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Faculty of Landscape Architecture, Horticulture and Crop Production Science

Enhancing rapeseed seedcake quality for feed and food using CRISPR-Cas RNP gene editing

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Abstract

Rapeseed is primarily grown for oil production, but its seed meal (RSM) is a valuable by-product rich in high-quality protein with potential for high-quality feed and food applications. However, its use in animal feed is limited by antinutritional factors, including glucosinolates (GSLs), sinapine, and phytic acid (PA), which impair nutrient uptake, reduce palatability, and negatively affect animal health. Due to the lack of natural genetic variation in these traits, traditional breeding has reached a plateau, while conventional genetic modification faces regulatory restrictions. To overcome these challenges and accelerate crop improvement, protoplast-based CRISPR RNP gene editing offers a promising transgene-free approach. However, a limitation in implementing this type of CRISPR gene editing in rapeseed is the regeneration of protoplasts into plants.

In this thesis, we developed an efficient regeneration protocol for rapeseed protoplasts, enabling the application of transgene-free CRISPR gene editing to reduce the levels of antinutritional factors. By targeting key genes involved in their biosynthesis and transport, we achieved high mutation efficiency and successfully edited multiple gene copies simultaneously. Knockout of GSL transporter genes (*BnGTR*s) reduced GSL content by up to 64%. Mutants of sinapine biosynthesis genes (*BnSGT, BnSCT,* and *BnREF1*) showed reductions of 45%, 31%, and 73%, respectively, with *BnREF1* emerging as the most effective target gene. Additionally, disruption of *BnITPK1* and *BnITPK4* in the PA biosynthesis pathway led to PA reductions of up to 32% and 62%, respectively. These findings establish a strong foundation for transgene-free gene editing in rapeseed and highlight its potential for enhancing RSM's nutritional value and agricultural viability.

Keywords: Rapeseed, protoplasts, CRISPR, RNP, transgene-free, antinutritional factors, glucosinolates, sinapine, phytic acid

Förbättring av rapsmjölskvalitet för foder och livsmedel med CRISPR-Cas RNP-genredigering

Abstrakt

Raps odlas främst för oljeproduktion, men dess frömjöl är en värdefull biprodukt rik på högkvalitativt protein med potential att användas som högkvalitativt foder och livsmedel. Användningen av frömjöl i djurfoder begränsas dock av antinutritionella faktorer, inklusive glukosinolater (GSL), sinapin och fytinsyra (PA), vilka försämrar näringsupptaget, försämrar smaken och påverkar djurhälsan negativt. På grund av bristen på naturlig variation i dessa egenskaper har traditionell växtförädling nått en platå, medan konventionell genetisk modifiering möter regulatoriska begränsningar. För att övervinna dessa utmaningar och påskynda förbättringen av grödor erbjuder protoplastbaserad CRISPR RNP-genredigering en lovande transgenfri metod. En begränsande faktor vid tillämpning av denna teknik i raps är dock regenereringen av protoplaster till hela växter.

I denna avhandling utvecklade vi ett regenereringsprotokoll för rapsprotoplaster, vilket möjliggjorde användningen av transgenfri CRISPR RNP-genredigering för att minska halter av antinutritionella faktorer. Genom att rikta in oss på nyckelgener involverade i deras biosyntes och transport uppnådde vi hög mutationseffektivitet och lyckades mutera flera genkopior samtidigt. Mutation av GSL-transportörgener (*BnGTR*-gener) minskade GSL-halten med upp till 64 %. Mutationer i gener för sinapinbiosyntes (*BnSGT*, *BnSCT* och *BnREF1*) resulterade i minskningar på 45 %, 31 % respektive 73 %, där *BnREF1* visade sig vara det mest effektiva målet. Dessutom ledde mutation av *BnITPK1* och *BnITPK4* i PA-biosyntesvägen till minskningar av PA på upp till 32 % respektive 62 %. Dessa resultat etablerar en stark grund för transgenfri genredigering i raps och belyser dess potential att förbättra frömjölets näringsvärde och agronomiska användbarhet.

Nyckelord: Raps, protoplaster, CRISPR, RNP, transgenfri, antinutritionella faktorer, glukosinolater, sinapin, fytinsyra

Dedication

To my parents,

Nothing you have done for me has gone unnoticed.
Nothing taken for granted.

“Man is the unnatural animal, the rebel child of nature, and more and more does he turn himself against the harsh and fitful hand that reared him.”

— H.G. Wells

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Li, X., Sandgrind, S., Moss, O., Guan, R., Ivarson, E., Wang, E. S., et al. (2021). **Efficient protoplast regeneration protocol and CRISPR/Cas9-mediated editing of glucosinolate transporter (*GTR*) genes in rapeseed (*Brassica napus* L.).** *Frontiers in Plant Science* 12

II. Moss, O., Li, X., Wang, E. S., Kanagarajan, S., Guan, R., Ivarson, E., & Zhu, L.-H. (2025). **Knockout of *BnaX.SGT.a* caused significant sinapine reduction in transgene-free rapeseed mutants generated by protoplast-based CRISPR RNP editing.** *Frontiers in Plant Science*, *15*

III. Moss, O., Li, X., Wang, E. S., Kanagarajan, S., Ivarson, E., & Zhu, L.-H. (2025). **Evaluation of *Bna.SCT* and *Bna.REF1* as CRISPR Targets to Reduce Sinapine in Rapeseed Seed Meal Using a DNA-Free RNP Approach** [submitted]

IV. Kanagarajan, S., Moss, O., Manikandan, A., Sandgrind, S., Wang, E. S., Li, X., Guan, R., & Zhu, L.-H. (2025). **DNA-free multiplex CRISPR/Cas9 editing of *BnGTR* reduces seed glucosinolate content in rapeseed (*Brassica napus* L.)** [manuscript]

V. Kanagarajan, S., Moss, O., Wang, E. S., Li, X., & Zhu, L.-H. (2025). **Reducing phytic acid levels in rapeseed (*Brassica napus* L.) by using CRISPR-Cas9 RNP gene editing** [manuscript]

The contribution of Oliver Moss to the papers included in this thesis was as follows:

I. Performed some of the tissue culture work together with XL. Designed and performed the protoplast transfections together with RG. Performed the mutant screening together with SK. Wrote the manuscript together with co-authors.

II. Designed the study together with L-HZ and SK. Cloned and sequenced the genes. Designed and generated the CRISPR/Cas9 gRNAs. Performed the protoplast transfections, regeneration of protoplasts together with XL. Screened putative mutants. Wrote the manuscript together with co-authors.

III. Designed the study together with L-HZ and SK. Cloned and sequenced the genes. Designed and generated the CRISPR/Cas9 gRNAs. Performed the isolation and transfection of protoplasts, regeneration of protoplasts together with XL. Screened putative mutants. Wrote the manuscript together with co-authors.

IV. Performed the isolation and transfection of protoplasts, and regeneration of protoplasts. Wrote the manuscript together with co-authors.

V. Performed the isolation and transfection of protoplasts, and regeneration of protoplasts. Wrote the manuscript together with co-authors.

Abbreviations

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DSB	Double-strand break
GFP	Green fluorescent protein
GSL	Glucosinolate
NGTs	New Genomic Techniques
PA	Phytic acid
PAM	Protospacer Adjacent Motif
PEG	Polyethylene glycol
PGRs	Plant growth regulators
RNP	Ribonucleoprotein
RSM	Rapeseed meal
TILLING	Targeting Induced Local Lesions in Genomes

1. Introduction

Rapeseed (*Brassica napus* L.) is one of the most widely cultivated oil crops globally, valued for its high-quality oil, which is used in food products, biofuels, and industrial applications (United States Department of Agriculture, 2023). As global demand for vegetable oils continues to rise, rapeseed plays a crucial role in this need, particularly in Europe, Asia, and North America (Gupta & Pratap, 2007). In addition to oil production, rapeseed yields a significant by-product: seed meal. Rich in high-quality protein and nutrients, rapeseed meal (RSM) is a valuable resource for animal feed (Cheng et al., 2022). However, its use is limited by antinutritional factors such as glucosinolates (GSLs), sinapine, and phytic acid (PA), which reduce its effectiveness as a feed source.

Utilising agricultural side-streams and increasing efficiency are fundamental objectives that should always be prioritised as part of farming practices, as they reduce waste and enhance profitability. However, the need to improve efficiency has become even more pressing in recent years. The growing global population puts additional pressure on agriculture, while climate change and rising global temperatures are expanding the range of pests and pathogens posing new risks to crops that are not adapted to these threats (Bebber et al., 2013; Jacobs et al., 2024). Moreover, unpredictable weather patterns can negatively affect crop yields, and the rapid pace of environmental change often outpaces the ability of plants to naturally adapt (Jacobs et al., 2024).

Rapeseed meal is primarily used in livestock, poultry, and aquaculture feed but also has potential applications in the food industry due to its protein content and beneficial properties, including emulsifying and antioxidative effects (Östbring et al., 2019; Zahari et al., 2022). Given the global scale of rapeseed cultivation, reducing its antinutritional factors would significantly enhance its value as a feed and food ingredient, and contribute to more sustainable livestock production systems.

To develop strategies for improving rapeseed meal, it is useful to consider the crop's history, which provides insights into its widespread cultivation, genetic characteristics, and limitations.

1.1 The history of rapeseed

Rapeseed (*Brassica napus*) belongs to the Brassicaceae family, which comprises many agriculturally important crops, such as various types of cabbage, as well as broccoli, cauliflower, and mustard. In 1935, Nagaharu U systematically crossed different *Brassica* species, elucidating their chromosomal biology and evolutionary relationships, and developed the Triangle of U (U, 1935). This study revealed that several *Brassica* species are diploid, namely *B. rapa* (A genome), *B. nigra* (B genome), and *B. oleracea* (C genome), while others are allotetraploid. The allotetraploid species include *B. napus* (AACC genome), *B. juncea* (AABB genome), and *B. carinata* (BBCC genome). U's research demonstrated that the allotetraploid species originated from the natural hybridisation of their diploid predecessors. *B. napus* originated from the natural hybridisation of *B. rapa* and *B. oleracea*. Crops with higher ploidy levels established themselves in agriculture long ago, due to humans favouring their performance in the field, even before the genetic complexities underlying their traits were fully understood.

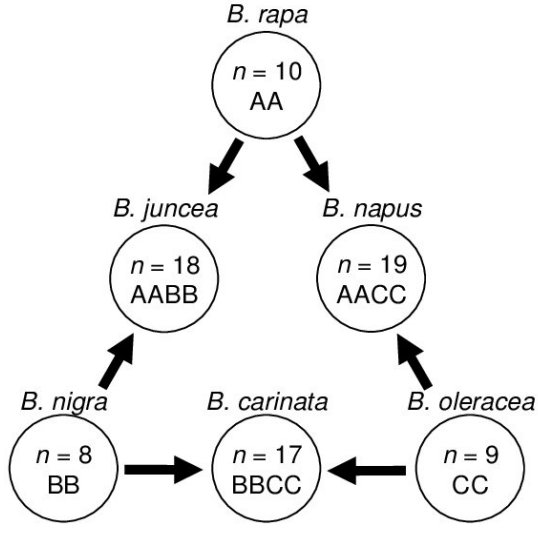

Fig. 1. Diagrammatic representation of the Triangle of U, illustrating the genetic relationships among six common *Brassica* species (Weerakoon, 2012).

Brassica species can be cultivated for oleiferous purposes (oil production), rapiferous purposes (root production), or foliar purposes (leafy vegetables). There can be significant vegetative variation within a single *Brassica* species, allowing its subspecies to be grown for multiple purposes. For example, subspecies of *Brassica rapa* can be cultivated for oleiferous, rapiferous, and foliar purposes. These subspecies include field mustard (*B. rapa* subsp. *oleifera*) for oil, turnip (*B. rapa* subsp. *rapa*) for its root, and several foliar crops such as bok choy (*B. rapa* subsp. *chinensis*) and napa cabbage (*B. rapa* subsp. *pekinensis*). While *B. napus* is commonly known as the oil crop rapeseed, the subspecies *B. napus* subsp. *rapifera* is commonly known as the vegetable rutabaga/swede (Simpson, 2010, pp. 357–360). However, in this thesis, the term *Brassica napus* refers specifically to *B. napus* subsp. *napus*, rapeseed.

The relationships within the Brassicaceae help to explain the common names for *B. napus*, including oilseed rape and rapeseed. The term 'rape' originates from the Latin *rapum*, meaning turnip, highlighting *B. napus*'s close relationship to *B. rapa* (turnip).

The hybridisation event that gave rise to *B. napus* occurred approximately 7,500 years ago, likely in the Mediterranean region (Gómez-Campo & Prakash, 1999). The origins of its cultivation and domestication remain uncertain, but rapeseed has been used as an oil crop in Europe since at least the Middle Ages. Initially, its oil was primarily used for lamps and machinery lubrication. Its effectiveness as an industrial lubricant stemmed from its high erucic acid content, which provided stability under extreme temperatures and pressures (Eskin, 2016). However, erucic acid was later found to have adverse health effects when consumed, reinforcing the negative perception of rapeseed due to its association with industrial uses (Gupta & Pratap, 2007).

Cultivation of rapeseed grew with the increase in demand for industrial lubricant during the industrial revolution, and grew more so during World War II, establishing it firmly as a crop throughout the world (Eskin, 2016). After WWII the demand as a lubricant declined, but the establishment as a crop, and food shortages in the post war era, led to rapeseed being used as a food oil. However, with this increase in consumption, a link to negative health effects was observed, which was confirmed in the 1950s by animal studies, to be due to the high levels of erucic acid (Przybylski & Mag, 2002; Eskin, 2016).

During this expansion of rapeseed oil, the defatted seed meal was trialled for use as an animal feed. However, farmers noticed that feeding large amounts of rapeseed meal to livestock caused problems such as poor growth, goitre, and reduced fertility (Gupta & Pratap, 2007; Eskin, 2016). These issues were later linked to the presence of GSLs, at approximately the same time as the issues associated with erucic acid were characterised. Researchers confirmed that, when metabolised, the breakdown products of GSLs were the cause of these problems (Gupta & Pratap, 2007). GSLs also impart a bitter taste on the seed meal, reducing palatability and food intake, and cause toxicity at high levels (Tripathi & Mishra, 2007; Felker et al., 2016; Lee & Woyengo, 2018). GSLs were not a major concern for humans, as they mostly remain in the seed meal after oil pressing, and so only low amounts are consumed with the oil.

The identification of these two major drawbacks, high erucic acid and GSLs, led to the initiation of breeding initiatives aimed at addressing these issues. Canadian scientists Dr. Baldur S. Stefansson and Dr. R. Keith Downey began their breeding efforts in the 1960s, and by 1978, they had developed a variety with erucic acid levels below 2%, down from 40-60% in previous cultivars (Downey & Harvey, 1963; Stefansson, 2012). This variety was named Canola, a portmanteau of the words "Canada," "oil," and "low acid," and was later trademarked. The name "Canola" became the common term for rapeseed in North America. However, Canola was not grown in Europe, instead breeders applied Stefansson and Downey's breeding techniques to cultivars better suited to European agriculture. This is why the crop is still referred to as rapeseed in Europe (Przybylski & Mag, 2002).

Following the development of low-erucic acid rapeseed, which made the oil safe—and even beneficial due to its cardioprotective qualities—for human consumption (Eskin, 2016), breeding efforts shifted toward improving the seed meal for animal feed. So-called "00" varieties were developed to reduce not only erucic acid but also GSL content in RSM (Kondra & Stefansson, 1970). These improvements allowed RSM, once a by-product of oil pressing, to be utilised as an animal feed.

Today, rapeseed is widely cultivated in temperate regions, with major producers including Canada, the European Union (particularly Germany, France, and Poland), China, and India, collectively accounting for millions of hectares of farmland annually (United States Department of Agriculture, 2023). Advancements in rapeseed breeding have been instrumental in

transforming it into a versatile and economically vital crop, meeting both nutritional and industrial demands. However, there is always room for improvement.

1.2 Antinutrients in rapeseed meal

The breeding efforts that led to the development of low erucic acid, low GSL '00' varieties made rapeseed suitable for both oil and seed meal production without causing significant health issues. However, the use of rapeseed seed meal (RSM) as animal feed remains limited due to the presence of antinutritional factors, which can hinder nutrient absorption, reduce feed palatability, and contribute to digestive inefficiencies. The primary antinutritional compounds—after GSLs—are sinapine (a phenolic compound), followed by phytic acid, and to a lesser extent, dietary fibre, tannins, and other phenolic compounds.

1.2.1 Glucosinolates

Before the breeding efforts of the 1970s, RSM contained GSLs at levels exceeding 100 μmol/g (Tripathi & Mishra, 2007). However, the discovery of the low-GSL cultivar *Bronowski* led to intensive breeding efforts that drastically reduced GSL levels in RSM, with varieties containing less than 30 μmol/g becoming the standard (Kondra & Stefansson, 1970; Hannoufa et al., 2014). While early breeding efforts significantly lowered GSL content, the remaining levels still cause issues when RSM is consumed.

GSLs are a diverse class of secondary metabolites found in brassicaceous plants. More than 100 GSLs have been identified, yet their precise functions remain debated. However, they are widely recognised for their role in plant defence, influencing insect behaviour as both attractants and repellents and exhibiting insecticidal, bactericidal, and fungicidal properties (Hannoufa et al., 2014).

Despite their benefits to the plant, GSLs pose challenges when rapeseed meal is used as animal feed. GSLs can cause health issues, particularly affecting the liver, kidneys, and thyroid (Tripathi & Mishra, 2007; Felker et al., 2016; Lee & Woyengo, 2018), albeit to a lesser extent than in earlier rapeseed varieties. Additionally, they impart a bitter taste to the seed meal, reducing palatability and consequently lowering feed intake (Tripathi &

Mishra, 2007). As a result, rapeseed meal is typically mixed with other feeds to dilute its GSL content and mitigate potential adverse effects.

Modern genetic technologies have been employed to further reduce GSL levels (Liu et al., 2011; Jhingan et al., 2023). However, results have been variable due to the complexity of this trait and the challenges of applying genetic strategies in a polyploid crop. Furthermore, since GSLs play a key role in plant defence, reducing their levels in vegetative tissues raises concerns about potential impacts on pest and pathogen resistance (Fan et al., 2008; Erb & Kliebenstein, 2020).

A promising approach is to target GSL transporter (*GTR*) genes to limit GSL accumulation in seeds while maintaining protective levels in vegetative tissues (Nour-Eldin et al., 2017). *GTR* genes have been linked to seed GSL accumulation in genome-wide association studies (GWAS), making them an attractive target for genetic intervention (He et al., 2022; Tan et al., 2021).

1.2.1 Sinapine

Sinapine is a secondary metabolite in rapeseed that serves as a storage compound for choline and contributes to the plant's UV-B protection (Yates et al., 2019; Sheahan, 1996). Brassicaceous oilseeds accumulate significantly more sinapine than most other crops, with rapeseed containing up to 30 times the amount found in soybean seed meal (Kozlowska et al., 1990). However, sinapine negatively affects the quality of rapeseed meal, imparting a bitter taste that reduces feed intake (Kozlowska et al., 1990); causing egg taint, whereby eggs from certain chicken breeds develop a fishy taste (Qiao et al., 2008; Ward et al., 2009); and forming complexes with amino acids, enzymes, and other compounds, which impairs nutrient uptake (Kozlowska et al., 1990).

Efforts to reduce sinapine levels in rapeseed through traditional breeding have had limited success due to the lack of natural genetic variation in rapeseed germplasm (Zum Felde et al., 2007). However, advances in molecular biology and biotechnology have provided insights into the biochemistry and genetics of sinapine biosynthesis, identifying key genes as potential targets for reduction strategies (Fig. 2) (Milkowski & Strack, 2010.

SGT (UGT84A9), encoding UDP-glucose:sinapate glucosyltransferase, is a central enzyme in sinapine biosynthesis (Milkowski et al., 2004). Targeting this gene has led to sinapine reductions of up to 72% through

genetic modification (Hüsken et al., 2005) and 57% through EMS (ethyl methanesulphonate) mutagenesis (Emrani et al., 2015). Sinapoylglucose:choline sinapoyltransferase (*Bna.SCT*) , another gene of interest due to its seed-specific expression (Weier et al., 2008), has been targeted in transgenic approaches, but genetic modification yielded only moderate reduction (52%) in sinapine (Bhinu et al., 2009). REDUCED EPIDERMAL FLUORESCENCE1 (*Bna.REF1*) has also been investigated as a target for sinapine reduction, using both transgenic (Mittasch et al., 2013) and mutagenic approaches (Emrani et al., 2015), yielding sinapine reductions of 58% and 71%, respectively.

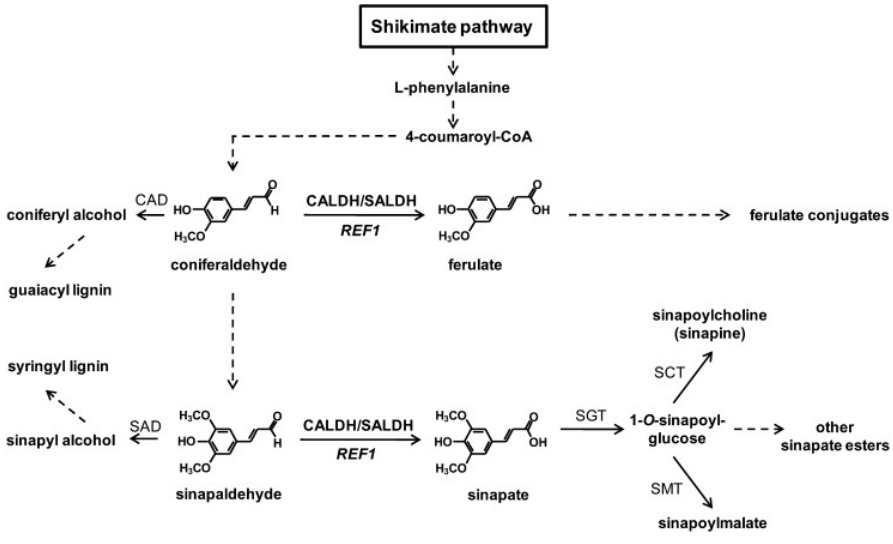

Fig. 2. Biosynthesis of sinapate esters, showing the positions of key genes in the pathway (Mittasch et al., 2013)

1.2.2 Phytic acid

Phytic acid (PA) is the primary storage form of phosphorus in rapeseed seeds (Raboy, 2020). It has a strong binding affinity for essential minerals, including calcium, iron, magnesium, and zinc, forming insoluble complexes that reduce mineral bioavailability in both livestock and humans (Raboy, 2020). Additionally, PA can interact with proteins and digestive enzymes, impairing protein digestion (Kies et al., 2006). These effects limit the nutritional efficiency of rapeseed meal as animal feed. Furthermore, monogastric animals lack phytase, the enzyme required to hydrolyse PA,

making phosphorus unavailable for absorption. As a result, undigested PA is excreted, contributing to eutrophication and environmental degradation (Holt et al., 1970; Kumar et al., 2010).

