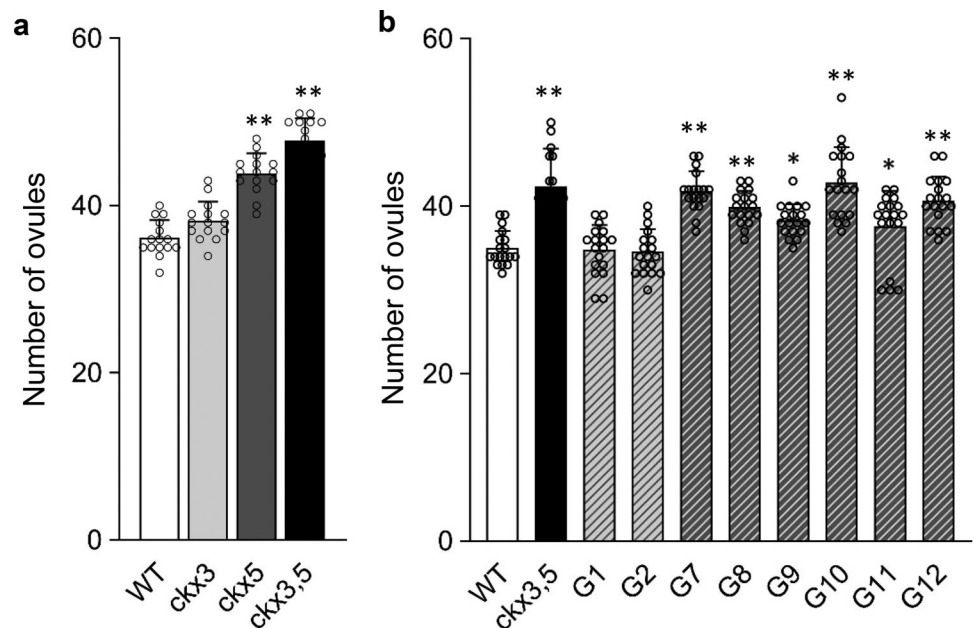


Fig. 3 Number of seeds per pod. **a** The number of seeds per pod in wild type (WT) and the mutant lines *Bnckx3*, *Bnckx5*, and *Bnckx3,5*. **b** The number of seeds per pod in different genotypes carrying various combinations of *Bnckx3* and *Bnckx5* mutant alleles. Plants were grown in a greenhouse. Data shown are mean values $\pm$ SD, $n = 15$ – 18 . The statistical significance of differences compared to WT was calculated using one-way ANOVA (Tukey's test). None of the mutant genotypes shows a difference from WT with $p \leq 0.05$

harbour the *ala2* and *a2c2* mutant combinations. They showed in all three independent trials an increase of ovule number between 17 and 28%, which is in the same range as the increase recorded for the *Bnckx3,5* mutant (Figs. 2b, S2). Also, the increase in ovule number of G12 (*a2c1*) was in all three trials highly significant, around 15%. In comparison, G8 (*c1c2*), G9 (*alc1*), and G11 (*alc2*) showed a lower increase in ovule number (Figs. 2b, S2). These results indicate a relatively higher importance of the *BnCKX3-A2* allele, which might be combined with one of the other three mutated *BnCKX3* alleles and mutated *BnCKX5* to achieve the highest increase in ovule number per gynoecium.

Seeds per pod

The increased number of ovules per gynoecium may eventually result in an increased number of seeds per pod. However, in the *Bnckx3,5* mutant, about one-third more ovules per gynoecium did not lead to a higher number of seeds per pod due to increased seed abortion (Schwarz et al. 2020). Also, in the four trials shown here, *Bnckx3,5* has either a similar or a lower number of seeds per pod compared to WT (Fig. 3, S3). Also, *Bnckx3* and *Bnckx5* mutants had a similar number of seeds per pod like WT, with a relatively large variability in all genotypes (Fig. 3a). The comparison of the eight mixed genotypes yielded a similar result. On average, the number of seeds per pod was comparable in all lines, including WT, and again the mutant genotypes showed a relatively large variability (Fig. 3b). In summary, this confirms the result of Schwarz et al. (2020) that not all of the additional ovules found in the mutants develop into mature seeds.

Seed oil and protein content

Important yield components that have not yet been analysed in *Bnckx3,5* mutants are the oil content of the seeds and the proportion of unsaturated fatty acids. Cytokinin is a factor regulating sink strength of tissues (Guivarc'h et al. 2002; Werner et al. 2008), raising the possibility that the oil content might change if CK metabolism genes are mutated. The analysis of the seeds of plants grown in the field by near-infrared spectroscopy (NIRS) detected an oil content between 37 and 40% and a protein content of 21–23% (Fig. 4).

