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Genome editing of oilseed species by CRISPR/Cas9 for trait improvement

Sjur Sandgrind

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Sjur Sandgrind
Faculty of Landscape Architecture, Horticulture and Crop Production Science
Department of Plant Breeding
Alnarp

SLU
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Cover: Rapeseed flowers, rapeseed seed cake, and field cress flowers
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Abstract

The demand for vegetable oils for food and other applications is rapidly increasing due to a growing population and an urgent need to replace fossil oils. Furthermore, there are several challenges facing agricultural production, such as a shortage of arable land, climate change, and water pollution due to nutrient leaching from extensive use of fertilizers and tillage. More sustainable agricultural systems, better use of side-streams, domestication of novel oil crops, and improvement of existing oil crops are important for global food security and the supply of raw materials for different industries. In Sweden, rapeseed is the main oil crop, but is mainly cultivated in the southern region of Sweden due to its weak cold tolerance. The seed cake is rich in protein, but is underutilized due to high levels of anti-nutritional compounds such as glucosinolates. Field cress (*Lepidium campestre*) is a novel cold-hardy and high-yielding oilseed crop that can be undersown with a spring cereal in cool climates as a cover crop for reducing nutrient leaching and soil erosion, while producing a valuable seed oil and improving the cereal yield. Development of the CRISPR/Cas9 genome editing system has revolutionized the field of biology and led to major advances in plant science. This novel breeding tool offers great opportunities for accelerated trait improvement of crops with high precision. In this thesis, we have developed efficient protocols for protoplast transfection and regeneration of field cress and rapeseed, and demonstrated their usefulness in genome editing via CRISPR/Cas9 by mutating multiple glucosinolate transporter genes in rapeseed and field cress for reducing seed glucosinolate contents, and by mutating key genes for improving the oil quality of field cress.

Keywords: CRISPR/Cas9, domestication, genome editing, glucosinolate, *Lepidium campestre*, oil quality, protoplast, rapeseed, seed cake.

Author's address: Sjur Sandgrind, Swedish University of Agricultural Sciences, Department of Plant Breeding, Alnarp, Sweden

Genomredigering av oljefröarter via CRISPR/Cas9 för förbättring av egenskaper

Abstrakt

Efterfrågan på vegetabiliska oljor för livsmedel och andra industriella tillämpningar ökar snabbt på grund av en växande befolkning och ett akut behov för att ersätta fossila oljor. Dessutom finns det flera utmaningar för jordbruket, såsom brist på odlingsbar mark, klimatförändring och vattenföroreningar på grund av näringsläckage från omfattande användning av konstgödsel och jordbearbetning. Mer hållbara jordbrukssystem, bättre användning av sidoströmmar, domesticering av nya oljegrödor och förbättring av befintliga oljegrödor är viktiga för den globala livsmedelssäkerheten och tillgången på råvaror för transport och industri. I Sverige är raps den viktigaste oljegrödan, men odlas främst i södra Sverige på grund av dess svaga köldtolerans. Frökakan är rik på protein, men är underutnyttjad på grund av höga halter av antinutrienter, t.ex. glukosinolater. Fältkrassing (*Lepidium campestre*) är en ny köldhärdig och högavkastande oljeväxt som kan undersås med en vårsäd i svala klimat som en täckgröda för att minska näringsläckage och jorderosion, medan den producerar en värdefull olja och förbättrar spannmålsavkastningen. Utvecklingen av genomredigeringssystemet CRISPR/Cas9 har revolutionerat biologiområdet och lett till stora framsteg i växtvetenskapen. Detta nya förädlingsverktyg erbjuder stora möjligheter för accelererad förbättring av egenskaper hos växtarter med hög precision. I den här avhandlingen har vi utvecklat effektiva protokoll för protoplasttransfektion och regenerering av fältkrassing och raps, och visat deras användbarhet i genomredigering via CRISPR/Cas9 genom att mutera glukosinolat transportörgener i raps och fältkrassing för att reducera fröglukosinolathalter och att mutera gener för att förbättra oljekvalitet i fältkrassing.

Nyckelord: *Lepidium campestre*, raps, CRISPR/Cas9, domesticering, oljekvalitet, protoplast, glukosinolat, frökaka, fältkrassing.

Författarens adress: Sjur Sandgrind, Sveriges lantbruksuniversitet, Institutionen för växtförädling, Alnarp, Sverige

Dedication

For Sondre. You would have loved this!

There's a point, around the age of twenty, when you have to choose whether to be like everybody else the rest of your life, or to make a virtue of your peculiarities.

Ursula K. Le Guin

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., Kanagarajan, S. and Zhu, L-H. (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 (1303).