Post-harvest treatment with phytase can reduce PA levels, but this approach is costly and labour-intensive (Żyla & Koreleski, 1993; Chen et al., 2024; Xiong et al., 2022). A more sustainable strategy is to develop low-phytate rapeseed varieties through genetic approaches. Reducing seed PA content not only improves nutrient bioavailability in feed, but also decreases reliance on non-renewable rock phosphate fertilisers due to a lower phosphorous demand by the crop (Lott et al., 2000).

Natural variation in PA levels is limited, with strong conservation of the genes involved, making traditional breeding strategies difficult in developing low-PA crops, and necessitating alternative genetic interventions (Sparvoli & Cominelli, 2015; Raboy, 2006). Research on PA reduction has primarily focused on staple food and feed crops such as maize, barley, rice, and soybean, while oilseed crops like rapeseed have received comparatively less attention (Sparvoli & Cominelli, 2015; Raboy, 2006).

However, recent advances have facilitated the elucidation of the roles of genes involved in the accumulation of PA in rapeseed seeds (Sashidhar et al. 2019; Sashidhar et al. 2020a Sashidhar et al. (2020b). One of the genes that shows potential as a genetic target is *ITPK* (encoding inositol 1,3,4-trisphosphate 5/6-kinase), which is involved in PA biosynthesis in the seed (Sashidhar et al. 2020b; Kim & Tai, 2014; Shi et al., 2003).

Genetic interventions have already been applied to reduce antinutrients in RSM, but these approaches often come with limitations that affect their effectiveness or adoption. The challenges associated with existing plant breeding techniques underscore the need for innovative strategies to meet the evolving demands of modern agriculture.

1.3 The need for improvement in plant breeding

There is a need in plant breeding to accelerate genetic improvement to ensure food security (Voss-Fels et al., 2019). Conventional breeding is constrained by the long time required, the limited availability of natural genetic variation, the risk of genetic bottlenecks (Louwaars, 2018), and the difficulty of

introducing a desired trait into a high-performing variety without linkage drag (Peng et al., 2014; Ishii & Yonezawa, 2007).

Many of the limitations of conventional breeding can be addressed through genetic modification, which typically involves the introduction of foreign DNA to confer desirable traits. Genetic modification allows for the rapid introduction of new traits by drawing from a broader genetic pool and circumventing issues such as linkage drag. However, genetically modified organisms (GMOs) often face poor public perception in many parts of the world due to concerns about safety, environmental impact, and ethics, leading to strict regulations (Gbadegesin et al., 2022; Paull, 2019; European Parliament, 2024).

TILLING (Targeting Induced Local Lesions in Genomes) is a method that involves inducing mutations in a population of a species, most commonly through the use of a chemical mutagen. These random mutations are then screened to identify beneficial genetic variations, which can be utilised through conventional breeding. TILLING provides an alternative means of generating genetic variation without falling under the same regulatory scrutiny as GMOs. However, TILLING has several major disadvantages. The process requires large plant populations, which must both be mutated and screened, making it resource-intensive. Additionally, a high number of off-target mutations are typically induced, often numbering in the thousands, necessitating extensive backcrossing to eliminate unwanted changes (Szurman-Zubrzycka et al., 2023). Another key challenge is obtaining plants with mutations in enough copies of the target gene to produce the desired phenotype (Sashidhar et al., 2020; Harloff et al., 2012; Emrani et al., 2015).

1.3.1 CRISPR

In the last two decades, the emergence of New Genomic Techniques (NGTs) have shown potential to overcome the major drawbacks of conventional breeding, genetic modification, and TILLING, with CRISPR-Cas9 gene editing being one such technique.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a bacterial immune system that protects against viral infections by storing fragments of viral DNA and using them to recognise and target future invaders. This system was adapted into a genome-editing tool by harnessing the CRISPR-associated protein Cas9, which functions as programmable molecular scissors (Jinek et al., 2012). In CRISPR gene editing, a guide RNA (gRNA) directs Cas9 to a specific DNA sequence, where it introduces a double-strand break (DSB). The cell utilises its own repair machinery to fix the break via non-homologous end joining (NHEJ), an error-prone process that introduces small insertions or deletions (indels) due to imprecise repair, often disrupting gene function (Jinek et al., 2012). Since CRISPR-Cas mutagenesis works exclusively with the host's existing genome without introducing foreign DNA, the term "gene editing" is used instead of "genetic modification." A key component of this system is the PAM (Protospacer Adjacent Motif), a short DNA sequence required for Cas9 to recognise and cleave the target DNA. The PAM is typically 2-6 base pairs long, with "NGG" being the most common sequence in *Streptococcus pyogenes* Cas9, and it enables Cas9 to bind and initiate the cut at the desired location (Jinek et al., 2012).

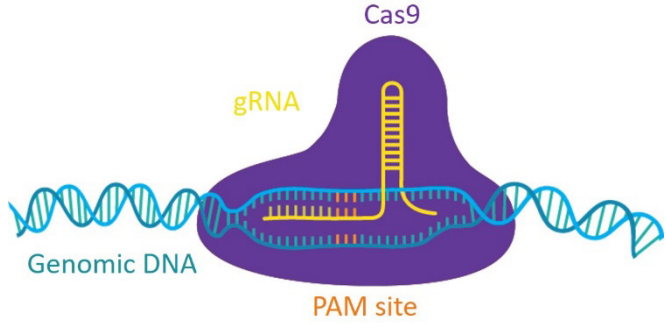

Fig. 3. Diagram depicting how a gRNA guides the Cas9 protein to a PAM site on the host's genomic DNA

Other programmable nucleases exist, including ZFNs (Zinc Finger Nucleases) and TALENs (Transcription Activator-Like Effector Nucleases) (Bibikova et al., 2001; Urnov et al., 2005; Christian et al., 2010) in biotechnology and genome editing. However, CRISPR offers several key advantages over ZFNs and TALENs, including its simpler design, greater

flexibility, and ease of use. Unlike ZFNs and TALENs, which require the engineering of specific protein domains to target DNA, CRISPR uses a single guide RNA to direct the Cas9 protein (Fig. 3), making it more versatile and efficient for genome editing across a wide range of organisms.

The mutations caused by CRISPR-Cas systems are similar to those that occur spontaneously in organisms during cell division, genetic recombination during reproduction, or those induced by environmental factors such as UV radiation, or through mutagenesis techniques like TILLING. However, CRISPR is typically introduced into plant cells using conventional genetic transformation methods, such as *Agrobacterium*-mediated delivery via floral dip or tissue culture-based approaches. These methods usually involve a DNA vector containing the sequences for the Cas9 protein, gRNA, promoter, terminator, selectable markers and T-DNA from *Agrobacterium*. Once inside the host cell, the genes in the vector are expressed, leading to the translation of the CRISPR components, enabling the Cas9 protein to introduce a double-strand break (DSB) at the desired genomic location (Zhang et al., 2021).

Despite the possibility of removing the foreign DNA through backcrossing—leaving the CRISPR-induced mutation as the only genetic alteration—the technique is still considered a form of genetic modification because it involves the introduction of foreign DNA during the process. This has sparked controversy, with some arguing that the use of genetic modification techniques, regardless of whether foreign DNA remains in the final organism, still classifies the resulting plant as a GMO.

1.3.2 Protoplasts

Protoplast-based gene editing offers a potential solution to this issue. Protoplasts are plant cells with their cell walls removed, leaving only the inner membrane, which is permeable to proteins and nucleic acids (Fig. 4) (Fehér & Dudits, 1994; Thorpe, 2012). This allows for the direct delivery of CRISPR-Cas components to the cell without the need for *Agrobacterium*-mediated integration of a DNA cassette into the genome (Yue et al., 2021; Andersson et al., 2017). This, coupled with the fact that the mutations induced by CRISPR based methods are similar to those that occur during natural processes, or those formed during less tightly regulated mutagenic practices (such as TILLING), make protoplast-based transgene-free

CRISPR-Cas mutagenesis much less controversial than other CRISPR-Cas delivery methods.

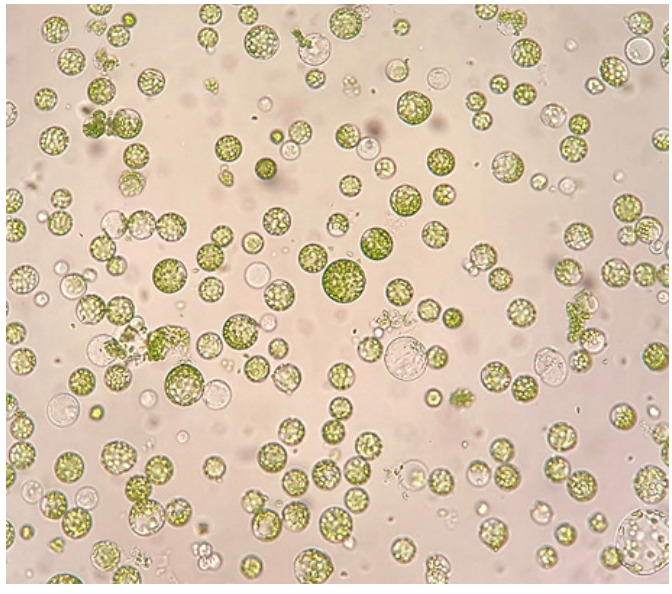

Fig. 4. Rapeseed protoplasts at 20× magnification

The mechanical isolation of protoplasts was reported as early as 1892, but their use in research only gained popularity in the 1960s, when enzymatic removal of the cell wall was demonstrated (Fehér & Dudits, 1994). This was further facilitated by the commercial availability of cell wall-degrading enzymes (Thorpe, 2012). Takebe et al. (1971) demonstrated that protoplasts could regenerate into complete plants, confirming their totipotency and expanding their potential applications in plant research. This opened the door for experiments such as somatic hybridisation and, later, genetic modification (Thorpe, 2012). However, protoplast-based methods faced significant challenges, particularly the difficulty of regenerating plants from single cells, which became a major bottleneck (Thorpe, 2012). The development of *Agrobacterium*- and biolistics-based methods for genetic modification, which could be applied to tissues and germline cells, circumvented these difficulties. As a result, protoplast-based approaches for genetic modification were largely sidelined (Thorpe, 2012).

However, in recent years, interest in protoplasts has resurged due to their potential for use in combination with CRISPR gene editing. This has

prompted research integrating CRISPR with protocols for regenerating agriculturally and scientifically significant plants, such as potato (*Solanum tuberosum*) (Andersson et al., 2017), *Nicotiana tabacum* (Lin et al., 2018), *Nicotiana benthamiana* (Hsu et al., 2021a), *Brassica oleracea* (Hsu et al., 2021b), and lettuce (*Lactuca sativa*) (Woo et al., 2015). This has also led to the development of a regeneration protocol for *Brassica napus* by my lab (Li et al., 2021), which is Paper I in this thesis and forms the foundation for the research presented in the other papers of the thesis.

2. Aims and objectives of the thesis

The overall aim of the thesis was to use CRISPR-Cas gene editing to create transgene-free mutants of rapeseed that had reduced levels of antinutritional factors in the seed.
The specific objectives were to:

1) Establish an efficient protocol for the regeneration of rapeseed protoplasts
2) Use CRISPR-Cas gene editing for the generation of transgene free mutants
3) Analyse target genotypic and phenotypic traits of mutants
4) Grow mutants for multiple generations to confirm stability of mutations

3. Key Results

3.1 Development of an efficient protoplast regeneration protocol, and CRISPR-Cas gene editing

In Paper I, we demonstrated a method for isolating and regenerating rapeseed protoplasts, addressing a major bottleneck in applying CRISPR-Cas9 gene editing technologies to rapeseed. We optimised a protoplast isolation protocol based on Arabidopsis (Yoo et al., 2007) for use in rapeseed, adjusting the enzyme solution concentration and incubation duration. A regeneration protocol was established by evaluating the effects of varying concentrations of plant growth regulators (PGRs) (Paper I, Tables 1–5) and culture durations (Paper 1, Tables 6–7) on the growth and development of rapeseed protoplasts. The final protocol involves five distinct media, each with specific nutrient, PGR, and sugar concentrations, along with defined culture durations to ensure regeneration. Paper I highlights the narrow tolerance ranges for PGR concentrations and culture durations, emphasising the sensitivity of rapeseed protoplasts.

Plasmid transfection was performed using polyethylene glycol (PEG). To estimate transfection efficiency, a green fluorescent protein (GFP) vector was used, enabling early detection of transient expression via confocal laser scanning microscopy (Fig. 5). This provided a rapid estimate of transfection efficiency, bypassing the need for labour-intensive protoplast sequencing to detect mutations. Transfection efficiencies of 40–80% were routinely observed.

To demonstrate the feasibility of CRISPR-Cas gene editing in these protoplasts, a CRISPR vector containing gRNAs targeting *BnGTR* genes was used. CRISPR-induced mutations were detected in 3 out of 16 regenerated plants, corresponding to an editing efficiency of 19%, with up to 5 out of 12 *BnGTR* genes mutated in a single plant. This study highlights the effectiveness of our optimised protoplast regeneration and transfection protocols for gene editing in rapeseed.

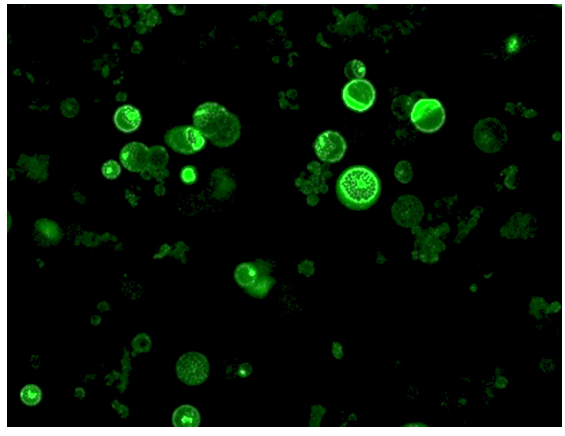

Fig. 5. Rapeseed protoplasts expressing the GFP vector, observed 10× magnification using confocal laser scanning microscopy.

In Papers II, III, and IV, we built on the CRISPR mutagenesis protocol developed in Paper I by using ribonucleoproteins (RNPs) instead of plasmids. RNPs offer several advantages over plasmids in CRISPR mutagenesis, including eliminating the need for plasmid preparation and avoiding concerns about spontaneous plasmid DNA integration into the host genome. Additionally, RNPs can enhance editing efficiency and reduce the likelihood of off-target mutations (Zhang et al., 2021; DeWitt et al., 2017). Paper IV provides a direct comparison between plasmid and RNP-based CRISPR delivery, as the same gRNAs were used to target *BnGTR* genes. Plasmid-based delivery achieved an editing efficiency of 19%, with a maximum of five genes edited in a single plant. In contrast, RNP-based delivery resulted in an editing efficiency of 81%, with up to twelve *BnGTR* genes mutated in a single plant. Similarly, in Papers II, III, and V, which also employed RNP-based CRISPR delivery, we observed higher editing efficiencies than with plasmid-based delivery, with efficiencies of 28–36% in Paper II, 22–63% in Paper III, and 64-100% in paper V.

The heritability of mutations was confirmed by the consistency of mutant phenotypes across generations and the presence of mutations in the most advanced generations of all mutant lines across the studies. In both plasmid- and RNP-based approaches, multiple gene copies were successfully mutated simultaneously in individual plants, demonstrating the benefits of CRISPR-Cas mutagenesis in polyploid crops, where targeting multiple gene copies is

necessary to overcome gene redundancy. However, the higher mutation efficiency and greater number of gene copies mutated in RNP-based CRISPR-Cas mutagenesis suggest it is the more effective approach for generating mutants.

3.2 Glucosinolates

In Papers I and IV, *GSL* transporter genes (*GTR* genes) were targeted using CRISPR-Cas9 mutagenesis to generate rapeseed mutants with reduced GSL levels in the seed. In Paper I, *BnGTR* genes were identified, and four sgRNAs were tested, revealing that sgRNA1 and sgRNA4 were the most effective at inducing mutations. Paper IV then employed these sgRNAs in RNP-mediated CRISPR mutagenesis, successfully generating mutations in multiple *BnGTR* genes simultaneously.

In the T_2 generation, four mutant lines were obtained, each carrying mutations in both *BnGTR1* and *BnGTR2*, with 10–12 edited alleles per plant. Among these, mutant line M9 was mutated in 12 copies of the *BnGTR* genes, the most of all the mutant lines, and exhibited the lowest GSL levels (7.5–10.34 μmol/g), compared to approximately 20.67 μmol/g in wild-type (WT) plants (Fig. 6). The mutations also altered the GSL profile (Fig. 6).
Paper IV (*BnGTR*) demonstrated that RNP-mediated CRISPR-Cas9 editing of *BnGTR1* and *BnGTR2* is an effective approach for generating heritable mutations, reducing seed GSL content in rapeseed without the use of transgenic techniques.

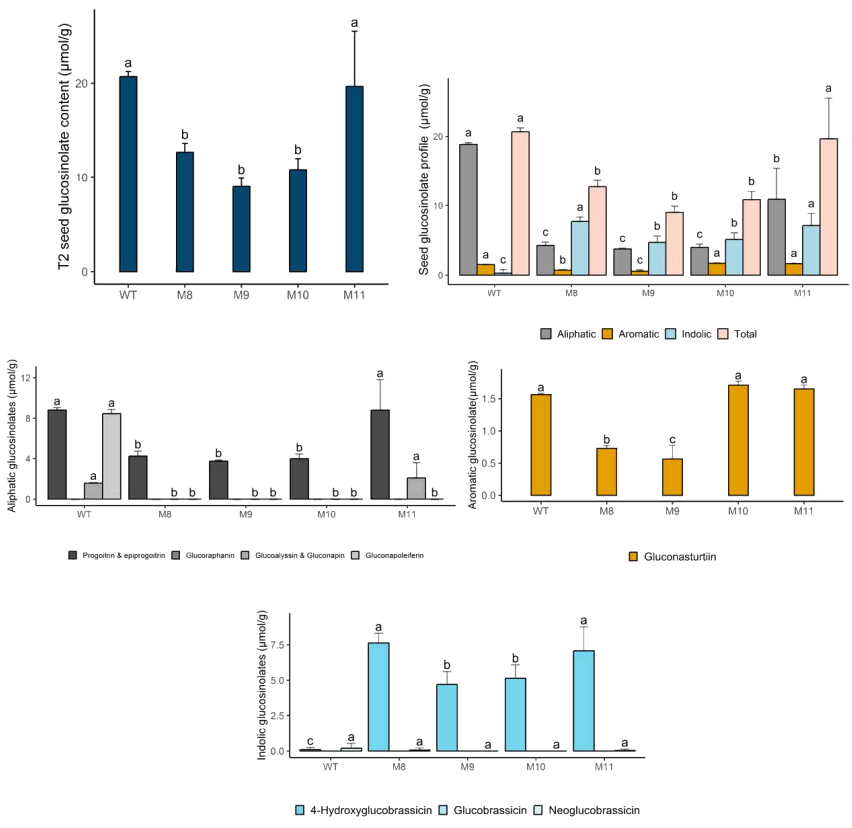

Fig. 6. Seed glucosinolate (GSL) contents and GSL composition of RNP-mediated gene editing generated mutants. Different letters above the bars represent significant differences at $p<0.05$. Error bars represent ± SD.

3.3 Sinapine

In Papers II and III, the roles of three key sinapine biosynthesis genes were investigated for their potential as genetic targets in the generation of low-sinapine rapeseed lines. These genes were *BnSGT* (Paper II), *BnSCT*, and *BnREF1* (Paper III).

3.3.1 BnSGT

Two gRNAs, sgRNA1 and sgRNA2, targeting different regions of *BnSGT*, were designed and used for mutagenesis in rapeseed protoplasts. Twenty-

five plants derived from protoplasts transfected with each gRNA were screened for CRISPR-induced mutations. Nine sgRNA1 mutants and seven sgRNA2 mutants were identified, corresponding to mutation efficiencies of 36% and 28%, respectively.

Ultimately, five mutant lines, all derived from sgRNA1, were selected for cultivation in the T_2 generation for assessment. Twelve plants per line were grown, and genetic analysis showed that both alleles of both paralogues of *BnaX.SGT.a* were mutated in all lines (double mutants), except for in line B5.1.9, which retained one wild-type allele. The T_2 line with the greatest sinapine reduction showed a 45% decrease (Fig. 7), while the individual plant with the highest reduction exhibited a 49% decrease. No differences in the growth and wellbeing of the mutant lines were observed in comparison to WT.

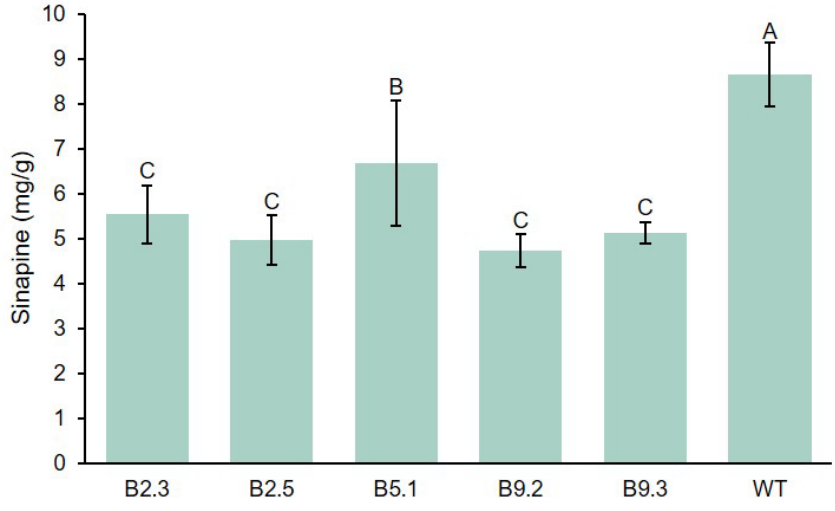

Fig. 7. Average sinapine content of the seeds of all plants from each *BnaX.SGT.a* mutant line in the T_2 generation in comparison with WT (n=4). Different letters above the bars represent significant differences at $p<0.05$. Error bars represent ± SD.