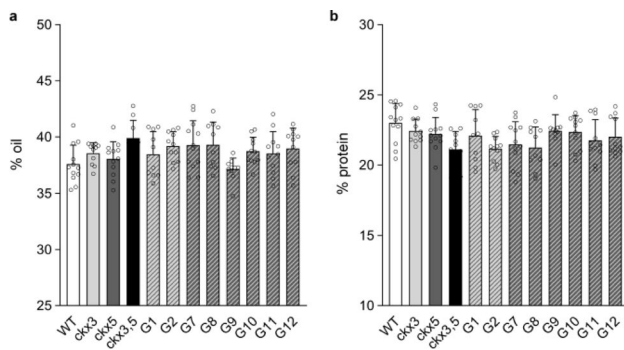


Fig. 4 Oil and protein content of seeds. **a** Percent of oil content in seeds. **b** Percent of protein content in seeds at 9% moisture content. Seeds were harvested from plants grown in the field, and data were recorded using NIRS. Data shown are mean values $\pm$ SD, $n=12$. The statistical significance of differences compared to WT was calculated using one-way ANOVA (Tukey's test). None of the mutant genotypes shows a difference from WT with $p \leq 0.05$

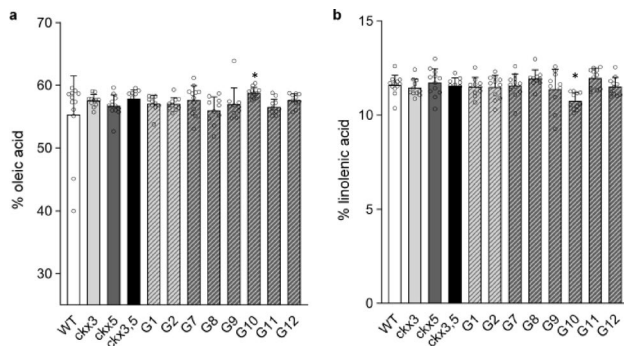


Fig. 5 Content of oleic acid and linolenic acid in seed oil. **a** Percent of oleic acid (18:1). **b** Percent of linolenic acid (18:3). Seeds were harvested from plants grown in the field, and data were recorded using NIRS. Data are given as percentages of total fatty acids and are mean values $\pm$ SD, $n=12$. The statistical significance of differences compared to WT was calculated using one-way ANOVA (Tukey's test). * $p \leq 0.05$

Generally, seeds having a higher oil content had a lower protein content. In this trial, seeds from *Bnckx3,5* contained 6% more oil than seeds from WT, i.e., 40% compared to 37%. The percentage of unsaturated fatty acids was around 60% for oleic acid (18:1) and around 12% for linoleic acid (18:3) for all genotypes (Fig. 5). The content of glucosinolate was between 16 and 20 μ mol per g seed for the different genotypes, with a significant increase compared to WT only in the *Bnckx5* mutant (Fig. S4).

The oil and protein content of seeds from plants of the same genotypes grown in a greenhouse was about 44% and 19%, respectively, with only small differences between the genotypes and irrespective of whether the seeds were harvested from the main stem or lateral stems (Fig. S5). The proportion of oleic acid was between ca. 58–62% in seeds from all genotypes and comparable for main stem and lateral branches. Seeds derived from *Bnckx3,5* and G10

showed a significant increase in the proportion of oleic acid compared to WT from both main stem and lateral branches, while this was limited to the main stem for other genotypes with mutated *BnCKX3* alleles (Fig. S6a,b). In this trial, the proportion of linolenic acid was lowest in the genotypes with the highest content of oleic acid, but the differences from WT were mostly not significant (Fig. S6c–d). The concentration of glucosinolate tended to be increased in several mutant genotypes, but also these changes were mostly not significant (Fig. S6e–f). It is noteworthy that the glucosinolate content of plants grown in the greenhouse was considerably lower than that of plants grown in the field (Fig. S4), highlighting the impact of growth conditions and underpinning the need for further field trials.

Discussion

The genetic analysis of the roles of individual *BnCKX3* and *BnCKX5* genes of the tetraploid *B. napus* in regulating yield components has extended earlier work (Schwarz et al. 2020) and revealed distinct contributions of these genes to two important yield components, the activity of the inflorescence meristem and the number of ovules. Not all *BnCKX* gene copies contributed equally to these traits; instead, a nuanced functional redundancy was found. A striking result has been the unexpectedly strong role of *BnCKX5* in regulating yield components. For *BnCKX3*, genetic analysis revealed a stronger contribution of genes from the A genome compared to the C genome of *B. napus*. Finally, increasing the activity of the inflorescence meristem and the number of ovules did not negatively influence seed oil content and fatty acid profile.