II. Sandgrind, S.†, Li, X.†, Ivarson, E., Ahlman, A. and Zhu, L-H. (2021). Establishment of an Efficient Protoplast Regeneration and Transfection Protocol for Field Cress (*Lepidium campestre*). Frontiers in Genome Editing, 3 (32).

III. Sandgrind, S., Li, X., Ivarson, E., Wang, E.S., Guan, R., Kanagarajan, S. and Zhu, L-H. Improved Fatty Acid Composition of Field Cress (*Lepidium campestre*) by CRISPR/Cas9-Mediated Genome Editing. Frontiers in Plant Science (under review)

IV. Sandgrind, S., Li, X., Ivarson, E., Wang, E.S., Guan, R., Kanagarajan, S. and Zhu, L-H. Knockout of Transporter Genes Resulted in Significant Reduction of the Glucosinolate Content in the Seeds of Field Cress (*Lepidium campestre*). (manuscript)

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†These authors share first authorship

The contribution of Sjur Sandgrind 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. Performed some of the tissue culture work together with XL. Designed and performed the protoplast transfections. Wrote the manuscript together with co-authors.

III. Designed the studies together with L-HZ and XL. Cloned the genes. Designed and generated the CRISPR/Cas9 vectors. Performed the protoplast transfections, mutant screening, and FA analysis. Wrote the manuscript together with co-authors.

IV. Designed the studies together with SK and L-HZ. Performed the protoplast transfections and mutant screening. Performed the glucosinolate analysis together with ESW. Wrote the manuscript together with co-authors.

1. Introduction

1.1 Importance of vegetable oils for food and other applications

Vegetable oils are the most energy-dense plant reserves and the second most important human food group after cereals, accounting for about 300 kcal/person/day (Bates et al., 2013; FAOSTAT, 2019b). As they are composed of long-chain hydrocarbons, they are also used as substitutes for fossil oils as fuel and feedstocks for various industrial applications. Production of oil crops has been the fastest growing subsector of global agriculture, and vegetable oil consumption is expected to increase even further in the future (Alexandratos and Bruinsma, 2012). The global production of vegetable oils was approximately 204 million metric tons in 2019, and the major sources were oil palm (36%), soybean (28%), rapeseed (12%), and sunflower (10%) (Figure 1). In addition to its importance as a food source, vegetable oils are also used for fuel and important feedstocks for a variety of products such as plastics, paints, detergents, soaps, surfactants, lubricants, and cosmetics. A valuable by-product of vegetable oil production is the seed cake (seed meal or press cake), residues that remain after oil extraction. The seed cake is typically rich in protein, fiber, and other nutrients. However, in Brassicaceae species, the nutritional value of the seed cake can be impaired by the presence of anti-nutritional compounds such as glucosinolates (GSLs), phytates, phenols, and sinapine (Bell, 1993; Wittkop et al., 2009). The protein content ranges from 15% to 50%, depending on species, growth conditions, and extraction method (Ramachandran et al., 2007). The seed cake is predominantly used as a protein source in animal feed, but the maximum recommended inclusion rate is limited by the content

of anti-nutritional compounds (Bell, 1993; Choi et al., 2015; Arefaine et al., 2019). The potential to use the seed cake for food purposes has also been explored (Fetzer et al., 2018; Kotecka-Majchrzak et al., 2020; Östbring et al., 2020; Singh et al., 2022).

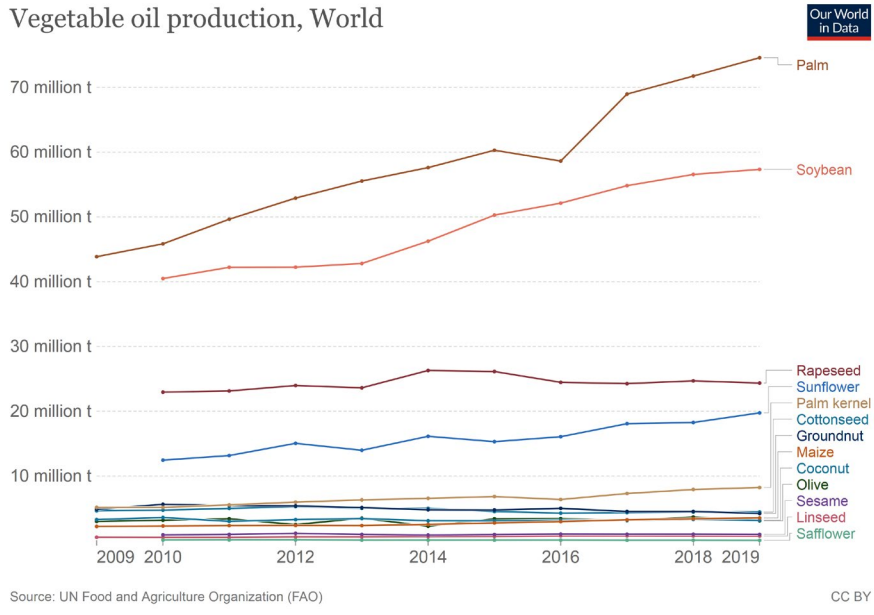

Figure 1. Global vegetable oil production. Data from FAOSTAT (2019a).