3.3.2 BnSCT

Mutation efficiency for *BnSCT* gRNAs was 22% for SCTsgRNA1 and 63% for SCTsgRNA2. Two mutant lines were grown to the T_2 generation for evaluation. Amplicon sequencing confirmed and characterised the CRISPR-induced mutations. One line (SCT2 11.3) carried mutations in all four alleles but retained a silent mutation in one, while the other line (SCT2 11.5) exhibited loss-of-function mutations (a stop codon or frameshift) in all four alleles.

As a result, sinapine levels were reduced by an average of 27% in SCT2 11.3 and 31% in SCT2 11.5 (Fig. 8). No significant difference was observed between the two lines. No differences in the growth and wellbeing of the mutant lines were observed in comparison to WT.

3.3.3 BnREF1

The mutation efficiency for *BnREF1* mutants was 38% for REF1sgRNA1 and 56% for REF1sgRNA2. Four mutant lines were grown to the T_1 generation for further analysis. Amplicon sequencing confirmed that three lines (REF1 3, REF1 4, and REF2 7) carried mutations in all four *BnREF1*

alleles, while plants within one line (REF1 4) exhibited mixed genotypes. Some plants had loss-of-function mutations in all four alleles, whereas others retained a functional allele.

This genotypic variation was reflected in sinapine content: double homozygous plants exhibited a low-sinapine phenotype, whereas heterozygous plants showed no reduction. This segregation indicated that a single mutated allele was insufficient to lower sinapine levels in *BnREF1* mutants.

The T_1 *BnREF1* mutant line with the lowest sinapine content was REF1 1.3, which exhibited an average sinapine reduction of 73% (Fig. 9)—the largest decrease observed among all sinapine-reducing mutant lines generated in rapeseed. No differences in the growth and wellbeing of the mutant lines were observed in comparison to WT.

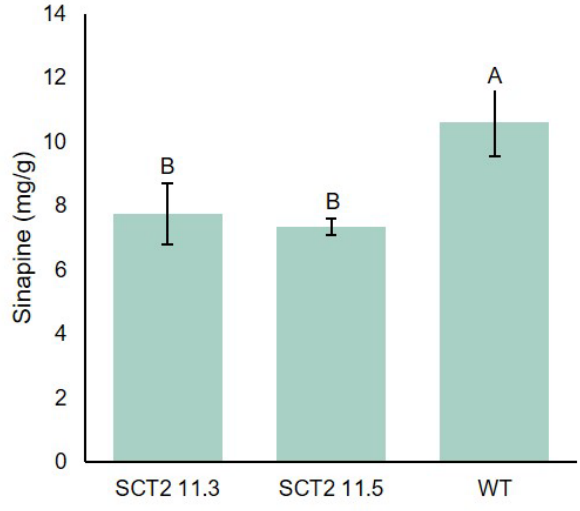

Fig. 8. Average sinapine content in the seeds of rapeseed *BnSCT* mutants and WT in T_2 generation. WT n=16. Different letters above the bars represent significant differences at $p<0.05$ Error bars represent ± SD

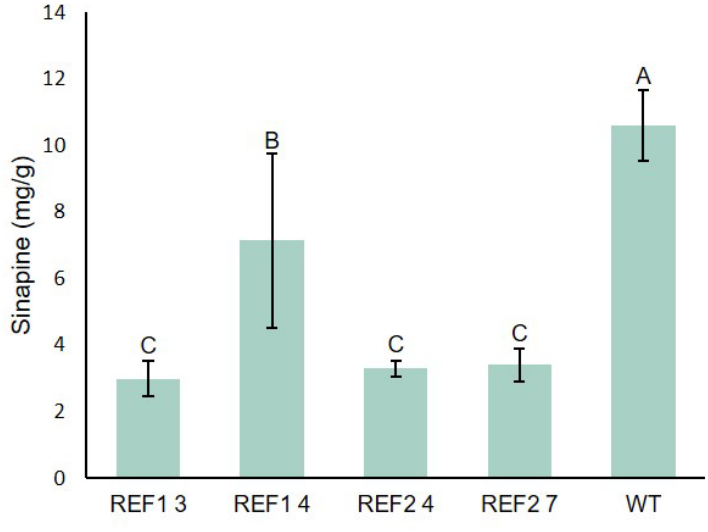

Fig. 9. Average sinapine content in the seeds of rapeseed *BnREF1* mutants and WT in the T_1 generation. WT n=16. Different letters above the bars represent significant differences at $p<0.05$ Error bars represent ± SD

3.4 Phytic acid

In Paper V, the roles of PA biosynthesis genes *BnITPK1* and *BnITPK4* in PA accumulation in the seeds of rapeseed were investigated using CRISPR gene editing. gRNAs were designed for each gene and used for protoplast transfection. Screening revealed that nine out of fourteen plants were successfully mutated with the *BnITPK1* gRNA, and four out of four plants were successfully mutated with the *ITPK4* gRNA, corresponding to mutation efficiencies of 64% and 100%, respectively.

Seven T_0 *BnITPK1* mutants and four T_0 *BnITPK4* mutants were grown to maturity and analysed for PA content in the seeds. The *BnITPK1* mutants displayed a wide range of changes in PA levels, from a 32% reduction to a 12% increase in PA compared to WT (Fig. 10). The *BnITPK4* mutants showed a more pronounced and consistent reduction in PA. ITPK4-M1, ITPK4-M2, and ITPK4-M3 had PA reductions of 59.21% to 62.26%. However, ITPK4-M4 only showed a reduction of 7.73%, likely due to incomplete gene knockout (Fig. 10).

Fig. 10. Phytic acid content in seeds of T_0 rapeseed *BnITPK4* (M1-4) and *BnITPK1* (M5-11) mutants, and WT.

4. Conclusions and future perspectives

Utilising agricultural side-streams is becoming increasingly important as the global population grows and climate change poses new challenges to food security and sustainability. Rapeseed meal (RSM) is one such side-stream with the potential to become a high-quality feed and even a food source. Although traditional plant breeding has been used to improve rapeseed, progress may have plateaued due to limited genetic variation—an issue that could affect other crops as well. To address these challenges, the genetic improvement of crops needs to be accelerated; however, existing breeding and genetic approaches each have limitations.

CRISPR-Cas gene editing offers a solution to these limitations, with its relative ease of use, precision, and potential for the rapid introduction of novel traits without the need for lengthy backcrossing processes. Although currently regulated in parts of the world, recognition of the advantages and minimal risks of transgene-free gene editing is increasing, contributing to its growing acceptance globally (Dionglay, 2024). This shift is evident in the European Union's changing approach to NGTs in plant breeding, with recent proposals seeking to ease restrictions on gene-edited crops. These regulatory developments indicate a promising outlook for the wider integration of NGTs in agriculture (European Parliament, 2024).

We developed a protocol for the efficient regeneration of rapeseed protoplasts, which facilitated the implementation of CRISPR-Cas gene editing. Using this protocol, we successfully created transgene-free mutant knockouts for key genes involved in the biosynthesis and transport of major nutritional factors in rapeseed, leading to a reduction in all targeted antinutritional factors: GSL, sinapine, and PA. This success can be attributed to the high editing efficiency of the RNP-based approach and its ability to simultaneously mutate multiple gene copies.

Assessment of the growth and agricultural properties of the mutant lines needs to be performed in order to evaluate their viability for agricultural use. However, minimal changes in growth and performance have been observed so far. Nonetheless, this thesis provides a strong foundation for the improvement of RSM through gene editing, marking a significant step forward for the utilisation of this underutilised side-stream.

The next step in this research is to implement CRISPR RNP editing to target multiple genes simultaneously, enabling the reduction of multiple

antinutritional factors within a single line. Experimentation in this direction has already begun, with promising results.

References

Andersson, M., Turesson, H., Nicolia, A., Fält, A.-S., Samuelsson, M., & Hofvander, P. (2017). Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. *Plant Cell Reports*, *36*(1), 117–128. https://doi.org/10.1007/s00299-016-2062-3

Bebber, D. P., Ramotowski, M. A. T., & Gurr, S. J. (2013). Crop pests and pathogens move polewards in a warming world. *Nature Climate Change*, *3*(11), 985–988. https://doi.org/10.1038/nclimate1990

Bhinu, V.-S., Schäfer, U. A., Li, R., Huang, J., & Hannoufa, A. (2009). Targeted modulation of sinapine biosynthesis pathway for seed quality improvement in *Brassica napus*. *Transgenic Research*, *18*(1), 31–44. https://doi.org/10.1007/s11248-008-9194-3

Bibikova, M., Carroll, D., Segal, D. J., Trautman, J. K., Smith, J., Kim, Y. G., & Chandrasegaran, S. (2001). Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. *Molecular and Cellular Biology*, *21*(1), 289–297. https://doi.org/10.1128/MCB.21.1.289-297.2001

Chen, Y., Tao, X., Hu, S., He, R., Ju, X., Wang, Z., & Aluko, R. E. (2024). Effects of phytase/ethanol treatment on aroma characteristics of rapeseed protein isolates. *Food Chemistry*, 431, 137119. https://doi.org/10.1016/j.foodchem.2023.137119

Cheng, H., Liu, X., Xiao, Q., Zhang, F., Liu, N., Tang, L., et al. (2022). Rapeseed meal and its application in pig diet: A review. *Agriculture* 12, 849. doi: 10.3390/agriculture12060849

Christian, M., Cermak, T., Doyle, E. L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A. J., & Voytas, D. F. (2010). Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics*, *186*(2), 757–761. https://doi.org/10.1534/genetics.110.120717

DeWitt, M. A., Corn, J. E., Carroll, D. (2017). Genome editing via delivery of Cas9 ribonucleoprotein. *Methods* 121–122, 9–15. doi: 10.1016/j.ymeth.2017.04.003

Dionglay, C. (2024, January 24). *Updates on Global Regulatory Landscape for Gene-Edited Crops*. Science Speaks. https://www.isaaa.org/blog/entry/default.asp?BlogDate=1/24/2024

Downey, R. K., & Harvey, B. L. (1963). Methods of breeding for oil quality in rape. *Canadian Journal of Plant Science*, *43*(3), 271–275. https://doi.org/10.4141/cjps63-054

Emrani, N., Harloff, H.-J., Gudi, O., Kopisch-Obuch, F., & Jung, C. (2015). Reduction in sinapine content in rapeseed (*Brassica napus* L.) by induced

mutations in sinapine biosynthesis genes. *Molecular Breeding*, *35*, 1–11. https://doi.org/10.1007/s11032-015-0236-2

Erb, M., & Kliebenstein, D. J. (2020). Plant Secondary Metabolites as Defenses, Regulators, and Primary Metabolites: The Blurred Functional Trichotomy. *Plant Physiology*, *184*(1), 39–52. https://doi.org/10.1104/pp.20.00433

Eskin, N. A. M. (2016). Rapeseed Oil/Canola. In B. Caballero, P. M. Finglas, & F. Toldrá (Eds.), *Encyclopedia of Food and Health* (pp. 581–585). Academic Press. https://doi.org/10.1016/B978-0-12-384947-2.00585-7

European Parliament. (2024). Texts adopted—Plants obtained by certain new genomic techniques and their food and feed—Wednesday, 7 February 2024. Available online at: https://www.europarl.europa.eu/doceo/document/TA-9-2024-0067_EN.html (Accessed October 20, 2024)

Fehér, A., & Dudits, D. (1994). Plant Protoplasts for Cell Fusion and Direct DNA Uptake: Culture and Regeneration Systems. In I. K. Vasil & T. A. Thorpe (Eds.), *Plant Cell and Tissue Culture* (pp. 71–118). Springer Netherlands. https://doi.org/10.1007/978-94-017-2681-8_4

Felker, P., Bunch, R., & Leung, A. M. (2016). Concentrations of thiocyanate and goitrin in human plasma, their precursor concentrations in brassica vegetables, and associated potential risk for hypothyroidism. *Nutrition Reviews*, *74*(4), 248–258. https://doi.org/10.1093/nutrit/nuv110

Gbadegesin, L. A., Ayeni, E. A., Tettey, C. K., Uyanga, V. A., Aluko, O. O., Ahiakpa, J. K., Okoye, C. O., Mbadianya, J. I., Adekoya, M. A., Aminu, R. O., Oyawole, F. P., & Odufuwa, P. (2022). GMOs in Africa: Status, adoption and public acceptance. *Food Control*, *141*, 109193. https://doi.org/10.1016/j.foodcont.2022.109193

Gómez-Campo, C., & Prakash, S. (1999). Origin and domestication. In C. Gómez-Campo (Ed.), *Biology of Brassica coenospecies* (pp. 33–58). Elsevier Science.

Gupta, S. K., & Pratap, A. (2007). History, Origin, and Evolution. In *Advances in Botanical Research* (Vol. 45, pp. 1–20). Academic Press. https://doi.org/10.1016/S0065-2296(07)45001-7

Hannoufa, A., Pillai, B. V. S., & Chellamma, S. (2014). Genetic enhancement of *Brassica napus* seed quality. *Transgenic Research*, *23*(1), 39–52. https://doi.org/10.1007/s11248-013-9742-3

Harloff, H.-J., Lemcke, S., Mittasch, J., Frolov, A., Wu, J. G., Dreyer, F., Leckband, G., & Jung, C. (2012). A mutation screening platform for rapeseed (*Brassica napus* L.) and the detection of sinapine biosynthesis mutants. *Theoretical and Applied Genetics*, *124*(5), 957–969. https://doi.org/10.1007/s00122-011-1760-z

He, Y., Yang, Z., Tang, M., Yang, Q.-Y., Zhang, Y., & Liu, S. (2022). Enhancing canola breeding by editing a glucosinolate transporter gene lacking natural

variation. *Plant Physiology*, *188*(4), 1848–1851. https://doi.org/10.1093/plphys/kiac021

Holt, R. F., Timmons, D. R., & Latterell, J. J. (1970). Accumulation of phosphates in water. *Journal of Agricultural and Food Chemistry, 18*(5), 781–784.

Hsu, C.-T., Lee, W.-C., Cheng, Y.-J., Yuan, Y.-H., Wu, F.-H., & Lin, C.-S. (2021a). Genome editing and protoplast regeneration to study plant–pathogen interactions in the model plant *Nicotiana benthamiana*. *Frontiers in Genome Editing, 2.* https://doi.org/10.3389/fgeed.2020.627803

Hsu, C.-T., Yuan, Y.-H., Lin, Y.-C., Lin, S., Cheng, Q.-W., Wu, F.-H., Sheen, J., Shih, M.-C., & Lin, C.-S. (2021b). Targeted gene editing in plants using CRISPR-Cas9, single-stranded DNA oligonucleotides, and protoplast regeneration [Preprint]. *bioRxiv*. https://doi.org/10.1101/2021.03.09.434087

Hüsken, A., Baumert, A., Strack, D., Becker, H. C., Möllers, C., & Milkowski, C. (2005). Reduction of Sinapate Ester Content in Transgenic Oilseed Rape (*Brassica napus*) by dsRNAi-based Suppression of BnSGT1 Gene Expression. *Molecular Breeding*, *16*(2), 127–138. https://doi.org/10.1007/s11032-005-6825-8

Ishii, T., & Yonezawa, K. (2007). Optimization of the Marker-Based Procedures for Pyramiding Genes from Multiple Donor Lines: I. Schedule of Crossing between the Donor Lines. *Crop Science*, *47*(2), 537–546. https://doi.org/10.2135/cropsci2006.06.0435

Jacobs, C., Berglund, M., Kurnik, B., Dworak, T., Marras, S., Mereu, V., et al. (2024). *Climate change adaptation in the agriculture sector in Europe* (European Environment Agency). Available at: https://www.eea.europa.eu/publications/cc-adaptation-agriculture (Accessed October 22, 2024)

Jhingan, S., Harloff, H. J., Abbadi, A., Welsch, C., Blümel, M., Tasdemir, D., & Jung, C. (2023). Reduced glucosinolate content in oilseed rape (*Brassica napus* L.) by random mutagenesis of *BnMYB28* and *BnCYP79F1* genes. *Scientific Reports, 13*(1), Article 28661. https://doi.org/10.1038/s41598-023-28661-6

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science (New York, N.Y.)*, *337*(6096), 816–821. https://doi.org/10.1126/science.1225829

Kies, A. K., De Jonge, L. H., Kemme, P. A., & Jongbloed, A. W. (2006). Interaction between Protein, Phytate, and Microbial Phytase. In Vitro Studies. *Journal of Agricultural and Food Chemistry*, *54*(5), 1753–1758. https://doi.org/10.1021/jf0518554

Kim, S.-I., & Tai, T. H. (2014). Identification of novel rice low phytic acid mutations via TILLING by sequencing. *Molecular Breeding*, *34*(4), 1717–1729. https://doi.org/10.1007/s11032-014-0127-y

Kondra, Z., and Stefansson, B. (1970). Inheritance of the major glucosinolates of rapeseed (*Brassica napus*) meal. *Canadian Journal of Plant Science* 50(6)**,** 643-647.

Kozlowska, H., Naczk, M., Shahidi, F., Zadernowski, R. (1990). "Phenolic acids and tannins in rapeseed and canola," in *Canola and Rapeseed: Production, Chemistry, Nutrition and Processing Technology*. Ed. Shahidi, F. (Boston, MA, US: Springer), 193–210. doi: 10.1007/978-1-4615-3912-4_11

Kumar, V., Sinha, A. K., Makkar, H. P. S., & Becker, K. (2010). Dietary roles of phytate and phytase in human nutrition: A review. *Food Chemistry*, *120*(4), 945–959. https://doi.org/10.1016/j.foodchem.2009.11.052

Lee, J. W., & Woyengo, T. A. (2018). Growth performance, organ weights, and blood parameters of nursery pigs fed diets containing increasing levels of cold-pressed canola cake. *Journal of Animal Science*, *96*(11), 4704–4712. https://doi.org/10.1093/jas/sky317

Li, L.-F., & Olsen, K. M. (2016). Chapter Three - To Have and to Hold: Selection for Seed and Fruit Retention During Crop Domestication. In V. Orgogozo (Ed.), *Current Topics in Developmental Biology* (Vol. 119, pp. 63–109). Academic Press. https://doi.org/10.1016/bs.ctdb.2016.02.002

Lin, C.-S., Hsu, C.-T., Yang, L.-H., Lee, L.-Y., Fu, J.-Y., Cheng, Q.-W., Wu, F.-H., Hsiao, H. C.-W., Zhang, Y., Zhang, R., Chang, W.-J., Yu, C.-T., Wang, W., Liao, L.-J., Gelvin, S. B., & Shih, M.-C. (2018). Application of protoplast technology to CRISPR/Cas9 mutagenesis: From single-cell mutation detection to mutant plant regeneration. *Plant Biotechnology Journal*, *16*(7), 1295–1310. https://doi.org/10.1111/pbi.12870

Liu, Z., Hammerlindl, J., Keller, W., McVetty, P. B. E., Daayf, F., Quiros, C. F., & Li, G. (2011). MAM gene silencing leads to the induction of C3 and reduction of C4 and C5 side-chain aliphatic glucosinolates in *Brassica napus*. *Molecular Breeding,* *27*(4), 467–478. https://doi.org/10.1007/s11032-010-9444-y

Lott, J. N. A., Ockenden, I., Raboy, V., & Batten, G. D. (2000). Phytic acid and phosphorus in crop seeds and fruits: A global estimate. *Seed Science Research*, *10*(1), 11–33. https://doi.org/10.1017/S0960258500000039

Louwaars, N. P. (2018). Plant breeding and diversity: A troubled relationship? *Euphytica*, *214*(7), 114. https://doi.org/10.1007/s10681-018-2192-5

Milkowski, C., & Strack, D. (2010). Sinapate esters in brassicaceous plants: Biochemistry, molecular biology, evolution and metabolic engineering. *Planta*, *232*(1), 19–35. https://doi.org/10.1007/s00425-010-1168-z

Milkowski, C., Baumert, A., Schmidt, D., Nehlin, L., & Strack, D. (2004). Molecular regulation of sinapate ester metabolism in Brassica napus: Expression of

genes, properties of the encoded proteins and correlation of enzyme activities with metabolite accumulation. *The Plant Journal: For Cell and Molecular Biology*, *38*(1), 80–92. https://doi.org/10.1111/j.1365-313X.2004.02036.x

Mittasch, J., Böttcher, C., Frolov, A., Strack, D., & Milkowski, C. (2013). Reprogramming the Phenylpropanoid Metabolism in Seeds of *Brassica napus* by Suppressing the Orthologs of REDUCED EPIDERMAL FLUORESCENCE1. *Plant Physiology*, *161*. https://doi.org/10.1104/pp.113.215491

Nour-Eldin, H. H., Madsen, S. R., Engelen, S., Jørgensen, M. E., Olsen, C. E., Andersen, J. S., Seynnaeve, D., Verhoye, T., Fulawka, R., Denolf, P., & Halkier, B. A. (2017). Reduction of antinutritional glucosinolates in Brassica oilseeds by mutation of genes encoding transporters. *Nature Biotechnology*, *35*(4), 377–382. https://doi.org/10.1038/nbt.3823

Östbring, K., Tullberg, C., Burri, S., Malmqvist, E., & Rayner, M. (2019). Protein Recovery from Rapeseed Press Cake: Varietal and Processing Condition Effects on Yield, Emulsifying Capacity and Antioxidant Activity of the Protein Rich Extract. *Foods*, *8* (12), 627. https://doi.org/10.3390/foods8120627

Paull, J. (2019). The failures of genetically modified organisms (GMOs): Resistance, regulation, and rejection. *AGROFOR International Journal, 4*(3), 139–150. https://doi.org/10.7251/AGRENG1903139P

Peng, T., Sun, X., & Mumm, R. H. (2014). Optimized breeding strategies for multiple trait integration: I. Minimizing linkage drag in single event introgression. *Molecular Breeding*, *33*(1), 89–104. https://doi.org/10.1007/s11032-013-9936-7

Primrose, S. B., & Twyman, R. M. (2013). Genetic modification of plants. In *Principles of gene manipulation and genomics* (8th ed., pp. 245–270). Wiley-Blackwell.

Przybylski, R., & Mag, T. (2002). Canola/rapeseed oil. In F. D. Gunstone (Ed.), *Vegetable oils in food technology: Composition, properties and uses* (pp. 98–127). Blackwell Publishing.

Qiao, H. Y., Dahiya, J. P., Classen, H. L. (2008). Nutritional and physiological effects of dietary sinapic acid (4-hydroxy-3,5-dimethoxy-cinnamic acid) in broiler chickens and its metabolism in the digestive tract. *Poultry Sci.* 87, 719–726. doi: 10.3382/ps.2007-00357

Raboy, V. (2006). Seed phosphorus and the development of low-phytate crops. In B. L. Turner, A. E. Richardson, & E. J. Mullaney (Eds.), *Inositol phosphates: Linking agriculture and environment* (pp. 111–132). CAB International.