A single *BnCKX5* allele is sufficient to maintain the activity of the inflorescence meristem at the WT level, even if, in addition, all *BnCKX3* alleles are mutated (Fig. 1). Further mutation of the second *BnCKX5* allele caused a strong increase in the frequency of flower formation, illustrating the relevance of *BnCKX5*. Moreover, the sole mutation of *BnCKX5* is sufficient to cause a significant increase in the number of ovules per gynoecium (Fig. 2), further underpinning the important role of this gene. Previously, *BnCKX3* had been suspected to be functionally more relevant as it is the closest homologue to both the yield genes *OsCKX2* of rice (Ashikari et al. 2005) and *AtCKX3* of Arabidopsis (Bartrina et al. 2011; Schwarz et al. 2020). For the four *BnCKX3* alleles, genetic analysis in the background of mutated *BnCKX5* has demonstrated a stronger role for the two gene copies of the A genome in regulating inflorescence meristem activity, as well as for the *BnCKX3-A2* allele in regulating ovule number. It would be interesting to study the consequences of individual *BnCKX3* gene mutations on the

expression of the other *BnCKX3* genes, in particular on the respective other *BnCKX3-A* and *BnCKX3-C* alleles, characterized by distinct expression profiles (Schwarz et al. 2020). The different relative functional importance of *CKX* genes in *Arabidopsis* and *B. napus* highlights that this can differ between species and even between a model plant and the closely related crop plant, underpinning the assessment that *Arabidopsis* is not an infallible model for gene expression in the Brassicaceae (Song et al. 2015).

What could be the reason(s) for *BnCKX5* being the major regulator of CK activity in the inflorescence meristem and the placenta? RNA-seq analysis revealed that all six *BnCKX* gene copies are expressed at a low level in vegetative tissues and exhibit distinct expression patterns in different reproductive tissues. However, a conspicuous, generally stronger expression of *BnCKX5* as compared to *BnCKX3* was not found (Schwarz et al. 2020). In situ hybridization detected expression of *BnCKX3* and *BnCKX5* in the organizing centers of the inflorescence and floral meristems, with *BnCKX5* covering larger domains (Schwarz et al. 2020). This is relevant as the regulation of meristem activity by cytokinin is exerted through the WUS-CLV circuit (Leibfried et al. 2005; Gordon et al. 2009; Bartrina et al. 2011). The larger expression area of *BnCKX5* covers most of the *CLV1* expression domain. Lack of *BnCKX5* activity, resulting in a higher concentration of cytokinin, could lead to a lower expression of the *CLV1* gene, which is negatively regulated by CK and functions to restrict the WUS domain, thus limiting meristem activity and size (Brand et al. 2000; Schoof et al. 2000; Lindsay et al. 2006; Gordon et al. 2009).

Furthermore, in situ hybridization has shown that *BnCKX5* is expressed in ovules and placenta of young gynoecia, where no expression of *BnCKX3* was detected (Schwarz et al. 2020). This is consistent with the main role of *BnCKX5* in regulating the activity of meristematic cells in the placenta, thus influencing ovule primordia formation. Cytokinin acts here as a positional cue regulating the distance between ovule primordia on the placental tissue. We hypothesize that loss of *BnCKX5* activity alters a cytokinin gradient, such that upon mutation of this gene, the cytokinin threshold concentration required for primordia formation is reached at a closer distance. Taken together, it is likely that the functional dominance of *BnCKX5* is at least partially due to the differences in gene expression, at least in the case of ovule formation.

Additional reasons for the dominance of *BnCKX5* could lie in differences in subcellular localisation of *CKX* proteins (Werner et al. 2003; Köllmer et al. 2014; Niemann et al. 2015; Liu et al. 2018), providing access to different cytokinin pools, and differences in substrate specificity (Galuszka et al. 2007; Kowalska et al. 2010). Interestingly, *BnCKX5-A1* is predicted to be transported into the endoplasmic

reticulum (ER) like the *Arabidopsis* orthologue, which may be expelled to the apoplast (Niemann et al. 2015). In contrast, *BnCKX5-C1* is predicted to be a soluble cytoplasmic protein (Ødum et al. 2024). This difference in *BnCKX5* proteins encoded by the two Brassica subgenomes requires experimental verification. Different subcellular localization of the proteins may provide access to different intra- and extracellular cytokinin pools feeding into distinct cytokinin signaling pathways (Romanov et al. 2018). Enhanced CK signaling through different pathways could be the basis for a stronger role of *BnCKX5* in oilseed rape as compared to *Arabidopsis*.