The rapidly increasing demand for vegetable oils for food, feed, fuel, and industrial applications, is driven by a growing global population and a desire to shift away from fossil oils. The global human population is expected to approach 8.5 billion people in 2030 and reach approximately 10.4 billion in the 2080s (United Nations, 2022). Unfortunately, yield trends are far below what are needed to meet the projected demands. Moreover, much of the food production base currently in use is showing signs of degradation, and large scale conversion of land reserves into cultivated land is problematic due to the risk of exacerbating climate change and causing harm to important ecosystems (Ray et al., 2013; Schmidt-Traub et al., 2019; Voss-Fels et al., 2019; Zheng et al., 2022). As shown in Figure 2, the global arable land is projected to only increase by approximately 5% from 2014 to 2050 (Alexandratos and Bruinsma, 2012). Furthermore, climate change can have

devastating impacts on global food security, depending on the degree of global warming (Wheeler and von Braun, 2013; Voss-Fels et al., 2019). There is overwhelming evidence that the emission of greenhouse gasses from the burning of fossil oils is a major driver of climate change. As vegetable oil is a renewable source of hydrocarbons, it has been proposed that fossil oils should be substituted with renewable vegetable oils to reduce the potentially catastrophic effects of climate change (IPCC, 2011). The biodiesel industry alone consumes about 15% of the vegetable oils produced (OECD, 2022), and the use of vegetable oils for industrial applications is expected to increase further as fossil oils are phased out. However, this could be a driver for deforestation and re-direct edible vegetable oils and other edible crops away from food use and towards fueling industries and transportation, thus potentially further straining global food security. The combination of various strategies, such as developing new high-yielding crops that can be included in sustainable and environmentally friendly cropping systems, general improvement and tailoring of existing crops for specific uses, and better utilization of side streams are critical for global food security and sustainable development of agriculture.

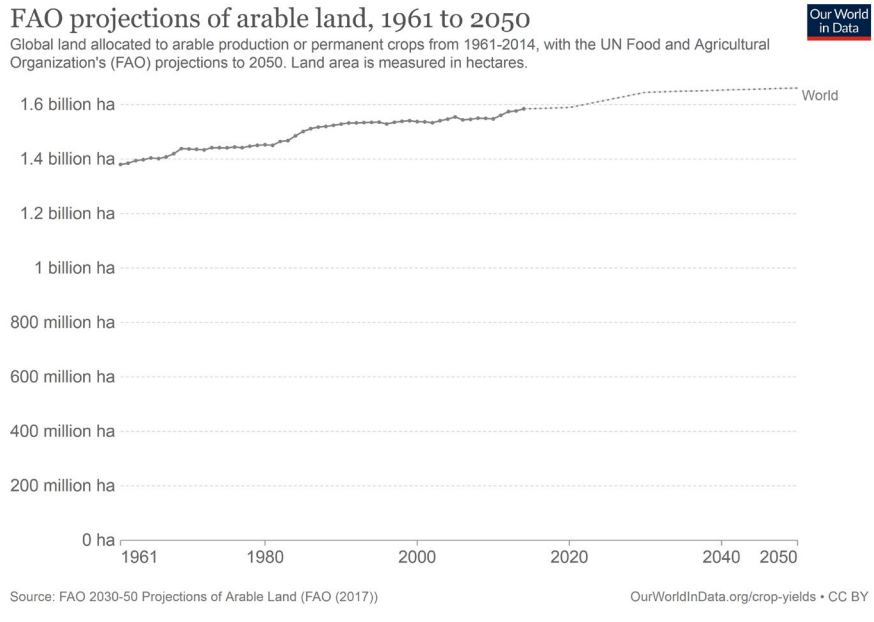

Figure 2. Projections for global land allocated to agriculture. Data from Alexandratos and Bruinsma (2012) and FAOSTAT (2017).