Raboy, V. (2020). Low phytic acid Crops: Observations Based on Four Decades of Research. *Plants*, *9*(2), Article 2. https://doi.org/10.3390/plants9020140

Sashidhar, N., Harloff, H. J., & Jung, C. (2020a). Knockout of MULTI-DRUG RESISTANT PROTEIN 5 genes lead to low phytic acid contents in oilseed rape. *Frontiers in Plant Science, 11*, Article 603. https://doi.org/10.3389/fpls.2020.00603

Sashidhar, N., Harloff, H. J., Potgieter, L., & Jung, C. (2020b). Gene editing of three *BnITPK* genes in tetraploid oilseed rape leads to significant reduction of phytic acid in seeds. *Plant Biotechnology Journal, 18*(11), 2241–2250. https://doi.org/10.1111/pbi.13380

Sashidhar, N., Harloff, H.-J., & Jung, C. (2019). Identification of phytic acid mutants in oilseed rape (*Brassica napus*) by large-scale screening of mutant populations through amplicon sequencing. *New Phytologist, 225*(5), 2022–2034. https://doi.org/10.1111/nph.16281

Sheahan, J. J. (1996). Sinapate Esters Provide Greater UV-B Attenuation than Flavonoids in *Arabidopsis thaliana* (Brassicaceae). *Am. J. Bot.* 83, 679–686. doi: 10.2307/2445845

Shi, J., Wang, H., Hazebroek, J., Ertl, D. S., & Harp, T. (2005). The maize low-phytic acid 3 encodes a myo-inositol kinase that plays a role in phytic acid biosynthesis in developing seeds. *The Plant Journal*, *42*(5), 708–719. https://doi.org/10.1111/j.1365-313X.2005.02412.x

Shi, J., Wang, H., Wu, Y., Hazebroek, J., Meeley, R. B., & Ertl, D. S. (2003). The Maize Low-Phytic Acid Mutant lpa2 Is Caused by Mutation in an Inositol Phosphate Kinase Gene. *Plant Physiology*, *131*(2), 507–515. https://doi.org/10.1104/pp.014258

Simpson, M. G. (2010). *Plant systematics* (2nd ed., pp. 357–360). Academic Press.

Sparvoli, F., & Cominelli, E. (2015). Seed Biofortification and Phytic Acid Reduction: A Conflict of Interest for the Plant? *Plants*, *4*(4), 728–755. https://doi.org/10.3390/plants4040728

Stefansson, B. R. (2012). The development of improved rapeseed cultivars. In J. K. G. Kramer (Ed.), *High and low erucic acid in rapeseed oils* (pp. 144–159). Academic Press.

Szurman-Zubrzycka, M., Kurowska, M., Till, B. J., & Szarejko, I. (2023). Is it the end of TILLING era in plant science? *Frontiers in Plant Science*, *14*. https://doi.org/10.3389/fpls.2023.1160695

Takebe, I., Labib, G., & Melchers, G. (1971). Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. *Naturwissenschaften*, *58*(6), 318–320. https://doi.org/10.1007/BF00624737

Tan, Z., Xie, Z., Dai, L., Zhang, Y., Zhao, H., Tang, S., Wan, L., Yao, X., Guo, L., & Hong, D. (2021). Genome- and transcriptome-wide association studies reveal the genetic basis and the breeding history of seed glucosinolate content in *Brassica napus*. *Plant Biotechnology Journal*, *20*(1), 211–225. https://doi.org/10.1111/pbi.13707

Thorpe, T. (2012). History of Plant Tissue Culture. In V. M. Loyola-Vargas & N. Ochoa-Alejo (Eds.), *Plant Cell Culture Protocols* (pp. 9–27). Humana Press. https://doi.org/10.1007/978-1-61779-818-4_2

Tripathi, M. K., & Mishra, A. S. (2007). Glucosinolates in animal nutrition: A review. *Animal Feed Science and Technology*, *132*(1), 1–27. https://doi.org/10.1016/j.anifeedsci.2006.03.003

U, N. (1935) Genome analysis in Brassica with special references to the experimental formation of *B. napus* and peculiar mode of fertilization. Jpn. J. Genet. 7, 389–452.

United States Department of Agriculture. (2023). *Production, supply and distribution online: Rapeseed*. Foreign Agricultural Service. Retrieved [13.02.23], from https://www.fas.usda.gov/data/production/commodity/2226000

Urnov, F. D., Miller, J. C., Lee, Y. L., Beausejour, C. M., Rock, J. M., Augustus, S., Jamieson, A. C., Porteus, M. H., Gregory, P. D., & Holmes, M. C. (2005). Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*, *435*(7042), 646–651. https://doi.org/10.1038/nature03556

Voss-Fels, K. P., Stahl, A., Hickey, L. T. (2019). Q&A: Modern crop breeding for future food security. *BMC Biol.* 17, 18. doi: 10.1186/s12915-019-0638-4

Ward, A. K., Classen, H. L., Buchanan, F. C. (2009). Fishy-egg tainting is recessively inherited when brown-shelled layers are fed canola meal. *Poultry Sci.* 88, 714–721. doi: 10.3382/ps.2008-00430

Weerakoon, S. (2012). Producing inter-specific hybrids between Brassica juncea (L.) Czern & Coss and B. oleracea (L.) to synthesize trigenomic (abc) Brassica. *Journal of Science of the University of Kelaniya Sri Lanka*, *6*. https://doi.org/10.4038/josuk.v6i0.4218

Woo, J. W., Kim, J., Kwon, S. I., Corvalán, C., Cho, S. W., Kim, H., Kim, S.-G., Kim, S.-T., Choe, S., & Kim, J.-S. (2015). DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nature Biotechnology*, *33*(11), 1162–1164. https://doi.org/10.1038/nbt.3389

Xiong, Z., Fu, Y., Yao, J., Zhang, N., He, R., Ju, X., & Wang, Z. (2022). Removal of anti-nutritional factors of rapeseed protein isolate (RPI) and toxicity assessment of RPI. *Food & Function*, *13*(2), 664–674. https://doi.org/10.1039/D1FO03217H

Yates, K., Pohl, F., Busch, M., Mozer, A., Watters, L., Shiryaev, A., et al. (2019). Determination of sinapine in rapeseed pomace extract: Its antioxidant and acetylcholinesterase inhibition properties. *Food Chemistry.* 276, 768–775. doi: 10.1016/j.foodchem.2018.10.045

Yoo, S.-D., Cho, Y.-H., & Sheen, J. (2007). *Arabidopsis* mesophyll protoplasts: A versatile cell system for transient gene expression analysis. *Nature Protocols*, *2*(7), 1565–1572. https://doi.org/10.1038/nprot.2007.199

Yue, J.-J., Yuan, J.-L., Wu, F.-H., Yuan, Y.-H., Cheng, Q.-W., Hsu, C.-T., & Lin, C.-S. (2021). Protoplasts: From Isolation to CRISPR/Cas Genome Editing Application. *Frontiers in Genome Editing*, *3*. https://doi.org/10.3389/fgeed.2021.717017

Zahari, I., Östbring, K., Purhagen, J. K., & Rayner, M. (2022). Plant-Based Meat Analogues from Alternative Protein: A Systematic Literature Review. *Foods*, *11*(18), 2870. https://doi.org/10.3390/foods11182870

Zhang, Y., Iaffaldano, B., Qi, Y. (2021). CRISPR ribonucleoprotein-mediated genetic engineering in plants. *Plant Communications.* 2, 100168. doi: 10.1016/j.xplc.2021.100168

Zum Felde, T., Baumert, A., Strack, D., Becker, H. C., & Möllers, C. (2007). Genetic variation for sinapate ester content in winter rapeseed (*Brassica napus* L.) and development of NIRS calibration equations. *Plant Breeding*, 126(3), 291–296. https://doi.org/10.1111/j.1439-0523.2007.01342.x

Żyla, K., & Koreleski, J. (1993). In-vitro and in-vivo dephosphorylation of rapeseed meal by means of phytate-degrading enzymes derived from *Aspergillus niger*. *Journal of the Science of Food and Agriculture*, *61*(1), 1–6. https://doi.org/10.1002/jsfa.2740610102

Popular science summary

Rapeseed is mostly grown for its oil, but the leftover seed meal (RSM) is a valuable source of protein that could be used in animal feed. However, RSM contains antinutritional compounds—glucosinolates, sinapine, and phytic acid—that make it less nutritious, less palatable, and harder for animals to digest. Traditional breeding has struggled to reduce these compounds, and genetic modification faces strict regulations.

In this research, we used a modern genetic technique called CRISPR-Cas gene editing to tackle this problem. CRISPR is commonly known as being similar to genetic scissors, because of its ability to make cuts in genes. The cuts caused effectively switch off genes, making it possible to alter the genetics of an organism, without introducing foreign DNA, as is done when genetic modification is used.

In order to implement CRISPR in rapeseed, we first needed to develop a method to regenerate rapeseed plants from individual cells. Then, we were able to precisely cut the genes responsible for producing the unwanted compounds using CRISPR. The cells with the edited genes were then grown to full plants again. Doing this we created plants with significantly lower levels of antinutritional compounds. For example, we reduced glucosinolate content by up to 64%, while modifying sinapine-related genes cut levels by up to 73%. We also reduced phytic acid in the seed by 62%.

This research opens the door for healthier, more sustainable animal feed while demonstrating a promising way to improve crops using gene editing without the need for traditional genetic modification.

Populärvetenskaplig sammanfattning

Raps odlas mest för sin olja, men det kvarvarande frömjölet är en värdefull proteinkälla som kan användas i djurfoder. Frömjölet innehåller dock antinutritionella föreningar–glukosinolater, sinapin och fytinsyra–som gör det mindre näringsrikt, mindre smakligt och svårare för djur att smälta. Traditionell förädling har haft svårt att minska dessa föreningar, och genetisk modifiering möter strikta regleringar.

I denna forskning använde vi en modern genetisk teknik som kallas CRISPR-Cas-genredigering för att tackla detta problem. CRISPR är vanligtvis känt som den "genetiska saxen", på grund av dess förmåga att klippa i gener. De klipp som görs stänger av gener, vilket gör det möjligt att ändra en organisms gener utan att introducera främmande DNA, som är fallet vid genetisk modifiering.

För att kunna använda CRISPR i raps, behövde vi först utveckla en metod för att regenerera rapsplantor från individuella celler. Därefter kunde vi noggrant klippa de gener som ansvarar för att producera de oönskade föreningarna med hjälp av CRISPR. Cellerna med de redigerade generna regenererades sedan till plantor. På detta sätt skapade vi plantor med betydligt lägre nivåer av antinutritionella föreningar. Till exempel minskade vi glukosinolathalten med upp till 64 %, medan modifiering av sinapinrelaterade gener sänkte nivåerna av sinapin med upp till 73 %. Vi minskade också fytinsyrahalten i fröet med 62 %.

Denna forskning öppnar dörren för ett hälsosammare och mer hållbart djurfoder samtidigt som den visar en lovande metod för att förbättra grödor genom genredigering utan att behöva använda traditionell genetisk modifiering.

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Efficient Protoplast Regeneration Protocol and CRISPR/Cas9-Mediated Editing of Glucosinolate Transporter (*GTR*) Genes in Rapeseed (*Brassica napus* L.)

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Difficulty in protoplast regeneration is a major obstacle to apply the CRISPR/Cas9 gene editing technique effectively in research and breeding of rapeseed (*Brassica napus* L.). The present study describes for the first time a rapid and efficient protocol for the isolation, regeneration and transfection of protoplasts of rapeseed cv. Kumily, and its application in gene editing. Protoplasts isolated from leaves of 3–4 weeks old were cultured in MI and MII liquid media for cell wall formation and cell division, followed by subculture on shoot induction medium and shoot regeneration medium for shoot production. Different basal media, types and combinations of plant growth regulators, and protoplast culture duration on each type of media were investigated in relation to protoplast regeneration. The results showed that relatively high concentrations of NAA (0.5 mg l^{-1}) and 2,4-D (0.5 mg l^{-1}) in the MI medium were essential for protoplasts to form cell walls and maintain cell divisions, and thereafter auxin should be reduced for callus formation and shoot induction. For shoot regeneration, relatively high concentrations of cytokinin were required, and among all the combinations tested, 2.2 mg l^{-1} TDZ in combination with auxin 0.5 mg l^{-1} NAA gave the best result with up to 45% shoot regeneration. Our results also showed the duration of protoplast culture on different media was critical, as longer culture durations would significantly reduce the shoot regeneration frequency. In addition, we have optimized the transfection protocol for rapeseed. Using this optimized protocol, we have successfully edited the *BnGTR* genes controlling glucosinolate transport in rapeseed with a high mutation frequency.

Keywords: *Brassica napus*, CRISPR/Cas9, gene editing, glucosinolate transporter, *GTR* gene, protoplast regeneration

INTRODUCTION

The CRISPR/Cas9 technology has now become a prevailing tool for plant genome editing owing to its high precision, efficiency and simplicity in use (Arora and Narula, 2017). Apart from its powerful role in functional genomics analysis, it has also revolutionized the strategy for crop breeding and improvement. So far the CRISPR/Cas9 system has been successfully applied to edit

genes in a number of plant species, such as *Arabidopsis thaliana* (Jiang et al., 2013), *Nicotiana tabacum* (Nekrasov et al., 2013), rice (Shan et al., 2013), maize (Liang et al., 2014), sorghum (Jiang et al., 2013), wheat (Shan et al., 2013), etc. However, the majority of these studies relied on stable transformation by *Agrobacterium tumefaciens* to deliver the CRISPR vectors. As stable transformation of plants normally results in regeneration of mutation lines with integration of foreign DNA into the plant genome, this gene editing system raise regulatory concerns related to genetically modified plants in some countries (Woo et al., 2015).

Polyethylene glycol (PEG)-mediated protoplast transfection is an alternative for delivery of CRISPR vectors or ribonucleoprotein complexes (RNPs) into plant cells, which can produce transgene-free mutation lines through transient gene expression. However, as protoplast regeneration remains a bottleneck for many plant species, gene editing through the protoplast approach for trait improvement has not been widely applied in most of the crop species. Application of the protoplast approach for gene editing in crop species reported so far were mainly for research purpose (Nicolia et al., 2015; Woo et al., 2015; Malnoy et al., 2016; Kim et al., 2017; Liang et al., 2017; Lin et al., 2018), while in most cases no protoplast regeneration results were reported. Development of an efficient and reliable protoplast regeneration method is thus essential for the application of all currently available CRISPR gene editing systems for directly producing transgene-free mutants for many plant species.

Rapeseed is an important oil crop, accounting for about 16% of the total global vegetable oil production (USDA, 2019). Cultivated rapeseed is an allotetraploid species (*B. napus*; *2n* = 38, AACC) that was formed by polyploidization of two diploids ancestors, *B. oleracea* (genome CC) and *B. rapa* (genome AA) (Chalhoub et al., 2014). Although the gene editing system of CRISPR/Cas9 has been used in rapeseed for trait improvement, the published results so far relied on stable transformation with *Agrobacterium* (Braatz et al., 2017; Li et al., 2018; Huang et al., 2020; Zheng et al., 2020). To the best of our knowledge, only a few studies reported using protoplasts for gene editing by CRISPR/Cas9 in rapeseed, while none of them have reported success in obtaining mutation lines, i.e. no protoplast regeneration after transfection. Murovec et al. (2018) reported using RNPs for gene editing of rapeseed, but no mutations were detected after protoplast transfection. Lin et al. (2018) reported that the rapeseed genome could be mutated by CRISPR/Cas9 using the protoplast approach, but no regenerated plants from the transfected protoplasts were reported. All the published results indicate that proof-of-concept protoplast regeneration protocols for CRISPR/Cas9 genome editing are still lacking for most crop species in general, including rapeseed.

Development of protoplast culture technology in *Brassica* species started in the 1970s, and received a great amount of attention in the early 1980s for a variety of applications, including mutant isolation, somatic hybridization and genetic transformation. Although intensive studies on protoplast culture conditions were conducted, protoplast regeneration remained at very low levels in most cases. Furthermore, the regeneration frequency is often species and genotype dependent, making method improvement very challenging (Kielkowska and Adamus, 2012). This is mainly because a large number of conditions need to be optimized in order to obtain reasonably high regeneration frequencies for each species. These conditions include protoplast isolation method, protoplast density for culture, nutrients, type and concentration of sugars, concentrations and combinations of plant growth regulators (PGRs) in culture media, culture conditions and the developmental stage of protoplast calli capable of shoot induction, etc.

Apart from providing edible oil, rapeseed also contains a large amount of high quality protein, which remains in the seedcake after oil extraction. The seedcake is currently used only as animal feed due to the presence of antinutritional factors, which make the seedcake taste bitter and undesirable for food uses (Nour-Eldin et al., 2012). One of such antinutritional factors is glucosinolates (GSLs). GSLs are synthesized in vegetative tissues and transported to seeds in *Brassica* species, and this transport is mainly regulated by glucosinolate transporter (*GTR*) genes (Nour-Eldin et al., 2012). Eliminating or reducing the quantity of GSLs in seedcake is thus necessary to improve the rapeseed seedcake for feed and food uses.

In this study, we report a rapid and efficient protoplast transfection and regeneration protocol for rapeseed gene editing using CRISPR/Cas9. Using this protocol, we have obtained high transfection and mutation frequencies, and successfully obtained mutated plants with the targeted mutations in the *BnGTR* genes.

MATERIALS AND METHODS

Plant Material

Seeds of spring rapeseed (*B. napus* L.) cv. Kumily, kindly provided by Lantmännen, Svalöf, Sweden, were used in this study.

In vitro Culture Conditions

All *in vitro* cultures in this study were maintained in a controlled climate chamber which has a temperature of 23 °C/18 °C (day/night) and 16 h photoperiod with a light intensity of 40 μmol m^{-2} s^{-1} (cool white fluorescent tubes).

Seed Germination

Seeds were surface sterilized using 15% (w/v) calcium hypochlorite ($CaCl_2O_2$) for 20 min, and then rinsed thoroughly with sterile water. Surface sterilized seeds were planted on germination medium in sterile plastic boxes. The germination medium contained half strength Murashige & Skoog (MS), 10 g l^{-1} sucrose, 7 g l^{-1} Bacto agar at pH 5.7. The boxes were placed in the climate chamber as stated above.

Protoplast Isolation

Protoplasts were isolated according to Yoo et al. (2007), with some modifications. About 40 fully opened young leaves from 3–4 weeks old seedlings were sliced into fine pieces on wetted filter paper in a sterile Petri dish and incubated in plasmolysis solution (0.4 M mannitol at pH 5.7) for 30 min at room temperature

(RT) in the dark. The leaf pieces were then treated with 10 ml enzyme solution and incubated for 14–16 h at RT in the dark with gentle shaking. The enzyme solution consisted of 1.5% (w/v) cellulase Onozuka^{TM} R-10 (Yakult Pharmaceutical Co., LTD., Tokyo, Japan), 0.6% (w/v) Macerozyme^{TM} R-10 (Yakult Pharmaceutical Co., Ltd.), 0.4 M mannitol, 10 mM MES, 0.1% (w/v) BSA, 1 mM $CaCl_2$ and 1 mM β-mercaptoethanol at pH 5.7.

The isolated protoplasts were filtered through a 40 μm nylon cell strainer into a 50 ml Falcon tube, diluted with 30 ml W5 solution (Menczel et al., 1981) and centrifuged at 100 *g* for 10 min. Pellets were re-suspended in 10 ml W5 solution and centrifuged at 100 *g* for 5 min, and this process was repeated twice. Pellets were then re-suspended in 5 ml W5 solution and incubated on ice in the dark for 30 min. The supernatant was discarded and the protoplasts were diluted with 5–10 ml W5 solution based on the size of pellets. Protoplast solution of 15 μl was loaded on a hemocytometer for counting protoplasts under light microscope. After centrifugation for 3 min at 100 *g*, the protoplast density was adjusted to 400 000 to 600 000 per ml using 0.5 M mannitol solution. Equal volume of the protoplast suspension and alginate solution were mixed for making alginate disks. The alginate-solution consisted of 2.8% (w/v) sodium alginate and 0.4 M mannitol according to Kielkowska and Adamus (2012). To produce alginate disks, about 500 μl of the mixed protoplast and alginate suspension were pipetted onto the calcium-agar plates (0.4 M mannitol, 2.2 g l^{-1} $CaCl_2$ and 10 g l^{-1} Phyto agar) and incubated at RT for 30 min. Thereafter, 2 ml of calcium-solution (50 mM $CaCl_2$, 0.4 M mannitol) was added onto each disk and incubated for 1 h at RT to complete polymerization. The disks were then transferred to the culture medium as described below.

Protoplast Culture in Liquid Medium

The prepared protoplast-alginate disks were cultured in 6-well tissue culture plates with one disk in each well and addition of 2–3 ml MI medium. Plates were covered with aluminium foil and kept at RT for 24 h, thereafter placed under fibre cloth without aluminium foil in the climate chamber under conditions as stated above. After 3–4 d, the MI medium was replaced by MII. MI medium consisted of 2.18 g l^{-1} Nitsch medium (Nitsch and Nitsch, 1969), 10 g l^{-1} sucrose, 10 g l^{-1} glucose, 100 g l^{-1} mannitol, 100 mg l^{-1} casein, 0.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg l^{-1} α-naphthaleneacetic acid (NAA) at pH 5.7. MII medium was the same as MI, but PGRs were changed to 1.1 mg l^{-1} thidiazuron (TDZ) and 0.05 mg l^{-1} 2,4-D instead. During this culture period, MII medium was renewed every 5–7 d.

Plant Regeneration, Growth and Rooting on Solid Medium

After 20–25 d, the protoplast calli from the alginate disks were directly spread on the shoot induction medium (SIM) in Petri dishes for shoot induction. The SIM medium consisted of full-strength MS, 30 g l^{-1} sucrose, 50 g l^{-1} mannitol, 1.1 mg l^{-1} or 2.2 mg l^{-1} TDZ, 0.05 mg l^{-1} NAA, 0.5 mg l^{-1} $AgNO_3$ and 2.5 g l^{-1} Gelrite at pH 5.7. After 10–20 d on the SIM medium, the protoplast calli were transferred to shoot regeneration medium (SRM) in Petri dishes for shoot regeneration. Different SRM media were designed, in which C- and N-sources, types and combinations of PGRs, as well as culture duration in MI, MII and on SIM medium were tested. The detailed experimental designs are presented in (**Tables 1**–**7**). The medium was renewed every 3–4 weeks during the shoot regeneration phase.