The expression of all four *BnCKX3* genes from the A and C genomes is mostly very similar and strongest in reproductive tissues (Chao et al. 2020; Schwarz et al. 2020). *BnCKX3-A1/C1* are most strongly expressed in open flowers, while *BnCKX3-A2/C2* expression is also found in young flowers, developing seeds, and pods (Schwarz et al. 2020). The dominant role for *BnCKX3-A2* in regulating ovule number could be due to its stronger expression in comparison to *BnCKX3-C2*, in young developing flower buds (Schwarz et al. 2020), where most of the ovules are formed (Qadir et al. 2021; Yu et al. 2022). Indeed, a large proportion of effective mutations are located in the *cis*-regulatory regions of genes, and even subtle changes in gene dosage can affect yield traits significantly (Eshed and Lippman 2019). A different functional contribution of genes from the A and C genomes, as we found for *BnCKX3*, has also been reported for other homoeologous Brassica genes showing functional divergence (Chalhoub et al. 2014; Wu et al. 2018; Gu et al. 2024).

All four *BnCKX3* proteins contain an N-terminal sequence predicting their import to the ER like their *Arabidopsis* counterparts (Schmülling et al. 2003; Werner et al. 2006; Niemann et al. 2015). Sequence analysis predicted with the highest probability that these are soluble ER proteins (Ødum et al. 2024). *BnCKX3-A1/C1* also have a high score for membrane association, again similar to *Arabidopsis* *CKX3* (Niemann et al. 2015), which is not the case for *BnCKX3-A2/C2*. Notably, *CKX* proteins show a dynamic glycosylation pattern, which may impact their subcellular localization (Motyka et al. 1996), enzymatic activity, or substrate preferences. Taken together, different possible factors may contribute to the differential activity of *BnCKX3* proteins encoded by the A and C genomes.

Importantly, we have found that the seed oil content as well as the composition and concentrations of unsaturated fatty acids in seeds from *BnCKX3,5* mutant plants were, in most cases, comparable to WT. There were, generally, also no major changes in the content of glucosinolates. This addresses the concern that yield gains might come at the expense of seed quality. The maintenance of oil content and unsaturated fatty acid levels indicates that enhancement of

yield components is not necessarily offset by a reduction in seed quality, which is encouraging for breeding programs targeting both yield and quality (Weselake et al. 2024).

Our study has yielded information that is important for use in targeted breeding. Only a subset of *BnCKX3/5* alleles needs to be mutated to enhance yield components. The reduction from six to four genes required to enhance yield components simplifies the path for marker-assisted or genome editing-based breeding approaches, particularly the introgression of the mutated loci in new germplasm. Targeting both *BnCKX5* alleles is critical for maximizing inflorescence meristem activity and ovule number, while mutating in addition only a subset of *BnCKX3* alleles is sufficient. Testing a *Bnckx5* double mutant with only one additional mutated *BnCKX3* gene of the A genome would be the logical next step. Of course, because of the limitations of greenhouse and small-scale field experiments (Khaipho-Burch et al. 2023; Flavell et al. 2025), it is necessary to test the performance of the different *BnCKX3,5* mutants in more detail in field trials at multiple locations and under different environmental conditions, and to study whether enhancement of the studied traits translates into higher seed yield.

Finally, we would like to mention that there are additional options to achieve yield enhancement in oilseed rape using *BnCKX* and other cytokinin genes. Recently, *BnCKX2* was shown to regulate seed size (Yan et al. 2023), and *BnCKX1* was identified as a candidate gene affecting seed yield and weight (Pal et al. 2021). Earlier, the expression of *BnCKX2* and *BnCKX4* suggested their role in seed development (Song et al. 2015). Transgenic expression of cytokinin biosynthesis genes has led to a higher seed yield in oilseed rape (Roedel et al. 1998; Kant et al. 2015). Together, this illustrates that there are numerous opportunities to use targeted manipulation of the cytokinin system to improve crop yield (Jameson and Song 2016, 2020). The enhancement of source strength could also be tested in *Bnckx3,5* mutants if their overall yield is not increased due to source limitation.

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Author contributions IS and TS designed the study and planned the experiments; IS performed experiments and statistical analyses; CM performed the NIRS analysis; IS and TS analysed data; IS and TS prepared the manuscript. All other authors read and approved the final manuscript.

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Data availability All data needed to evaluate the conclusions in the paper are present in the paper and supporting information.

Declarations

Conflict of interest The authors declare no competing interests.

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