The regenerated shoots were transferred to the shoot growing medium consisting of fullstrength MS, 20 g l^{-1} sucrose, 0.05 mg l^{-1} 6-benzyladnine (BAP), 0.03 mg l^{-1} gibberellic acid 3 (GA_3) and Bacto agar 7.5 g l^{-1} at pH 5.7.

The elongated shoots were transferred to the rooting medium consisting of half strength MS, 20 g l^{-1} sucrose, 0.05 mg l^{-1} NAA and Bacto agar 7.5 g l^{-1} at pH 5.7. The rooted shoots were then planted in soil in the biotron with standard management. The growth conditions in the biotron were 21°C/16°C (day/night), 16 h photoperiod with a light intensity of 250 μmol m^{-2} s^{-1} and 60% humidity.

TABLE 1 | Effect of PGRs in MI medium on protoplast growth and development of rapeseed.

PGR conc. (mg l^{-1})	Viability of protoplasts (%)*	PGR conc. (mg l^{-1})	Viability of protoplasts (%)*
TDZ 1.1 2,4-D 1.0	0.0 c	NAA 0.5 2,4-D 0.5	80.0 a
TDZ 1.1 2,4-D 0.5	0.0 c	BAP 2.0 NAA 0.5	0.0 c
TDZ 1.1 2,4-D 0.25	0.0 c	Zeatin 1.0 NAA 0.5	0.0 c
TDZ 0.55 2,4-D 0.5	0.0 c	BAP 2.0 2,4-D 0.5 Zeatin 1.0 2,4-D 0.5	20.3 b 13.3 b

*Medium I composition: 2.18 g l^{-1} Nitsch medium, 10 g l^{-1} sucrose, 10 g l^{-1} glucose, 100 g l^{-1} mannitol, 100 mg l^{-1} casein at pH 5.7. *Percentage of protoplasts maintained round and compact in form, and green in color, observed under light microscope 7 d after protoplast culture. Values followed by the same letter were not statistically different at $p = 0.05$ ($n = 3$).*

TABLE 2 | Effect of PGRs in MII medium on protoplast development of rapeseed.

PGR conc. (mg l^{-1})	Callus formation (%)*	PGR conc. (mg l^{-1})	Callus formation (%)*
BAP 1.0 NAA 0.5	0.0 b	TDZ 1.1 NAA 0.1	0.0 b
BAP 1.0 NAA 0.1	0.0 b	TDZ 1.1 NAA 0.05	75.0 a
BAP 2.0 NAA 0.1	0.0 b	TDZ 1.1 2,4-D 0.1	0.0 b
TDZ 2.2 NAA 0.1	0.0 b	TDZ 1.1 2,4-D 0.05	80.0 a

*The protoplasts were cultured in MI medium before being transferred to MII medium. Medium II composition: 2.18 g l^{-1} Nitsch medium, 10 g l^{-1} sucrose, 10 g l^{-1} glucose, 100 g l^{-1} mannitol, 100 mg l^{-1} casein at pH 5.7. *The results were recorded when protoplast colonies were about 0.1 mm in diameter after 30 d in the MII medium. Values followed by the same letter were not statistically different at $p = 0.05$ ($n = 3$).*

TABLE 3 | Effect of PGRs in shoot induction medium (SIM) on protoplast regeneration of rapeseed.

PGR conc. (mg l^{-1})	Regeneration (%)*	PGR conc. (mg l^{-1})	Regeneration (%)*
TDZ 1.1 NAA 0.05	0.0 b	TDZ 2.2 NAA 0.05	0.0 b
TDZ 1.1 NAA 0.05	35.0 a	TDZ 2.2 NAA 0.05	40.0 a
Mannitol 50,000		Mannitol 50,000	

*SIM medium composition: Full strength MS, sucrose 30 g l^{-1}, 0.5 mg l^{-1} $AgNO_3$, 2.5 g l^{-1} Gelrite at pH 5.7. *The results were recorded after one month on the SIM media. Values followed by the same letter were not statistically different at $p = 0.05$ ($n = 3$).*

TABLE 4 | Effect of PGRs in shoot regeneration medium (SRM) on protoplast regeneration of rapeseed.

PGR conc. (mg l^{-1})	Regeneration (%)*	PGR conc. (mg l^{-1})	Regeneration (%)
BAP 2.0 NAA 0.1	0.0 c	Kinetin 2.0 NAA 0.1	0.0 c
BAP 3.0 NAA 0.2	0.0 c	TDZ 0.5 NAA 0.1	0.0 c
BAP 5.0 NAA 0.5	1.0 c	TDZ 1.1 NAA 0.1	5.0 c
Zeatin 1.0 NAA 0.1	0.0 c	TDZ 2.2 NAA 0.5	45.0 a
Zeatin 2.0 NAA 0.2	0.0 c	TDZ 2.2 NAA 1.0	22.0 b

*SRM medium composition: Full strength MS, sucrose 20 g l^{-1}, 0.5 mg l^{-1} $AgNO_3$, 2.5 g l^{-1} Gelrite at pH 5.7. *The results were recorded after one month on the SRM medium. Values followed by the same letter were not statistically different at $p = 0.05$ ($n = 3$).*

TABLE 5 | Effect of C-sources in shoot regeneration medium (SRM) on protoplast regeneration of rapeseed.

Sugar conc. (g l^{-1})	Regeneration (%)*	Sugar conc. (g l^{-1})	Regeneration (%)
Sucrose 15	30.6 b	Glucose 10	11.3 c
Sucrose 20	41.0 a	Glucose 20	10.0 c
Sucrose 30	31.4 ab		

*SRM composition: Full strength MS, 2.2 mg l^{-1} TDZ, 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} $AgNO_3$, 2.5 g l^{-1} Gelrite at pH 5.7. *The results were recorded after one month on the SRM medium. Values followed by the same letter were not statistically different at $p = 0.05$ ($n = 3$).*

TABLE 6 | Effect of culture duration in MI and MII media on protoplast regeneration of rapeseed.

Duration	Regeneration (%)*						
	3d	5d	10d	15d	20d	30d	40d
In MI	35.0 a	15.0 b	0.0 c	0.0 c	0.0 c	-	-
In MII	-	-	0.0 c	20.0 a	40.0 b	15.0 c	0.0 c

*Medium I composition: 2.18 g l^{-1} Nitsch medium, 10 g l^{-1} sucrose, 10 g l^{-1} glucose, 100 g l^{-1} mannitol, 100 mg l^{-1} casein, 2.2 mg l^{-1} NAA, 0.5 mg l^{-1} 2,4-D at pH 5.7. Medium II composition: 2.18 g l^{-1} Nitsch medium, 10 g l^{-1} sucrose, 10 g l^{-1} glucose, 100 g l^{-1} mannitol, 100 mg l^{-1} casein, 1.1 mg l^{-1} TDZ, 0.05 mg l^{-1} 2,4-D at pH5.7. *The results were recorded after one month on the SRM medium, which consisted of full strength MS, 2.2 mg l^{-1} TDZ, 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} $AgNO_3$, 2.5 g l^{-1} Gelrite at pH 5.7. Values followed by the same letter were not statistically different at $p = 0.05$ ($n = 3$).*

TABLE 7 | Effect of culture duration on shoot induction medium (SIM) on protoplast regeneration of rapeseed.

Duration	Regeneration (%)*						
	15d	20d	25d	30d	40d	50d	60d
SIM1	17.0 c	39.7 a	40.0 a	26.0 b	14.0 c	5.0 d	0.0 d
SIM2	17.0 bc	45.0 a	45.0 a	20.0 b	10.0 c	8.0 cd	0.0 d

*SIM1 composition: Full strength MS, 30 g l^{-1} sucrose, 50 g l^{-1} mannitol, 1.1 mg l^{-1} TDZ, 0.05 mg l^{-1} NAA, 0.5 mg l^{-1} $AgNO_3$, 2.5 g l^{-1} Gelrite at pH 5.7. SIM2 composition: Full strength MS, 30 g l^{-1} sucrose, 50 g l^{-1} mannitol, 2.2 mg l^{-1} TDZ, 0.05 mg l^{-1} NAA, 0.5 mg l^{-1} $AgNO_3$, 2.5 g l^{-1} Gelrite at pH 5.7. *The results were recorded after two months on the SRM medium. Values followed by the same letter were not statistically different at $p = 0.05$ ($n = 3$).*

Identification and Cloning of *GTR* Genes, sgRNA Design and Vector Construction

Two known *BnGTR* orthologs from *A. thaliana*, *AtGTR1* (*AT3G47960*) and *AtGTR2* (*AT5G62680*) were used for a BLAST query in the NCBI database against the rapeseed reference genome cv. ZS11 (Bra_napus_v2.0) and six paralogs of *BnGTR1* (*LOC106397267*, *LOC106408997*, *LOC106410496*, *LOC106414122*, *LOC106445255* and *LOC111202315*) and six paralogs of *BnGTR2* (*LOC106347844*, *LOC106366161*, *LOC106369007*, *LOC106405453*, *LOC106411192* and *LOC106424883*) were found (**Table 8**). Genomic and full-length open reading frames of six *BnGTR1* and six *BnGTR2* paralogs were amplified from genomic DNA and cDNA of cv. Kumily, respectively, using gene specific primers according to published protocols (Kim et al., 2020; Muthusamy et al., 2020), with minor modifications, and confirmed by sequencing. Genomic DNA sequences of different paralogs from the *BnGTR1* and *BnGTR2* were aligned to find conserved target sites among the paralogs of each gene. Based on the location in the target gene sequence, off target potential and the GC content, two target sequences for all six *BnGTR1* paralogs (one in exon 2 and one in exon 3) and two target sequences for all six *BnGTR2* paralogs (both in exon 2) (**Table 9**) were designed using CRISPR MultiTargeter (Prykhozhij et al., 2015). All the chosen target sequences were 20 bp and tested for their off-target potential in the rapeseed genome using Cas-Offinder (Bae et al., 2014). Each target sequence was integrated into a single guide RNA (sgRNA) expression cassette (Addgene plasmids# 66201, 66198, 66202, 66203) using the primers listed in **Supplementary Table S1**. Thereafter, all four sgRNA expression cassettes were sequentially ligated into the pYLCRISPR/Cas9P_{ubi}-N vector according to the protocol described by Ma et al. (2015), resulting in a vector designated as pYLCRISPR/Cas9P_{ubi}-GTR **Supplementary Figure S1**. Moreover, in order to examine if the transgene integration happened or not in the mutants, PCR was performed on the *Cas9* and *nptII* genes in the pYLCRISPR/Cas9P_{ubi}-GTR vector

using the gene specific primers (**Supplementary Table S2**). The PCR analyses were performed using Phusion High-Fidelity PCR Master Mix with GC Buffer (Thermo Scientific^{TM}) according to the manufacturer's recommendations. The PCR conditions were 98°C for 3 min, followed by 30 cycles at 98°C for 10 s, 63°C for 30 s, 72°C for 30 s, with a final extension at 72°C for 8 min and the PCR products were separated on a 1% agarose gel.

Protoplast Transfection

For approximation of transfection efficiency, protoplasts were transfected with the vector pCW498-35S-GFiP-OcsT (14 743bp) harboring the *green fluorescent protein* gene (*GFP*) (Wood et al., 2009). For inducing mutations in the *BnGTR1* and *BnGTR2* genes, protoplasts were transfected with the pYLCRISPR/Cas9P$_{ubi}$-GTR vector (18537 bp).

After isolating and washing protoplasts as described above, ∼120 000 protoplasts were re-suspended in 200 µl freshly prepared MMG solution (0.5 M mannitol, 15 mM $MgCl_2$, 4 mM MES) in a 2 ml Eppendorf tube. The solution was mixed with 40 µg pCW498-35S-GFiP-OcsT vector or pYLCRISPR/Cas9P$_{ubi}$-GTR vector DNA and equal volume of freshly prepared PEG-calcium solution (25% (w/v) PEG 4000, 0.5 M mannitol and 0.1 M $CaCl_2$). The reaction was stopped after 5 min by addition of 1.5 ml W5 and mixed by inversion of the tubes, followed by centrifugation at 100 g for 3 min and immediate removal of supernatant.

Protoplasts transfected with the pCW498-35S-GFiP-OcsT vector DNA were re-suspended in 1 ml MI, transferred to 12-well tissue culture plates and incubated in the dark at RT. The protoplasts transfected with the pYLCRISPR/Cas9P$_{ubi}$-GTR vector DNA were re-suspended in 200 µl 0.5 M mannitol and embedded in alginate disks as described above.

Detection of *GFP* Gene Expression and Identification of *BnGTR* Mutants

For estimation of transfection efficiency, the protoplasts transfected with the *GFP* vector were observed after 48 h with Zeiss LSM 880 Airyscan confocal laser scanning microscope using an EC-Plan-Neofluar 10x/0.30 M27 objective for validation of GFP expression. Excitation wavelength was 488 nm and detection wavelength was 490–585 nm. Non-transfected protoplasts were used as control to verify that no auto-fluorescence could be observed.

To identify mutations in the *BnGTR* genes, genomic DNA was extracted from the regenerated shoots using Phire Plant Direct PCR kit (Thermo Scientific^{TM}) and used as template for PCR amplification of the target sequences with fluorescently labeled forward primers using Phusion High-Fidelity PCR Master Mix with GC Buffer (Thermo Scientific^{TM}) (**Supplementary Table S3**). The PCR amplicons were subjected to high-resolution fragment analysis (HRFA) as described by Andersson et al. (2017). For confirmation of the mutations by sequencing, PCR amplicons with non-labeled primers were ligated into the pJET1.2/blunt cloning vector (Thermo Scientific^{TM}) and transformed into Stellar^{TM} chemically competent cells of *E. coli* (Takara Bio, Shiga, Japan). Randomly selected single colonies were analyzed by Sanger sequencing (Eurofins Genomics, Konstanz, Germany).

Statistical Analysis

For the protoplast viability test, protoplast solution was loaded on a Hemocytometer and five 1 mm^2 squares were observed under light microscope seven days after culture, and this was repeated three times. For the callus and shoot regeneration tests, each treatment consisted of 40–50 protoplast colonies, and was repeated three times. The regeneration results were recorded about 1–3 months after shoots started to appear, depending on experiment. The detailed information is presented at the bottom of each corresponding table in the result section. Data was analyzed with ANOVA and Tukey's test using the statistical software Minitab (LLC) version 19.2020.1.

RESULTS

Effect of PGRs in MI Medium on Protoplast Viability at the Initial Stage

Protoplasts are very fragile and sensitive to the growth environment when they are freshly isolated due to lacking the cell wall. The medium composition, especially PGRs, is crucial to the initial protoplast culture. We thus tested several PGR combinations in MI medium, and found that the combination of 0.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} NAA gave the best result in terms of protoplast viability among all PGR combinations tested. In this medium, most protoplasts remained viable, as they were observed under a light microscope to be round and compact in form and green in color (**Figure 1A**) 7 d after protoplast culture. The protoplasts in the MI medium containing other PGR combinations became inviable (**Table 1**), namely shrunk and pale or brownish in color. This result is in agreement with the results from a previous report, which indicated that 2,4-D was essential for cell wall formation and initial protoplast growth (Glimelius, 1984). Moreover, our results showed that addition of cytokinin, like TDZ, BAP or zeatin, in combination with auxin in MI medium did not improve protoplast viability or growth compared with auxin alone.

Effect of PGRs in MII Medium on Protoplast Growth and Development

After the cell wall has formed, the protoplasts would undergo a rapid cell division (**Figures 1B, C**), and a suitable PGR combination in MII medium was found to be essential during this stage. We investigated different PGR combinations in MII medium. The results showed that the combinations of 1.1 mg l^{-1} TDZ with 0.05 mg l^{-1} 2,4–D and 1.1 mg l^{-1} TDZ with 0.05 mg l^{-1} NAA gave better results than the other PGR combinations tested, as the protoplasts divided rapidly and formed multiple protoplast colonies on these two media (**Table 2**), indicating that a relatively lower concentration of auxin was necessary for protoplast growth and further development during this stage. The results also showed that TDZ as cytokinin source was much more

TABLE 8 | Features of the *BnGTR* paralogs used in this study.

Arabidopsis orthologs	*B. napus* genes	Locus number	Genomic sequence length (bp)	Number of exons	Coding region (bp)
AtGTR1	*BnGTR1*	*LOC106397267*	2798	4	1905
	BnGTR1	*LOC106408997*	2673	4	1848
	BnGTR1	*LOC106410496*	2649	4	1848
	BnGTR1	*LOC106414122*	2666	4	1848
	BnGTR1	*LOC106445255*	2988	4	1905
	BnGTR1	*LOC111202315*	2685	4	1848
AtGTR2	*BnGTR2*	*LOC106347844*	2842	4	1839
	BnGTR2	*LOC106366161*	2755	4	1839
	BnGTR2	*LOC106369007*	8538	4	1839
	BnGTR2	*LOC106405453*	2453	4	1839
	BnGTR2	*LOC106411192*	2868	4	1836
	BnGTR2	*LOC106424883*	2754	4	1839

TABLE 9 | CRISPR target sequences (sgRNAs).

Name	Sequence (5′-3′)	Target gene
sgRNA1	AATGAGACATTTGAGAAGAT	*BnGTR1*
sgRNA2	GAATCAACAGTTTCTTCAAC	*BnGTR1*
sgRNA3	TTTGAGAAGCTTGGGATCAT	*BnGTR2*
sgRNA4	TTCCTTTGCGACACTTACTT	*BnGTR2*

efficient than BAP for facilitating the normal growth of the protoplasts.

Effect of Mannitol in SIM Medium on Protoplast Regeneration

Our results showed that culture of the protoplasts in MII medium longer than 20 d would result in brownish and inviable protoplasts (**Table 6**), likely due to inhibitory effect of high concentration of mannitol (100 g l^{-1}) on growth. To solve this problem, the protoplasts were transferred to SIM medium after 20 d, which contained half amount of mannitol compared to MII. As shown in (**Table 3**), the presence of mannitol in the SIM medium was still necessary for callus growth (**Figure 1D**), and thereby facilitating shoot regeneration (**Figure 1E**). Otherwise, the calli could become brownish, and no shoot regeneration would occur. This suggests that osmotic protection by mannitol was needed for maintaining the normal growth and development of protoplasts during this stage of protoplast culture.

Effect of PGRs in SRM Medium on Protoplast Regeneration

In this study, we found that the combination of TDZ as cytokinin-source and NAA as auxin-source in SRM medium gave the best result with regards to shoot regeneration among all the combinations tested (**Table 4**). Relatively high concentrations of cytokinin and auxin gave better effect on shoot regeneration, in which 2.2 mg l^{-1} TDZ in combination with 0.5 mg l^{-1} NAA gave the highest regeneration frequency, while all other PGR combinations resulted in a significantly decreased regeneration frequency.

Effect of C-Source in SRM Medium on Protoplast Regeneration

Sugar plays an important role in protoplast growth and development. We tested two types of sugars commonly used in protoplast culture as carbon source in the SRM media. The results showed that sucrose resulted in better shoot regeneration frequency than glucose, which seemed to be less effective in promoting shoot regeneration (**Table 5**). When comparing different concentrations of sucrose, we found that 20 g l^{-1} sucrose resulted in 41.0% regeneration frequency after two months, compared to 31.4% for 30 g l^{-1}.

Effect of Culture Duration in MI, MII and SIM Media on Protoplast Growth and Regeneration

We found that the culture duration in MI and MII media at the early stage of protoplast development was critical for shoot regeneration. The results in (**Table 6**) show that the culture duration in MI medium should not be longer than 5 d, while 15–20 d in MII medium was the most suitable duration for shoot regeneration. After 30 d in MII medium, the regeneration percentage decreased rapidly.

The culture duration on SIM medium also seemed to be important for shoot regeneration, as shown in (**Table 7**). The duration of 20–25 d on SIM medium was shown to be the most suitable duration among all durations tested for shoot regeneration. After 30 d, the regeneration percentage was significantly decreased.

Cloning of *BnGTR* Paralog Genes

All the 12 paralogs were amplified in cv. Kumily in this study, and the gene sequences were submitted in the GenBank database under the accession numbers, MW759464 to MW759475. The homology between different paralogs of the same gene family ranged between 86% to 99% for *BnGTR1* and 88% to 98% for *BnGTR2*.

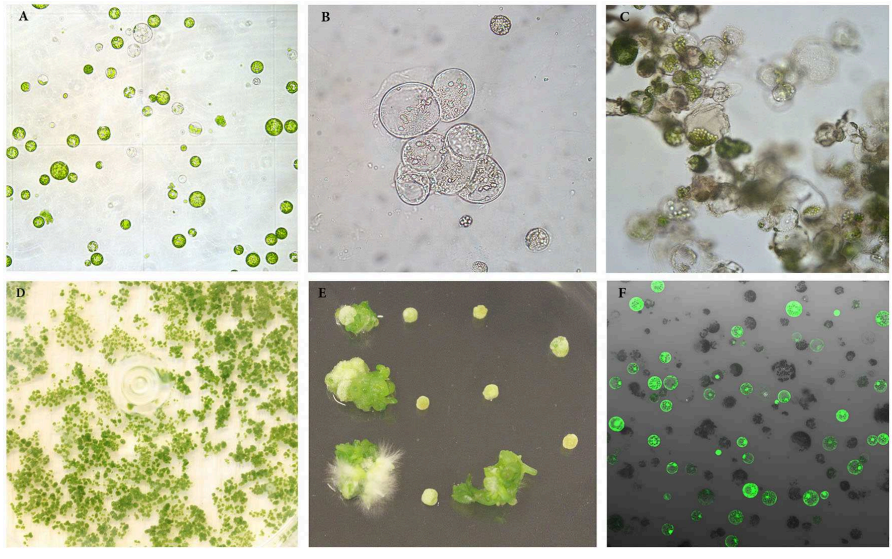

FIGURE 1 | Isolation, regeneration and transfection of protoplasts of rapeseed. **(A)** Freshly isolated protoplasts. **(B, C)** Protoplasts undergoing cell divisions and multiplication. **(D)** Protoplast colonies. **(E)** Shoot regeneration from protoplast colonies. **(F)** Transfected protoplasts expressing GFP protein observed under confocal laser scanning microscope.

Protoplast Transfection Efficiency

In order to estimate the efficiency of protoplast transfection, we transfected protoplasts with a vector harboring the *GFP* gene. The results showed that transfection efficiencies ranging from approximately 40 to 80% could routinely be observed, as measured by intact protoplasts exhibiting GFP fluorescence (**Figure 1F**) 48 h after transfection. This suggests that a large proportion of the protoplasts can express the transgene for a sustained time-period, and that the transfection protocol is working well for rapeseed under our culture conditions.

Identification of Mutation in the *BnGTR* Genes

We designed four highly conserved 20 bp target sequences (sgRNAs) for *BnGTR1* (sgRNA1 and sgRNA2) and *BnGTR2* (sgRNA3 and sgRNA4), for knocking out all paralogs of the two gene families. The sgRNA1 and sgRNA4 sequences shared 100% identity with the target regions in four paralogs of *BnGTR1* and *BnGTR2*, but had a single nucleotide mismatch 14 bp upstream of the PAM site in two paralogs of each targeted gene family (**Figure 2**). The sgRNA2 and sgRNA3 sequences had 100% identity in five paralogs of *BnGTR1* and *BnGTR2*, but had a single nucleotide mismatch 12 bp upstream of the PAM site in one paralog of *BnGTR1* and one nucleotide mismatch in one paralog of *BnGTR2* 17 bp upstream of the PAM site.

Using the above optimized protoplast regeneration and transfection protocols and the CRISPR vector harboring the four sgRNAs, we have successfully mutated multiple *BnGTR* genes.

Out of 50 calli, 16 shoots were regenerated, resulting in a regeneration frequency of over 30%. Out of the 16 regenerated shoots, 3 were found to be mutated, giving a mutation efficiency of over 18%. The results were based on three biological replicates. The sequencing results revealed various types of mutations consisting of single base insertions, 1-13 bp deletions and a substitution among the three mutant lines analyzed (**Figure 2**), indicating successful gene editing using our optimized protoplast protocol. No mutations at the target sites of sgRNA2 and sgRNA3 were detected. Sequencing results revealed that the mutations in deletion and insertion could lead to frameshift mutations and introduce premature stop codons to disrupt the open-reading frames. The PCR results showed no presence of the *Cas9* and *nptII* genes in the three mutants (**Supplementary Figure S2**).

DISCUSSION

Creation of more genetic variation is necessary to improve important agronomic traits of rapeseed, as the natural gene pool of the species has a low genetic diversity (Bus et al., 2011). Apart from crossbreeding, induced mutations has been used to increase genetic variation of the species. In recent years, the CRISPR/Cas9 technology has been proven to be a powerful tool for plant genetic modification, while its great potential has not been explored fully yet for trait improvement, and this is particularly true for rapeseed. One of the main reasons for this is the lack of an efficient method for delivering CRISPR vectors or complexes into plant cells for production of transgene-free mutation lines.

sgRNA1 targeting *BnGTR1* genes

	Sequence (5'-3')	Sequence (5'-3')	Sequence (5'-3')
sgRNA1	AATGAGACATTTGAGAAGAT	AATGAGACATTTGAGAAGAT	AATGAGACATTTGAGAAGAT
	Mutant 1	**Mutant 2**	**Mutant 3**
BnGTR1	**Target sequence 1 (5'-3') PAM**	**Target sequence 1 (5'-3') PAM**	**Target sequence 1 (5'-3') PAM**
Wildtype *Allele 1*	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**
Wildtype *Allele 2*	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**
LOC106397267 Allele 1	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGA---T**AGG**	AATGAGACATTTGAGAAGAT**AGG**
LOC106397267 Allele 2	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAATAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**
LOC106414122 Allele 1	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**
LOC106414122 Allele 2	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**
LOC106445255 Allele 1	AATGAGACATTTGAGA---T**AGG**	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**
LOC106445255 Allele 2	AATGAGACATTTGAGA---T**AGG**	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**
LOC111202315 Allele 1	AATGAGACATTTGAGA---T**AGG**	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**
LOC111202315 Allele 2	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**	AATGAGACATTTGAGAAGAT**AGG**

	Sequence (5'-3')	Sequence (5'-3')	Sequence (5'-3')
sgRNA1	AATGAGACATTTGAGAAGAT	AATGAGACATTTGAGAAGAT	AATGAGACATTTGAGAAGAT
	Mutant 1	**Mutant 2**	**Mutant 3**
BnGTR1	**Target sequence 1 (5'-3') PAM**	**Target sequence 1 (5'-3') PAM**	**Target sequence 1 (5'-3') PAM**
Wildtype *Allele 1*	AATGAAACATTTGAGAAGAT**AGG**	AATGAAACATTTGAGAAGAT**AGG**	AATGAAACATTTGAGAAGAT**AGG**
Wildtype *Allele 2*	AATGAAACATTTGAGAAGAT**AGG**	AATGAAACATTTGAGAAGAT**AGG**	AATGAAACATTTGAGAAGAT**AGG**
LOC106408997 Allele 1	AATGAAACATTTGAGA-GAT**AGG**	AATGAAACATTTGAGAAGAT**AGG**	AATGAAACATTTGAGAAGAT**AGG**
LOC106408997 Allele 2	AATGAAACATTTGAGA-GAT**AGG**	AATGAAACATTTGAGAAGAT**AGG**	AATGAAACATTTGAGAAGAT**AGG**
LOC106410496 Allele 1	AATGAAACATTTGAGA-GAT**AGG**	AATGAAACATTTGAGAAGAT**AGG**	AATGAAACATTTGAGAAGAT**AGG**
LOC106410496 Allele 2	AATGAAACATTTGAGAAGAT**AGG**	AATGAAACATTTGAGAAGAT**AGG**	AATGAAACATTTGAGAAGAT**AGG**

sgRNA4 targeting *BnGTR2* genes

	Sequence (5'-3')	Sequence (5'-3')	Sequence (5'-3')
sgRNA4	TTCCTTTGCGACACTTA CTT	TTCCTTTGCGACACTTA CTT	TTCCTTTGCGACACTTACTT
	Mutant 1	**Mutant 2**	**Mutant 3**
BnGTR2	**Target sequence 1 (5'-3') PAM**	**Target sequence 1 (5'-3') PAM**	**Target sequence 1 (5'-3') PAM**
Wildtype *Allele 1*	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTACTT**CGG**
Wildtype *Allele 2*	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTACTT**CGG**
LOC106366161 Allele 1	TTCCTTTGCGACACTTA --T**CGG**	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTACTT**CGG**
LOC106366161 Allele 2	TTCCTTTGCGACACTTAACTT**CGG**	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTACTT**CGG**
LOC106369007 Allele 1	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTACTT**CGG**
LOC106369007 Allele 2	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTACTT**CGG**
LOC106405453 Allele 1	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTAACTT**CGG**	TTCCTTTGCGACACTTACTT**CGG**
LOC106405453 Allele 2	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTACTT**CGG**
LOC106411192 Allele 1	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTACTT**CGG**
LOC106411192 Allele 2	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTA CTT**CGG**	TTCCTTTGCGACACTTACTT**CGG**

	Sequence (5'-3')	Sequence (5'-3')	Sequence (5'-3')
sgRNA4	TTCCTTTGCGACACTTACTT	TTCCTTTGCGACACTTACTT	TTCCTTTGCGACACTTACTT
	Mutant 1	**Mutant 2**	**Mutant 3**
BnGTR2	**Target sequence 1 (5'-3') PAM**	**Target sequence 1 (5'-3') PAM**	**Target sequence 1 (5'-3') PAM**
Wildtype *Allele 1*	TTCCTCTGCGACACTTACTT**CGG**	TTCCTCTGCGACACTTACTT**CGG**	TTCCTCTGCGACACTTACTT**CGG**
Wildtype *Allele 2*	TTCCTCTGCGACACTTACTT**CGG**	TTCCTCTGCGACACTTACTT**CGG**	TTCCTCTGCGACACTTACTT**CGG**
LOC106347844 Allele 1	TTCCTCTGCGACACT--CTT**CGG**	TTCCTCT----------**CGG**	TTCCTCT----------**CGG**
LOC106347844 Allele 2	TTCCTCTGCGACACTTACTT**CGG**	TTCCTCTGCGACACTTACTT**CGG**	TTCCTCTGCGACACTTACTT**CGG**
LOC106424883 Allele 1	TTCCTCTGCGACACT--CTT**CGG**	TTCCTCT----------**CGG**	TTCCTCTGCGACACTTACTT**CGG**
LOC106424883 Allele 2	TTCCTCTGCGACACTTACTT**CGG**	TTCCTCTGCGACACTTACTT**CGG**	TTCCTCTGCGACACTTACTT**CGG**

FIGURE 2 | Types of mutations in the *BnGTR1* and *BnGTR2* genes detected in the three mutants in comparison with wild type of rapeseed cv. Kumily, determined by DNA sequencing. PAM sites are highlighted in bold letters. Mismatches with the sgRNAs are highlighted in green. Mutated nucleotides were highlighted in different colors, in which deletions are shown with hyphens in blue, substitution and insertions are highlighted in red and pink, respectively.

The protoplast transient transfection system is a promising approach for delivering CRISPR complexes, but the bottleneck of this approach is the difficulty in protoplast regeneration.

Protoplasts are plant cells that lack the cell wall, but possess plasma membrane and all other cellular components. The first developmental stage of protoplasts is formation of the cell wall, followed by cell divisions. The cell wall formation starts within a few hours after isolation, and may take several days to complete (Kartha et al., 1974). In this period, the protoplasts are very fragile and sensitive to the culture conditions and surrounding environment. It has been reported that for the culture of rapeseed hypocotyl protoplasts, the auxins 2,4-D and NAA were both necessary for cell wall formation and cell division. The ratio of NAA to 2,4-D content that stimulates protoplast colony growth best appears to be species- and even genotype-dependent. It has been reported that, in one case, a higher level of NAA than 2,4-D was either similar or better in stimulating protoplast colony growth of all genotypes tested (Glimelius, 1984), while in another study, higher levels of 2,4-D than NAA was reported to be beneficial for hypocotyl protoplast development in rapeseed (Barsby et al., 1986). In this study, we used identical quantities of 2,4-D and NAA, and it turned out to work well in this case.

Osmotic pressure must be maintained at the initial stage of protoplast culture. The isolated and cultured protoplasts require osmotic protection until they have developed cell walls (Kao and Seguin-Swartz, 1987), while the osmolarity should be gradually reduced to a normal level in order to maintain normal growth and development. In this study, mannitol was used to maintain osmotic pressure. We first used a high concentration of mannitol (100 g l^{-1}) in MI and MII media, which was then reduced to 50 g l^{-1} in SIM until the protoplasts became small colonies, and thereafter removed completely in the SRM medium. If mannitol was removed from the medium too early, the protoplasts would become brownish and eventually die. On the other hand, if the mannitol was removed from medium too late, the growth and regeneration of protoplasts would be negatively affected. The reason could be that continuous presence of mannitol would form an inappropriate cell environment for normal growth, e.g., affecting negatively the uptake of nutrients and water.

The culture density of protoplasts is also an important factor affecting protoplast growth and development. Some studies suggested that higher culture densities would promote the growth and division of protoplast cells (Chuong et al., 1985; Kielkowska and Adamus, 2012). The reason for this could be that cultured protoplasts stimulate growth and mitotic division of adjacent cells by releasing growth factors into the surrounding medium (Davey et al., 2005). In this study, we also found that a low density of protoplasts could result in poor cell division and thus reduced callus formation. However, too high density of protoplasts would result in brownish colonies, likely because of rapidly depleted available nutrients that caused a large number of protoplasts to fail to undergo divisions (Chuong et al., 1985). The most suitable plating density in this study was 0.4 million protoplasts per ml for rapeseed, while up to 1 million per ml also lead to regeneration of plants in many cases.

Low regenerative capacity is the major obstacle affecting the application of protoplasts for rapeseed. With induction and appropriate manipulations, the protoplasts are able to undergo a series of differentiation stages, and finally form whole plants under optimal or suitable conditions. Among all factors affecting protoplast regeneration, PGRs is thought to be the most important one. A general concept is that high auxin to cytokinin ratio is suitable to stimulate cell divisions and cell wall formation of protoplasts, and high cytokinin to auxin ratio is required for shoot regeneration. However, this ratio varies a lot from species to species (Kao and Seguin-Swartz, 1987), and thus needs to be optimized for each crop. We found in our study that TDZ gave the best shoot regeneration among all types of cytokinin tested. Moreover, high concentration of cytokinin in combination with a relatively high level of auxin (2.2 mg l^{-1} TDZ and 0.5 mg l^{-1} NAA) had a great positive effect on protoplast regeneration in rapeseed. Although BAP is widely used for many crops for *in vitro* cultures, it did not seem to be effective for protoplast regeneration in rapeseed, as shown in this study.

We also found in this study that the culture duration in different culture media at different developmental stages played an important role in protoplast regeneration of rapeseed, in which prolonged culture durations at earlier stages of development would reduce regeneration rapidly. For instance, the culture duration in MI medium should not be longer than 5 d, the duration in MII should be shorter than 30 d and not more than 20 d in SIM medium. These findings suggest that it is crucial to transfer protoplast cultures into the successive media in a timely manner.

In this study, the *BnGTR* genes were successfully edited by CRISPR/Cas9 in rapeseed using our optimized protoplast regeneration and transfection protocols, demonstrating for the first time the high capacity of the protoplast approach in genetic improvement of rapeseed by CRISPR/Cas9. We believe that this optimized protoplast regeneration protocol will be beneficial to other researchers working with rapeseed or other *Brassica* species. We are still working on generating more mutation lines in order to get desirable and more homozygous mutation lines. It should be kept in mind that modern widely cultivated cultivars are allotetraploid. This allopolyploidization leads to multiple homologs of most genes controlling the same traits in the rapeseed genome compared with the related diploid model species *A. thaliana* (Chalhoub et al., 2014). In order to develop a knockout mutant in rapeseed, it is imperative to edit all paralogous sequences of the *BnGTR* genes. Therefore, selfing for a couple of generations might be needed to obtain homozygous mutation lines in all paralogs of the *BnGTR* genes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

L-HZ led the research and, together with XL and SK, designed the studies. XL, SS, and SK performed the most of the experiments. XL, L-HZ, SK, and SS wrote the manuscript. OM, RG, EI, and EW contributed to the protoplast transfection and regeneration studies. All authors have read the manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.680859/full#supplementary-material

REFERENCES

Andersson, M., Turesson, H., Nicolia, A., Falt, A. S., Samuelsson, M., and Hofvander, P. (2017). Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. *Plant Cell Rep.* 36, 117–128. doi: 10.1007/s00299-016-2062-3

Arora, L., and Narula, A. (2017). Gene editing and crop improvement using CRISPR-Cas9 system. *Front Plant Sci.* 8:1932. doi: 10.3389/fpls.2017.01932

Bae, S., Park, J., and Kim, J. S. (2014). Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics* 30, 1473–1475. doi: 10.1093/bioinformatics/btu048

Barsby, T. L., Yarrow, S. A., and Shepard, J. F. (1986). A rapid and efficient alternative procedure for the regeneration of plants from hypocotyl protoplasts of *Brassica napus*. *Plant Cell Rep.* 5, 101–103. doi: 10.1007/BF00269244

Braatz, J., Harloff, H. J., Mascher, M., Stein, N., Himmelbach, A., and Jung, C. (2017). CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different homoeologous gene copies in polyploid oilseed rape (*Brassica napus*). *Plant Physiol.* 174, 935–942. doi: 10.1104/pp.17.00426

Bus, A., Korber, N., Snowdon, R. J., and Stich, B. (2011). Patterns of molecular variation in a species-wide germplasm set of *Brassica napus*. *Theor. Appl. Genet.* 123, 1413–1423. doi: 10.1007/s00122-011-1676-7

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I.,a,. P., Tang, H., Wang, X., et al. (2014). Early allopolyploid evolution in the post-neolithic *Brassica napus* oilseed genome. *Science* 345, 950–953. doi: 10.1126/science.1253435

Chuong, P. V., Pauls, K. P., and Beversdorf, W. D. (1985). A simple culture method for *Brassica* hypototyl protoplasts. *Plant Cell Rep.* 4, 4–6. doi: 10.1007/BF00285492

Davey, M. R., Anthony, P., Power, J. B., and Lowe, K. C. (2005). Plant protoplasts: status and biotechnological perspectives. *Biotechnol. Adv.* 23, 131–171. doi: 10.1016/j.biotechadv.2004.09.008

Glimelius, K. (1984). High growth rate and regeneration capacity of hypocotyl protoplasts in some Brassicaceae. *Physiol. Plant* 61, 38–44. doi: 10.1111/j.1399-3054.1984.tb06097.x

Huang, H., Cui, T., Zhang, L., Yang, Q., Yang, Y., Xie, K., et al. (2020). Modifications of fatty acid profile through targeted mutation at *BnaFAD2* gene with CRISPR/Cas9-mediated gene editing in *Brassica napus*. *Theor. Appl. Genet.* 133, 2401–2411. doi: 10.1007/s00122-020-03607-y

Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B., and Weeks, D. P. (2013). Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in arabidopsis, tobacco, sorghum and rice. *Nucleic Acids Res.* 41:e188. doi: 10.1093/nar/gkt780

Kao, H. M., and Seguin-Swartz, G. (1987). Study of factors affecting the culture of *Brassica napus* L. and *B. juncea Coss.* mesophyll protoplasts. *Plant Cell Tissue Organ Cult.* 10, 79–90. doi: 10.1007/BF00035906

Kartha, K. K., Michayluk, M. R., Kao, K. N., Gamborg, O. L., and Constabel, F. (1974). Callus formation and plant regeneration from mesophyll protoplasts of rape plants (*Brassica napus* L. cv. *Zephyr). Plant Sci. Lett.* 3, 265–271. doi: 10.1016/0304-4211(74)90097-2

Kielkowska, A., and Adamus, A. (2012). An alginate-layer technique for culture of *Brassica oleracea* L. protoplasts. *In Vitro Cell Dev. Biol. Plant* 48, 265–273. doi: 10.1007/s11627-012-9431-6

Kim, H., Kim, S. T., Ryu, J., Kang, B. C., Kim, J. S., and Kim, S. G. (2017). CRISPR/Cpf1-mediated DNA-free plant genome editing. *Nat. Commun.* 8:14406. doi: 10.1038/ncomms14406

Kim, S.-Y., Bengtsson, T., Olsson, N., Hot, V., Zhu, L.-H., and Åhman, I. (2020). Mutations in two aphid-regulated β-1,3-glucanase genes by CRISPR/Cas9 do not increase barley resistance to *Rhopalosiphum padi* L. *Front. Plant Sci.* 11:1403. doi: 10.3389/fpls.2020.01043

Li, C., Hao, M., Wang, W., Wang, H., Chen, F., Chu, W., et al. (2018). An efficient CRISPR/Cas9 platform for rapidly generating simultaneous mutagenesis of multiple gene homoeologs in allotetraploid oilseed rape. *Front. Plant Sci.* 9:442. doi: 10.3389/fpls.2018.00442

Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., et al. (2017). Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat. Commun.* 8:14261. doi: 10.1038/ncomms14261

Liang, Z., Zhang, K., Chen, K., and Gao, C. (2014). Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. *J. Genet. Genomics* 41, 63–68. doi: 10.1016/j.jgg.2013.12.001

Lin, C. S., Hsu, C. T., Yang, L. H., Lee, L. Y., Fu, J. Y., Cheng, Q. W., et al. (2018). Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration. *Plant Biotechnol. J.* 16, 1295–1310. doi: 10.1111/pbi.12870

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., et al. (2015). A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Mol. Plant* 8, 1274–1284. doi: 10.1016/j.molp.2015.04.007

Malnoy, M., Viola, R., Jung, M. H., Koo, O. J., Kim, S., Kim, J. S., et al. (2016). DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. *Front. Plant Sci.* 7:1904. doi: 10.3389/fpls.2016.01904

Menczel, L., Nagy, F., Kiss, Z. R., and Maliga, P. (1981). Streptomycin resistant and sensitive somatic hybrids of *Nicotiana tabacum* + *Nicotiana knightiana*: correlation of resistance to *N. tabacum* plastids. *Theor. Appl. Genet.* 59, 191–195. doi: 10.1007/BF00264975

Murovec, J., Gucek, K., Bohanec, B., Avbelj, M., and Jerala, R. (2018). DNA-free genome editing of *Brassica oleracea* and *B. rapa* protoplasts using CRISPR-Cas9 ribonucleoprotein complexes. *Front. Plant Sci.* 9:1594. doi: 10.3389/fpls.2018.01594

Muthusamy, S., Vetukuri, R. R., Lundgren, A., Ganji, S., Zhu, L.-H., Brodelius, P. E., et al. (2020). Transient expression and purification of β-caryophyllene synthase in *Nicotiana benthamiana* to produce β-caryophyllene *in vitro*. *PeerJ* 8:e8904. doi: 10.7717/peerj.8904

Nekrasov, V., Staskavicz, B., Wigel, D., Jones, J. D. G., and Kamoun, S. (2013). Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* 31, 691–693. doi: 10.1038/nbt.2655

Nicolia, A., Proux-Wera, E., Ahman, I., Onkokesung, N., Andersson, M., Andreasson, E., et al. (2015). Targeted gene mutation in tetraploid potato through transient TALEN expression in protoplasts. *J. Biotechnol.* 204, 17–24. doi: 10.1016/j.jbiotec.2015.03.021

Nitsch, J. P., and Nitsch, C. (1969). Haploid plants from pollen grains. *Science* 163, 85–87. doi: 10.1126/science.163.3862.85

Nour-Eldin, H. H., Andersen, T. G., Burow, M., Madsen, S. R., Jorgensen, M. E., Olsen, C. E., et al. (2012). NRT/PTR transporters are essential for translocation of glucosinolate defence compounds to seeds. *Nature* 488, 531–534. doi: 10.1038/nature11285

Prykhozhij, S. V., Rajan, V., Gaston, D., and Berman, J. N. (2015). CRISPR multitargeter: a web tool to find common and unique CRISPR single guide RNA targets in a set of similar sequences. *PLoS ONE* 10:e0119372. doi: 10.1371/journal.pone.0119372

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., et al. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat. Biotechnol.* 31, 686–688. doi: 10.1038/nbt.2650

USDA (2019). *Oil Crops Yearbook*. Available online at: https://www.ers.usda.gov/data-products/oil-crops-yearbook/ (accessed April 19, 2019).

Woo, J. W., Kim, J., Kwon, S. I., Corvalan, C., Cho, S. W., Kim, H., et al. (2015). DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nat. Biotechnol.* 33, 1162–1164. doi: 10.1038/nbt.3389

Wood, C. C., Petrie, J. R., Shrestha, P., Mansour, M. P., Nichols, P. D., Green, A. G., et al. (2009). A leaf-based assay using interchangeable design principles to rapidly assemble multistep recombinant pathways. *Plant Biotechnol. J.* 7, 914–924. doi: 10.1111/j.1467-7652.2009.00453.x

Yoo, S. D., Cho, Y. H., and Sheen, J. (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.* 2, 1565–1572. doi: 10.1038/nprot.2007.199

Zheng, M., Zhang, L., Tang, M., Liu, J., Liu, H., Yang, H., et al. (2020). Knockout of two *BnaMAX1* homologs by CRISPR/Cas9-targeted mutagenesis improves plant architecture and increases yield in rapeseed (*Brassica napus* L.). *Plant Biotechnol. J.* 18, 644–654. doi: 10.1111/pbi.13228

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Knockout of *BnaX.SGT.a* caused significant sinapine reduction in transgene-free rapeseed mutants generated by protoplast-based CRISPR RNP editing

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Rapeseed (*Brassica napus* L.) is known for its high-quality seed oil and protein content. However, its use in animal feed is restricted due to antinutritional factors present in the seedcake, with sinapine being one of the main compounds that reduces palatability. Attempts to develop rapeseed germplasm with lower sinapine levels through traditional breeding methods have shown limited progress. Genetic transformation methods could create new genotypes with reduced sinapine levels by silencing key genes involved in sinapine biosynthesis, though these methods often result in transgenic or genetically modified plants. The recent development of CRISPR-Cas technology provides a precise and efficient approach to crop improvement, with the potential to generate transgene-free mutants. In this study, we targeted the *BnaX.SGT.a* genes for knockout using CRISPR-Cas editing. By utilizing our newly established protoplast regeneration and transfection protocol for rapeseed, we demonstrated that DNA-free CRISPR editing via protoplast-based ribonucleoprotein (RNP) delivery was highly effective. We achieved successful knockout of the *BnaX.SGT.a* paralogues, with an average mutation efficiency of over 30%. Sequencing results revealed a variety of mutation types, from 1 bp insertions to 10 bp deletions, with most mutants exhibiting frameshift mutations that led to premature stop codons. The mutants displayed no visible phenotypic differences in growth patterns or flowering compared to the wild type. Importantly, sinapine content was significantly reduced in all T_2 generation mutants analysed, while seed weight remained comparable between mutants and the wild type.

KEYWORDS

rapeseed and canola, genome editing by CRISPR-Cas, protoplast-based CRISPR RNP, DNA-free, transgene-free mutants, seedcake quality, antinutritional factor or compound

Introduction

Rapeseed (*Brassica napus* L.) is the third largest source of vegetable oil in the world, after palm and soybean, and it is the main oil crop grown in Europe (Shahbandeh, 2024). The seedcake remaining after oil extraction is rich in high-quality protein with a favourable amino acid profile (Cheng et al., 2022). This gives rapeseed significant potential as a plant protein source for feed and food applications, a role which is primarily fulfilled by imported soybean meal in Europe at present. Currently, the use of rapeseed seedcake is limited in animal feed due to the presence of antinutritional factors. Sinapine is one such factor; it reduces palatability, inhibits nutrient absorption, and causes the eggs of certain species of chicken to have a fishy taste (Kozlowska et al., 1990; Qiao et al., 2008; Ward et al., 2009). In rapeseed, sinapine functions as a storage compound for choline, while also offering antioxidant properties and protection against UV-B radiation (Yates et al., 2019; Sheahan, 1996).

Reducing sinapine levels in rapeseed seeds could transform rapeseed meal from a low-value by-product into a high value protein source. This shift is particularly important in the face of a changing climate, as extreme weather events put increasing pressure on agriculture. With rising temperatures, new areas are becoming suitable for certain pests and pathogens, creating challenges for crops that are not adapted to these threats. Additionally, unpredictable weather patterns can negatively affect crop yields, and the rapid speed of environmental changes outpaces the ability of plants to naturally adapt (Jacobs et al., 2024). Given these challenges, maximizing the utility of the side streams of agricultural crops is crucial. For rapeseed, this could mean expanding its value beyond oil production to include high-quality protein, thereby adding economic value and enhancing sustainability by utilising the entire crop—including the seedcake, a current side stream of rapeseed oil production.

There are several methods for inducing mutations in target genes to improve traits, each with distinct advantages and limitations. Spontaneous mutations arise naturally without external intervention, maintaining the organism's native genetic background. However, their unpredictable nature and minor phenotypic effects often necessitate extensive screening to identify beneficial mutations. TILLING combines mutagenesis with targeted screening, enabling the identification of mutations in specific genes. This method is highly scalable, allowing the simultaneous screening of many individuals. Nevertheless, TILLING relies on random mutagenesis, which can produce nonspecific or off-target mutations that may be harmful or impair overall plant viability, and is very labour intensive. RNAi technology enables the silencing of specific genes, resulting in desired phenotypic traits. However, its application leads to genetically modified organisms (GMOs), which can face regulatory and public acceptance challenges.

The modern site-directed mutagenesis technology, such as CRISPR-Cas gene editing, provides promising solutions for modern plant breeding challenges. This technology enables the rapid and precise introduction of mutations in the host genome without integrating foreign DNA, and it has a low risk of off-target effects (DeWitt et al., 2017). Additionally, recent EU proposals suggest a potential relaxation of regulations on new genomic technologies (NGTs), including CRISPR-Cas-based approaches (Dionglay, 2024). Consequently, CRISPR-Cas-mediated mutation breeding addresses the limitations associated with traditional breeding, TILLING, and genetic engineering approaches, genetic engineering approaches, and TILLING, providing a more precise, efficient, and flexible approach for gene knockout. The estimated need to double the rate of genetic improvement to meet changing environmental demands aligns well with the potential of CRISPR-Cas mutagenesis (Voss-Fels et al., 2019). By reducing breeding cycles, CRISPR-Cas can produce new lines in half the time required by conventional breeding methods (May et al., 2023). This relationship highlights the critical role of CRISPR-Cas technology in modern plant breeding for meeting the urgent challenges faced in food supply from a growing global population in a changing climate.

Protoplast-based gene editing approaches are an efficient tool for achieving transgene-free gene editing, as they allow the direct delivery of CRISPR components across permeable cell membranes. This can be accomplished either by transiently expressing CRISPR components from plasmids or by introducing a ribonucleoprotein (RNP) complex composed of the Cas9 nuclease and a single guide RNA (sgRNA). Although both methods can generate transgene-free mutants, the RNP-based approach offers distinct advantages over plasmid-based methods. RNPs enable DNA-free editing, eliminating the need for plasmid vector preparation and removing concerns about plasmid DNA integration into the host genome. Additionally, RNPs enhance editing efficiency and reduce the likelihood of off-target mutations (Zhang et al., 2021; DeWitt et al., 2017). The primary challenge of protoplast-based methods lies in the difficulty of protoplast regeneration, which can vary significantly depending on the plant species or even the specific genotype. Recently, Li et al. (2021) developed an efficient protocol for regenerating rapeseed protoplasts and demonstrated the successful delivery of CRISPR plasmids to create mutant lines.

SGT (UGT84A9) (UDP-glucose:sinapate glucosyltransferase) is identified as a key enzyme in sinapine biosynthesis (Milkowski et al., 2004). There are two *SGT* genes in the *B. napus* genome, *BnaX.SGT.a* and *BnaX.SGT.b*, each located on both the A-genome and the C-genome, resulting in four loci: *BnaA.SGT.a*, *BnaA.SGT.b*, *BnaC.SGT.a*, and *BnaC.SGT.b* (Mittasch et al., 2010) *BnaX.SGT.a* is predominately expressed in developing seeds, while *BnaX.SGT.b* has negligible expression, apart from in flowers (Mittasch et al., 2010). Silencing *BnaX.SGT.a* through RNA interference (RNAi) has successfully reduced sinapine accumulation in rapeseed seeds (Hüsken et al., 2005; Wolfram et al., 2010). Furthermore, *BnaX.SGT.a* knockout lines generated via TILLING-based EMS mutagenesis demonstrate the potential for reducing sinapine accumulation through mutagenesis (Emrani et al., 2015). These findings underscore the role of *BnaX.SGT.a* in sinapine

accumulation. Importantly, research investigating the effects of *SGT* silencing has shown no adverse impacts on agricultural traits, including yield and oil content, as well as seed development, germination, or sensitivity to UV-B radiation (Hüsken et al., 2005; Wolfram et al., 2010; Hettwer et al., 2016). In this study, we aimed to target the two *BnaX.SGT.a* paralogues for reducing the sinapine levels in rapeseed seed of transgene-free mutants by protoplast-based CRISPR RNP editing.

Materials and methods

Plant material

The seeds used in this study were spring rapeseed (*B. napus* L.) cv. Kumily, a doubled haploid, provided by Lantmännen, Sweden.

Seed sterilisation

Seeds were surface sterilized by gentle shaking in 70% ethanol for 15 minutes, followed by gentle shaking in 20% kitchen bleach for 15 minutes. The seeds were then washed in sterile water four times.

In vitro seedling growth and growth conditions

Sterilized seeds were grown on the germination medium in single-use sterile plastic boxes. The germination medium contained half strength Murashige & Skoog (MS), 10 g l^{-1} sucrose, 7 g l^{-1} Bacto agar at pH 5.7. The boxes were placed in a climate chamber, which had a 16 hr photoperiod, with a light intensity of 40 µmol m^{-2} s^{-1} (cool white fluorescent tubes). The temperatures were 23°C/18°C for day and night respectively.

Sequencing of *BnaX.SGT* paralogues

For sequencing the *BnaX.SGT.a* and *BnaX.SGT.b* paralogues, DNA was extracted from the leaves of rapeseed cv. Kumily using the GeneJet Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA). PCR was performed using primers (Table 1), targeting all four paralogues of the two genes, designed using the sequences for *BnaX.SGT.a* (UGT84A9-1) and *BnaX.SGT.b* (UGT84A9-2) from NCBI. The PCR product was purified using NucleoSpin Gel and PCR Clean-up, Mini kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. The purified PCR product was then cloned into the pJET1.2/blunt vector using the CloneJET PCR Cloning Kit (Thermo Scientific, USA). Transformation was conducted using Stellar™ Competent Cells (Takara Bio, Japan) to propagate the recombinant plasmid. Plasmid DNA was subsequently purified from bacterial cultures with the NucleoSpin Plasmid Mini Kit (Macherey-Nagel), yielding high-purity plasmid DNA suitable for downstream applications. The recombinant plasmids of 16 colonies were then sent to Eurofins (Germany) for Sanger sequencing of the genes.

Design of sgRNAs

Geneious Prime 2024.0.5 was used to predict sgRNA sites, and its off-target checker tool was employed to assess potential off-target effects. Additionally, the online tool Cas-OFFinder (Bae et al., 2014) was used to further evaluate off-target sites (available at http://www.rgenome.net/cas-offinder/). Two sgRNAs (sgRNA1 at bp 425-442 and sgRNA2 at bp 735-758 of the gene) were selected based on high activity scores and absence of off-target effects, targeting on conserved functional domains as well as location in regions of high sequence homology. The two sgRNAs (Table 1) meeting these criteria most effectively were chosen for this study.

Protoplast isolation, transfection and plant regeneration

Leaves from 18-21 day old seedlings were used for protoplast isolation using the method described by Li et al. (2021). Approximately 120,000 isolated and washed protoplasts were re-suspended in 200 µl freshly prepared MMG solution (0.5 M mannitol, 15 mM $MgCl_2$, 4 mM MES) in a 2 mL Eppendorf tube for transfection. The solution was mixed with 20 µl RNP complex solution (4 µl gRNA (0.1 nmol/µl), 4 µl Cas9 (5 µg/µl), and 12 µl H_2O), and 220 µl freshly prepared PEG-calcium solution (40% (w/

TABLE 1 CRISPR target sequences and primers for gene sequencing and target site amplicon sequencing.

CRISPR target sequence (CTS) or primer	Sequence *	Purpose
CTS1 (sgRNA1)	GGACCCAGAGAACAGCACAG**GGG**	Gene knockout
CTS2 (sgRNA2)	ATTTTGTAAAGCGGTCCGAG**CGG**	Gene knockout
Forward primer	AGCACACAGAAGAGAACCCC	Sanger gene sequencing
Reverse primer	TCAGGATTTGCAGAAAACAAACA	Sanger gene sequencing
Forward primer	GCTGGTCGGACAACAAGAGA	Target site amplicon sequencing
Reverse primer	CTGCGAGTCTAACCACTCCA	Target site amplicon sequencing

*The sequence orientation of the CTSs is 3'-5' with the PAM sites in bold.

v) PEG 4000, 0.5 M mannitol, 0.1 M $CaCl_2$). The reaction was stopped after 6 min by addition of 1.5 ml W5 and gentle mixing by inversion of the tubes, followed by centrifugation for 3 min at 100 g and immediate removal of the supernatant. Protoplasts were then embedded in alginate discs and cultured in 6-well microplates, as described by Li et al. (2021). The embedded protoplasts were cultured for shoot regeneration according to the optimized protocol described in the same study. The *in vitro* regenerated shoots or putative mutants were rooted on the rooting medium, as described by Li et al. (2021). Once the shoots formed roots, they were transferred to soil pots and grown in the biotron growth chambers, where growth conditions were 21°C/16°C (day/night), 16 h photoperiod with a light intensity of 250 µ mol m^{-2} s^{-1} and 60% humidity.

Identification of mutant lines

Leaf tissue was taken from *in vitro* regenerated shoots of putative mutants and crushed with a pipette tip in Phire Dilution Buffer (Thermo Fisher Scientific, USA). The supernatant was used as a template for a PCR reaction using Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA) and gene specific primers (Table 1) to amplify the target region containing the sgRNA site. The PCR products were purified using GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific, USA) and sent for Sanger sequencing (Azenta Life Sciences, USA).

The seeds harvested from T_0 were sewn in pots and grown in the biotron. Seeds from T_1 plants were analysed for sinapine content and the lines with lower sinapine contents were sown for obtaining T_2 seeds. Two mutants from each T_2 line, as well as two WT, were sequenced using amplicon sequencing in order to acquire accurate sequences from all targeted alleles, and to elucidate the type of mutations caused. Genomic DNA was extracted from plants using the GeneJet Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA), and PCR was performed using Illumina adapter-linked primers to amplify the target region, which was then purified using GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific, USA). The samples were then sent for amplicon sequencing at Eurofins Genomics (Germany).

Extraction of sinapine

Sinapine was extracted from defatted rapeseed meal according to the method described by Wang et al. (1998) with modifications. In summary, 150 mg of pooled seeds from each plant in each generation (detailed numbers of plants for each generation are shown in Figures in the result section) were frozen at -80°C and milled using a Retsch MM 400 steel ball mill (Fisher Scientific, USA) at 30 Hz for 2 minutes. Sinapine extraction was then performed on 50 mg aliquots with 3 replicates per sample. For the defatting of samples, 1 ml of heptane (analytical grade) was added to each tube and the tubes were vortexed for 10 seconds and allowed to stand for 30 minutes at room temperature. The tubes were centrifuged at 10 000 rpm for 3 minutes and the supernatant was discarded. The defatting procedure was repeated once more with the standing time reduced to 5 minutes and the pellet was dried to completion in a vacuum desiccator (Concentrator Plus, Eppendorf, Germany). Sinapine was extracted from the defatted meal pellet by the addition of 1 ml of 70% methanol to each tube. The samples were vortexed for 10 seconds and placed in ultrasonic bath for 5 minutes. The tubes were then placed in a preheated heat block set to 75°C for 30 minutes. The tubes were centrifuged at 10,000 rpm for 2 minutes and the supernatant containing the crude sinapine extract was transferred to a new 2 ml screw-capped tube. To ensure complete extraction, another 1 ml of 70% methanol was added to the remaining pellet and the tube was vortexed for 10 seconds and allowed to stand at room temperature for 5 minutes. The tubes were centrifuged again at 10,000 rpm for 2 minutes and the supernatant was transferred to and mixed with the earlier collect. The samples were kept at -80°C until HPLC analysis.

HPLC analysis of sinapine

Samples were prepared for HPLC analysis by mixing 1 ml of the crude sinapine extract with 1 ml of eluent (10 mM sodium acetate, pH 4.0, with 13.5% acetonitrile). The mixture was centrifuged at 13,500 rpm for 5 minutes to pellet any residual particles, and 600 µl of the supernatant was transferred to an HPLC vial. Samples were analysed on an Agilent 1260 Series HPLC system, with separation of sinapine achieved using an Eclipse Plus C18, 3.0 x 100 mm, 3.5 µm column (Agilent, USA). A 6-minute isocratic elution was applied at a flow rate of 1 ml/min with a single eluent (10 mM sodium acetate, pH 4.0, with 13.5% acetonitrile). Sinapine was detected photometrically using a variable wavelength detector (VWD) at a signal wavelength of 330 nm and quantified based on its retention time with respect to a certified sinapine external standard (ChemFaces, China).

Phenotypic observations

Apart from regular visual observations on growth, flowering, seed setting etc., mature plants were photographed at harvest using a mirrorless interchangeable-lens camera (MILC; X-T3, Fuji). 100-seed weight was measured using a microbalance (RE 1614, Sauter).

Statistical analysis

Data for sinapine content and 100-seed weight were analysed with ANOVA and Tukey's test, with the significance level set at $p = 0.05$ using the Minitab statistical program (Minitab, LLC (USA), version 21.4.2.

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Results

Gene sequencing

The target genes were sequenced using Sanger sequencing with homology-based primers (Table 1). The sequencing results confirmed the presence of four *Bna.SGT* paralogues in rapeseed cv. Kumily. These paralogues fall into two distinct sequence types: *BnaX.SGT.a* and *BnaX.SGT.b*. Both sequence types are found in the A and C genomes of rapeseed, referred to as *BnaA.SGT.a*, *BnaA.SGT.b*, *BnaC.SGT.a*, and *BnaC.SGT.b*. The sequences of all *Bna.SGT* paralogues were obtained in cv. Kumily with the sequence-specific primers, which had 100% identity with the sequences from the NCBI database for all paralogues.

Protoplast transfection and screening of regenerated plants

The RNP transfected protoplasts regenerated well *in vitro* with a large number of shoots formed. Fifty T_0 shoots were screened for mutations on the target gene using Sanger sequencing of PCR products amplifying the sgRNA target regions. Of the 50 T_0 shoots sequenced, 25 were derived from protoplasts transfected with the RNP-sgRNA1 complex, and 25 from the RNP-sgRNA2 complex. Nine sgRNA1 and seven sgRNA2 mutants were identified via Sanger sequencing of PCR product, indicating mutation efficiencies of 36% and 28%, respectively. The *in vitro* rooted plantlets of all mutants were grown in the biotron growth chambers until harvest for phenotypic evaluation and sinapine analysis in the seeds. Since the five mutant lines (B2, B5, B9, B15, and B20) from sgRNA1 in T_0 were obtained first, they were chosen for further analysis. The selection of mutant lines for further evaluation in subsequent generations was based on sinapine content in comparison with WT.

For the T_1 generation, at least 5 seeds from each of the 5 T_0 mutant lines (Figure 1) were planted and grown in the biotron in order to confirm the stable inheritance of mutations. All plants were harvested, and their seeds were analysed for sinapine content

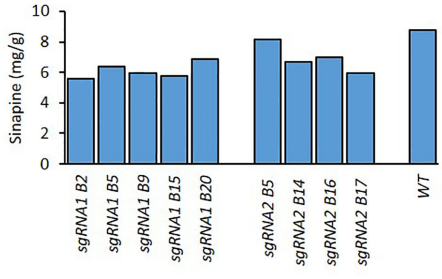

FIGURE 1
Sinapine content in the seeds of WT and *BnaX.SGT.a* mutants in the T_0 generation of rapeseed.

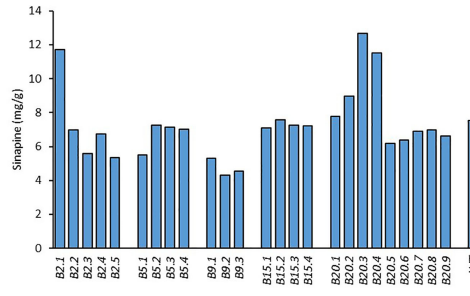

FIGURE 2
Sinapine content in the seeds of *BnaX.SGT.a* mutant lines in the T_1 generation and WT of rapeseed.

(Figure 2). The 5 T_1 mutant lines (B2.3, B2.5, B5.1, B9.2 and B9.3) with the lowest sinapine levels were selected, and 12 seeds from each line were grown in the biotron in the T_2 generation for phenotypic and genotypic analysis for individual plants.

Genotyping by amplicon sequencing

Two out of the twelve T_2 plants from each line were genotyped using amplicon sequencing to confirm the types of mutations. The results showed that all plants were mutated in both alleles of both paralogues of *BnaX.SGT.a* (double mutants), apart from B5.1.9, which maintained one wild type allele (Figure 3).

A variety of mutations were induced by the CRISPR RNP editing, ranging from 1 bp insertions, to 10 bp deletions. All of the mutations caused a frameshift, apart from that of B9.2.6 *BnaA.SGT.a* allele 2, which had an in-frame nonsense mutation (Figure 3). All mutations led to premature stop codons, disrupting the predicted active site, homodimer interface, and TDP-binding site on conserved domain GT1_Gtf-like domain of the gene.

Sinapine content

We measured the sinapine content in mature seeds of the mutants and WT in each plant of all generations. As shown in Figures 1, 2 and 4, an obvious reduction in sinapine content was detected in the majority of the lines analysed as early as the T_0 generation (Figure 1). This reduction in sinapine content persisted in T_1 (Figure 2), indicating stable inheritance of mutations. In the T_2 generation, all mutant lines were shown to have significantly lower sinapine than WT (Figures 4, 5), suggesting that homozygous lines were obtained. When comparing the efficacy of sgRNA1 and sgRNA2 in reducing sinapine content in T_0 seeds, no significant difference in sinapine content was found between the two mutation sites.

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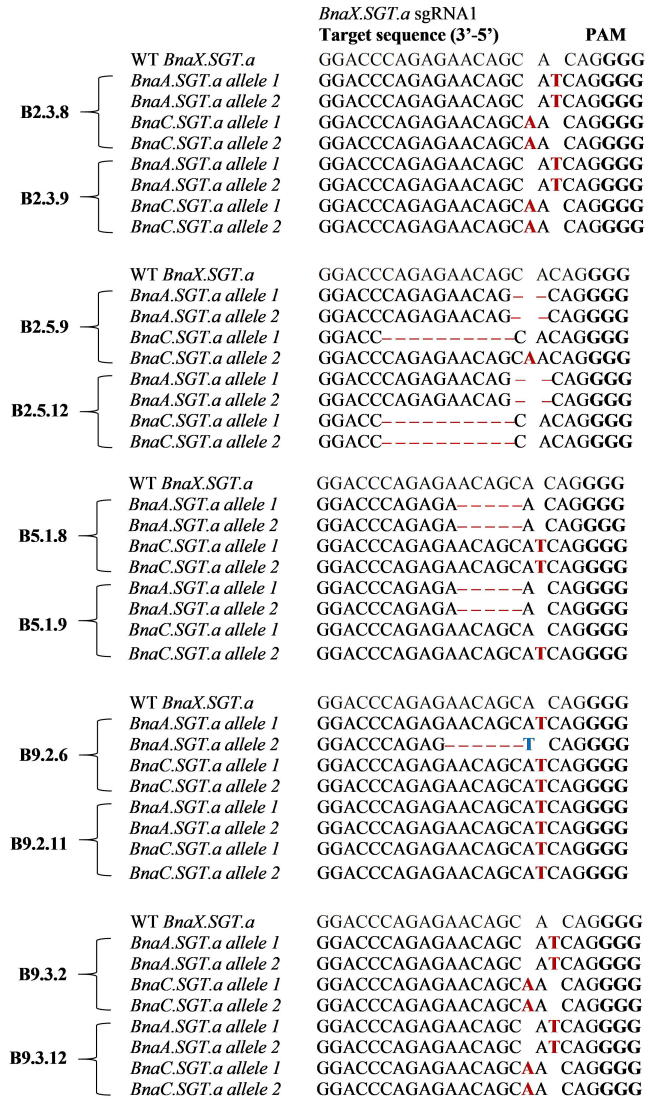

FIGURE 3
Types of mutations in the *BnaX.SGT.a* genes detected in the T_2 mutants in comparison with WT of rapeseed, determined by amplicon sequencing. Mutations are indicated by red letters (insertions), '–' (deletions), or blue letters (substitutions). PAM sites are highlighted in bold letters.

The average sinapine content across all T_2 double mutants was 5.08 mg/g, compared to 8.64 mg/g in the WT plants, reflecting a 41% reduction in the mutants. Among the T_2 double mutants, line B9.2 exhibited the lowest average sinapine content at 4.72 mg/g, corresponding to a 45% reduction (Figure 5). The individual plant with the lowest sinapine content was B2.3.9, measured at 3.92 mg/g, representing a 49% reduction (Figure 4).

Phenotypic observation

All of the homozygous T2 plants had visually normal growth and morphology (Figure 6). Fertility and flowering time did not visually differ from those of WT. The 100-seed weight result showed no significant difference between the mutant lines and WT, while the line B.2.3 showed a significantly lower seed weight than the other mutant lines (Figure 7).

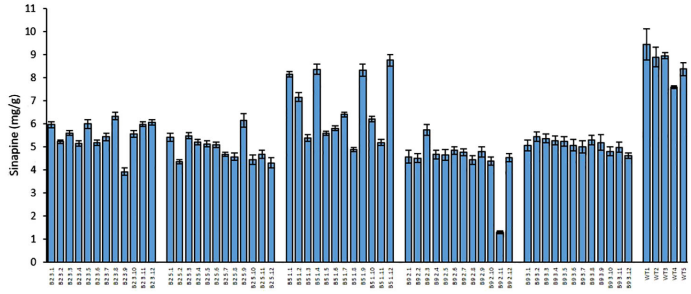

FIGURE 4
Sinapine content in the seeds of each individual plant in the T_2 generation and WT of rapeseed. Error bars represent ± SD (n=3).

Discussion

The present study demonstrated the possibility of creating transgene free, low sinapine, mutants of rapeseed by knocking out the sinapine biosynthesis gene *SGT* using a protoplast-based CRISPR RNP approach. The success of this was made possible by a highly efficient protoplast protocol for rapeseed, which was developed in our lab (Li et al., 2021). In contrast to CRISPR mutagenesis via *Agrobacterium*-transformation, this approach enables a rapid production of transgene-free mutants with improved traits without the need for backcrossing to eliminate transgenic DNA. This distinction can be significant with regard to regulatory scrutiny and public perception of crops generated using NGTs. The benefits and low risks associated with transgene-free gene editing are gaining recognition, leading to growing acceptance worldwide (Dionglay, 2024). This trend is reflected in the European Union's evolving stance regarding NGTs in plant breeding, with recent proposals aiming to relax regulations for gene-edited crops. Such regulatory adjustments signal a promising future for the broader adoption and use of NGTs in agriculture (European Parliament, 2024).

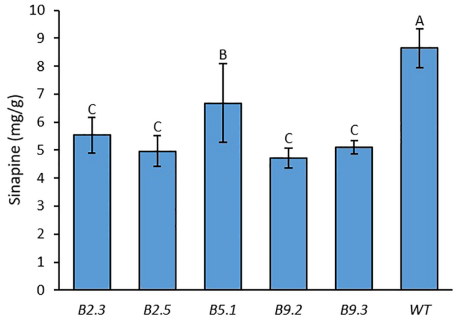

FIGURE 5
Average sinapine content of the seeds of all plants from each *BnaX.SGT.a* mutant line in the T_2 generation in comparison with WT of 4 plants of rapeseed. Different letters above the bars represent significant differences at $p<0.05$. Error bars represent ± SD.

Sequencing of *Bna.SGT* in cv. Kumily showed that four paralogues of the gene exist, as reported by Mittasch et al. (2010). *BnaX.SGT.a* is the main paralogue expressed in rapeseed, and show increased expression during seed maturation. On the other hand the paralogue *BnaX.SGT.b* is only expressed at levels similar to *BnaX.SGT.a* in flowers, but has minimal expression in other tissues (Mittasch et al., 2010). We designed sgRNAs to target *BnaX.SGT.a* due to its predominant role in sinapine accumulation in the seeds of rapeseed, as was done in other studies (Hüsken et al., 2005; Wolfram et al., 2010; Emrani et al., 2015).

In the present study, we achieved an average editing efficiency of 36% and 28% for the sgRNA1 and sgRNA2, respectively. These mutation efficiencies are clearly higher than the DNA-vector induced mutation efficiency in rapeseed, in which 18% of mutation efficiency was obtained from our earlier studies using the same protoplast regeneration method (Li et al., 2021).

We observed a relatively uniform reduction in sinapine levels across the double homozygous lines, indicating that all plants within each line carry functionally similar loss-of-function mutations. In contrast, in the B5.1 line, some individuals showed a significant reduction in sinapine levels, while others had levels comparable to those in the WT. Sequencing results confirmed that this line is segregating: individuals with no reduction in sinapine levels carried knockout mutations in three of the four *BnaX.SGT.a* alleles, with one allele remaining unmutated, while those with significantly reduced sinapine levels had nonsense mutations in all alleles. This suggests that all four *BnaX.SGT.a* alleles need to be mutated to achieve a significant reduction in sinapine content. The maintenance of the WT sinapine phenotype when only one functional allele remains can potentially be sustained through compensatory mechanisms where the remaining functional allele is upregulated via a feedback loop, or through haplosufficiency where a single functional allele is sufficient for normal sinapine synthesis.

Conventional biotechnological approaches have previously been employed to silence *SGT* gene to reduce the seed sinapine level of rapeseed. Hüsken et al. (2005) used RNA interference (RNAi) to downregulate *SGT* expression and achieved a sinapine

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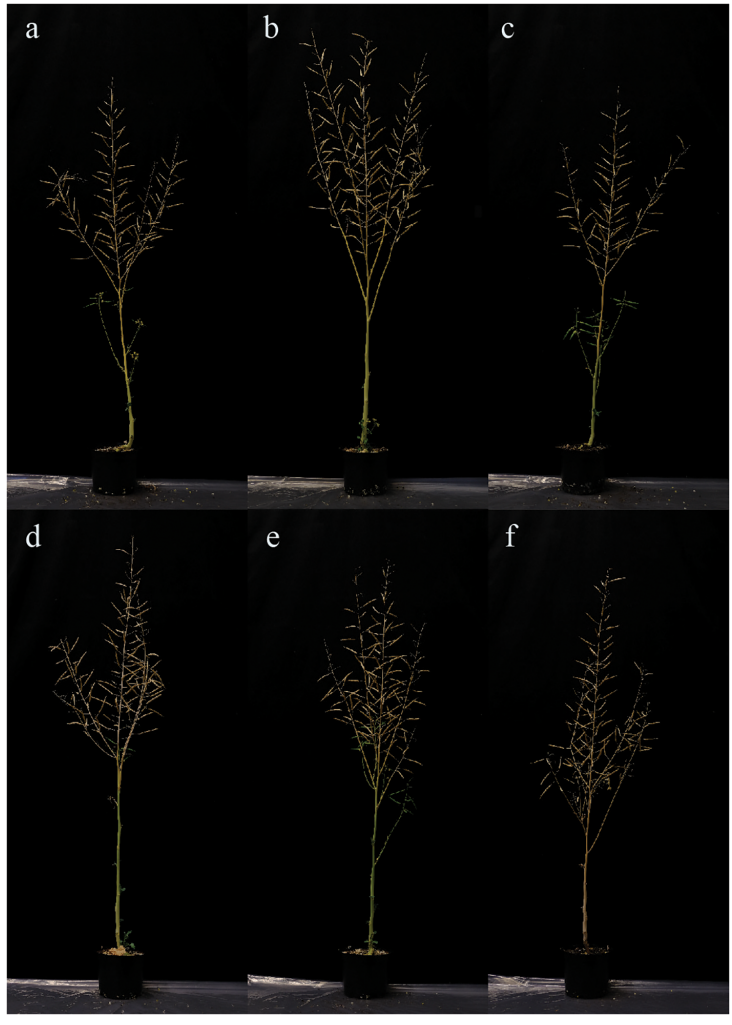

FIGURE 6
Photographs taken of *BnaX.SGT.a* mutants in the T_2 generation and WT of rapeseed at harvest: **(A)** B2.3.8, **(B)** B2.5.9, **(C)** B5.1.8, **(D)** B9.2.6, **(E)** B9.3.2, **(F)** WT.

content of 2.7 mg/g, a 72% reduction compared to WT. Emrani et al. (2015) utilized EMS mutagenesis to create *SGT* knockouts, reaching 3.3 mg/g, a 57% reduction in comparison to WT. In the present study, we attained a sinapine content of 3.9 mg/g, a 49% reduction in comparison to WT.

The mutants developed by Hüsken et al. (2005) are classified as GMOs, while those generated by Emrani et al. (2015) through TILLING exhibited unintended phenotypic effects, such as severe changes in leaf morphology, shifts in flowering time, and reduced fertility and seed production. In contrast, the CRISPR-Cas9 mutants produced in the current study via protoplast-based CRISPR RNP editing showed no adverse effects on growth or development under biotron conditions, indicating that our approach is a promising tool for efficient and precise crop improvement without transgene integration, and without unintended off-target effects.

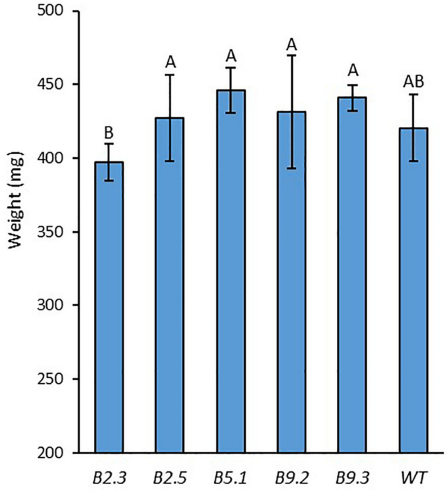

FIGURE 7
100-seed weight of *BnaX.SGT.a* mutants in the T_2 generation and WT of rapeseed. Different letters above the bars represent significant differences at $p<0.05$. Error bars represent ± SD.

In gene knockout studies, a major concern is the potential for unintended effects arising from the removing of a gene that may be involved in other biochemical pathways or affect the plant's growth and overall health. We did not visually observe any negative phenotypic changes in the *SGT* knockout lines, which is in concurrence with previous studies that have evaluated the effect of silencing *SGT* (Hüsken et al., 2005; Wolfram et al., 2010; Emrani et al., 2015; Hettwer et al., 2016). Hettwer et al. (2016) concluded that suppression of *SGT* resulted in reduced sinapine and sinapate ester accumulation, with no adverse effect on seed germination, seedling development, or response to UV-B radiation (Hettwer et al., 2016). Additionally, no negative effects were observed on key agricultural traits such as oil content, fatty acid composition, or protein content in the seeds (Hüsken et al., 2005).

In this study the CRISPR-edited mutants showed significant reduction in sinapine levels, further confirming the importance of *BnaX.SGT.a* in sinapine biosynthesis in rapeseed. However, the sinapine levels in the mutants did not reach levels as low as those reported in previous studies on *BnaX.SGT.a* (Hüsken et al., 2005; Wolfram et al., 2010; Emrani et al., 2015). This discrepancy may be attributed to mutations occurring in different positions in the gene, leading to alternative protein variants that influence sinapine biosynthesis in different ways. Another possible explanation for the lesser reduction in sinapine levels is that we screened fewer lines for sinapine content, which may have led us to overlook low-sinapine variants. Significant variability exists among different mutant lines, and prior studies have screened a larger number of mutants, potentially enabling the identification of variants with lower sinapine levels (Hüsken et al., 2005; Emrani et al., 2015).

To further reduce sinapine levels using our protoplast-based CRISPR RNP editing method, several strategies can be considered. One approach is to screen a larger number of mutants to identify those with lower sinapine content. Another approach would be to design multiple sgRNAs to simultaneously target all four *SGT* loci may be beneficial, as the less-expressed *BnaX.SGT.b* genes, which were not targeted in the current study, could be compensating for the inactivity of the more highly expressed *BnaX.SGT.a* genes.

The most promising strategy for achieving significantly lower sinapine levels may be to target more than one gene simultaneously. For instance, the highest reduction in sinapine has been achieved by concurrently silencing two genes, *FAH* and *SCT*, through RNAi, resulting in a 90% reduction in seed sinapine levels (Bhinu et al., 2009). In the present study we achieved up to a 49% reduction in sinapine content, reaching 3.9 mg/g. However, this level remains higher than the 2 mg/g target recommended as the major breeding goal (Harloff et al., 2012).

In conclusion, we successfully generated transgene-free rapeseed mutants with significantly reduced sinapine levels by knocking out *SGT* using our protoplast-based CRISPR RNP editing approach. This demonstrates the feasibility of rapidly creating new plant varieties with stable, heritable traits that remain transgene-free throughout all stages of production. The approach is notably faster and more cost-effective than conventional breeding methods. Additionally, CRISPR-generated mutants are likely to encounter less stringent regulatory oversight than conventional GMOs in the near future, providing a promising strategy for efficiently reducing sinapine and other anti-nutritional factors in crops.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

OM: Data curation, Writing – original draft. XL: Data curation, Writing – review & editing. EW: Data curation, Writing – review & editing. SK: Writing – review & editing. RG: Writing – review & editing, Data curation. EI: Writing – review & editing. LZ: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Bae, S., Park, J., and Kim, J.-S. (2014). Cas-OFFinder: A fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics* 30, 1473–1475. doi: 10.1093/bioinformatics/btu048

Bhinu, V.-S., Schäfer, U. A., Li, R., Huang, J., and Hannoufa, A. (2009). Targeted modulation of sinapine biosynthesis pathway for seed quality improvement in *Brassica napus*. *Transgenic Res.* 18, 31–44. doi: 10.1007/s11248-008-9194-3

Cheng, H., Liu, X., Xiao, Q., Zhang, F., Liu, N., Tang, L., et al. (2022). Rapeseed meal and its application in pig diet: A review. *Agriculture* 12, 849. doi: 10.3390/agriculture12060849

DeWitt, M. A., Corn, J. E., and Carroll, D. (2017). Genome editing via delivery of Cas9 ribonucleoprotein. *Methods* 121–122, 9–15. doi: 10.1016/j.ymeth.2017.04.003

Dionglay, C. (2024). "Updates on Global Regulatory Landscape for Gene-Edited Crops," in *Science Speaks*. Available at: https://www.isaaa.org/blog/entry/default.asp?BlogDate=1/24/2024. Science Speaks is an online blog by ISSSA Inco. The Philippines.

Emrani, N., Harloff, H.-J., Gudi, O., Kopisch-Obuch, F., and Jung, C. (2015). Reduction in sinapine content in rapeseed (Brassica napus L.) by induced mutations in sinapine biosyntheis genes. *Mol. Breed.* 35, 1–11. doi: 10.1007/s11032-015-0236-2

European Parliament. (2024). Texts adopted—Plants obtained by certain new genomic techniques and their food and feed—Wednesday, 7 February 2024. Available online at: https://www.europarl.europa.eu/doceo/document/TA-9-2024-0067_EN.html (Accessed October 20, 2024)

Harloff, H.-J., Lemcke, S., Mittasch, J., Frolov, A., Wu, J. G., Dreyer, F., et al. (2012). A mutation screening platform for rapeseed (Brassica napus L.) and the detection of sinapine biosynthesis mutants. *Theor. Appl. Genet.* 124, 957–969. doi: 10.1007/s00122-011-1760-z

Hettwer, K., Böttcher, C., Frolov, A., Mittasch, J., Albert, A., von Roepenack-Lahaye, E., et al. (2016). Dynamic metabolic changes in seeds and seedlings of *Brassica napus* (oilseed rape) suppressing *UGT84A9* reveal plasticity and molecular regulation of the phenylpropanoid pathway. *Phytochemistry* 124, 46–57. doi: 10.1016/j.phytochem.2016.01.014

Hüsken, A., Baumert, A., Strack, D., Becker, H. C., Möllers, C., and Milkowski, C. (2005). Reduction of Sinapate Ester Content in Transgenic Oilseed Rape (Brassica napus) by dsRNAi-based Suppression of BnSGT1 Gene Expression. *Mol. Breed.* 16, 127–138. doi: 10.1007/s11032-005-6825-8

Jacobs, C., Berglund, M., Kurnik, B., Dworak, T., Marras, S., Mereu, V., et al. (2024). *Climate change adaptation in the agriculture sector in Europe* (European Environment Agency). Available at: https://www.eea.europa.eu/publications/cc-adaptation-agriculture (Accessed October 22, 2024)

Kozlowska, H., Naczk, M., Shahidi, F., and Zadernowski, R. (1990). "Phenolic acids and tannins in rapeseed and canola," in *Canola and Rapeseed: Production, Chemistry, Nutrition and Processing Technology*. Ed. F. Shahidi (Boston, MA, US: Springer), 193–210. doi: 10.1007/978-1-4615-3912-4_11

Li, X., Sandgrind, S., Moss, O., Guan, R., Ivarson, E., Wang, E. S., et al. (2021). Efficient protoplast regeneration protocol and CRISPR/Cas9-mediated editing of glucosinolate transporter (GTR) genes in rapeseed (Brassica napus L.). *Front. Plant Sci.* 12. doi: 10.3389/fpls.2021.680859

May, D., Paldi, K., and Altpeter, F. (2023). Targeted mutagenesis with sequence-specific nucleases for accelerated improvement of polyploid crops: Progress, challenges, and prospects. *Plant Genome* 16, e20298. doi: 10.1002/tpg2.20298

Milkowski, C., Baumert, A., Schmidt, D., Nehlin, L., and Strack, D. (2004). Molecular regulation of sinapate ester metabolism in Brassica napus: Expression of genes, properties of the encoded proteins and correlation of enzyme activities with metabolite accumulation. *Plant Journal: For Cell Mol. Biol.* 38, 80–92. doi: 10.1111/j.1365-313X.2004.02036.x

Mittasch, J., Mikolajewski, S., Breuer, F., Strack, D., and Milkowski, C. (2010). Genomic microstructure and differential expression of the genes encoding UDP-glucose:sinapate glucosyltransferase (UGT84A9) in oilseed rape (Brassica napus). *Theor. Appl. Genet.* 120, 1485–1500. doi: 10.1007/s00122-010-1270-4

Qiao, H. Y., Dahiya, J. P., and Classen, H. L. (2008). Nutritional and physiological effects of dietary sinapic acid (4-hydroxy-3,5-dimethoxy-cinnamic acid) in broiler chickens and its metabolism in the digestive tract. *Poultry Sci.* 87, 719–726. doi: 10.3382/ps.2007-00357

Shahbandeh, M. (2024). Vegetable oils consumption worldwide 2023/24. Available online at: https://www.statista.com/statistics/263937/vegetable-oils-global-consumption/.

Sheahan, J. J. (1996). Sinapate Esters Provide Greater UV-B Attenuation than Flavonoids in Arabidopsis thaliana (Brassicaceae). *Am. J. Bot.* 83, 679–686. doi: 10.2307/2445845

Voss-Fels, K. P., Stahl, A., and Hickey, L. T. (2019). Q&A: Modern crop breeding for future food security. *BMC Biol.* 17, 18. doi: 10.1186/s12915-019-0638-4

Wang, S. X., Oomah, B. D., and McGregor, D. I. (1998). Application and evaluation of ion-exchange UV spectrophotometric method for determination of sinapine in *brassica* seeds and meals. *J. Agric. Food Chem.* 46, 575–579. doi: 10.1021/jf9705577

Ward, A. K., Classen, H. L., and Buchanan, F. C. (2009). Fishy-egg tainting is recessively inherited when brown-shelled layers are fed canola meal. *Poultry Sci.* 88, 714–721. doi: 10.3382/ps.2008-00430

Wolfram, K., Schmidt, J., Wray, V., Milkowski, C., Schliemann, W., and Strack, D. (2010). Profiling of phenylpropanoids in transgenic low-sinapine oilseed rape (*Brassica napus*). *Phytochemistry* 71, 1076–1084. doi: 10.1016/j.phytochem.2010.04.007

Yates, K., Pohl, F., Busch, M., Mozer, A., Watters, L., Shiryaev, A., et al. (2019). Determination of sinapine in rapeseed pomace extract: Its antioxidant and acetylcholinesterase inhibition properties. *Food Chem.* 276, 768–775. doi: 10.1016/j.foodchem.2018.10.045

Zhang, Y., Iaffaldano, B., and Qi, Y. (2021). CRISPR ribonucleoprotein-mediated genetic engineering in plants. *Plant Commun.* 2, 100168. doi: 10.1016/j.xplc.2021.100168

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Antinutritional factors such as glucosinolates, sinapine, and phytic acid limit the use of rapeseed meal as feed. We developed a protocol for the isolation and regeneration of rapeseed protoplasts and used CRISPR-Cas gene editing to target genes involved in the biosynthesis and transport of these compounds. This resulted in transgene-free mutants with reduced levels of all targeted antinutritional factors, providing a strong foundation for the improvement of rapeseed meal for use as high-quality feed.

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