1.2 Oilseed crops

1.2.1 Field cress (*Lepidium campestre*) – a novel oilseed crop

Modern civilization depends on only a few domesticated crop species of the more than 300 000 land plant species that are known, and these crop species have in most cases been domesticated over thousands of years. (Olsen and Wendel, 2013). The reliance on a few crops renders the global food supply vulnerable to the impact of climate change and biotic and abiotic stresses, which pose additional challenges to scaling food production to meet the rapid growth in demand. Increasing agrobiodiversity by domesticating new species can improve the robustness of the global food supply, potentially improve the utilization of marginal lands, increase land productivity, improve the sustainability of cropping systems, and lead to novel uses for plant-based products (Thrupp, 2000; Mayes et al., 2011; Ortiz et al., 2020).

The domestication of plants was initiated about 10 000 years ago when human societies began to transition from hunting and gathering to agriculture (Doebley et al., 2006). This transition was made possible by the gradual domestication of wild plants over time by intentional and unintentional human selection. A specific suite of traits termed "domestication syndrome" traits are typically selected for at the early stages of domestication (Harlan et al., 1973; Sedbrook et al., 2014). These domestication syndrome traits include larger fruits or seeds, loss of seed dormancy, loss of natural seed dispersal, and more determinate growth. In many cases, modern food crops are remarkably distinguished from their wild ancestors and so completely dependent on humans that they are no longer capable of propagating themselves in nature (Doebley et al., 2006). However, the domestication of plants has been a very slow, labor-intensive, and inefficient process where breeders have relied on phenotypes for crop improvement, which are influenced by environmental factors and management. The use of modern breeding tools, such as molecular markers, enables selection on genotypes, which has resulted in improved breeding efficiency and shortened breeding cycles, as plants with desirable traits can be preselected before the traits are expressed (Geleta et al., 2020).

With the goal of domesticating a novel oilseed species as a cover crop for the Nordic climate, researchers at the Swedish University of Agricultural Sciences started to domesticate field cress in the 1980s. A large number of accessions of mainly wild cruciferous species were collected and evaluated, focusing on plant type, oil quality, and cold tolerance (Eriksson, 2009). Several species from the *Lepidium* and *Barbarea* genera were selected for field trials, and the nutritional and technological qualities of the seeds were evaluated. Based on the agronomical performance and the seed oil composition, field cress (*Lepidium campestre*) was eventually selected for further domestication efforts (Merker and Nilsson, 1995; Nilsson et al., 1998; Andersson et al., 1999).

Field cress is a wild biennial oilseed species that belongs to the Brassicaceae family. It has several good agronomic traits, such as excellent cold tolerance, high seed yield potential, suitable seed size, branching only in the upper part of upright stems, relatively synchronous flowering, and resistance to the pollen beetle (Merker and Nilsson, 1995; Merker et al., 2010). It is a self-fertilizing diploid species ($2n = 2x = 16$), which enables straightforward breeding of homozygosity in traits of interest. Furthermore, it is a relative of the model species *Arabidopsis thaliana*, which simplifies gene discovery and molecular research, such as gene cloning and editing, even without any available genome sequence database. Due to its cold tolerance, it can be grown in regions such as northern Scandinavia where rapeseed cannot be cultivated, thus considerably expanding the potential planting region for high-yielding oilseed crops.

Undersowing field cress with a spring cereal has been shown to have a positive effect on the yield of the cereal, while simultaneously functioning as a cover crop that produces a high seed yield in the following spring (Merker et al., 2010). This cropping system could enable vegetable oil production on land already used for cereal production with beneficial effects, such as an increase in cereal yield, and reduced nutrient leaching and soil erosion (Ulen and Aronsson, 2018). As a wild species, field cress has some important agronomic traits that need to be improved before it can be considered an economically viable oil crop. In the past 30 years, some important traits, such as pod shatter, fatty acid (FA) profile, seed yield etc., have been improved with the help of modern breeding tools, including gene technology, considerably accelerating the domestication process of the

species (Ivarson et al., 2016; Ivarson et al., 2017; Gustafsson et al., 2018; Geleta et al., 2020).

1.2.2 Rapeseed (*Brassica napus*) – a major oilseed crop

Rapeseed (*Brassica napus*) is a major oilseed allopolyploid ($2n = 4x = 38$, AACC) crop, derived from interspecific crosses between *Brassica rapa* ($2n = 2x = 20$, AA) and *Brassica oleracea* ($2n = 2x = 18$, CC) approximately 1900 – 7200 years ago (Lu et al., 2019). Rapeseed cultivation began in Europe during the Middle Ages and thereafter spread worldwide (Chalhoub et al., 2016). Rapeseed is the third most important oil crop worldwide and the dominating oil crop in Sweden (FAOSTAT, 2019a; Jordbruksverket, 2021). Due to its relatively weak cold tolerance, it is mainly cultivated in the southern region of Sweden.

Throughout history, rapeseed oil has been mainly used as a lamp oil, for lubrication, and soap production, although it has also been used for margarine production to some extent (Appelquist, 1972). The large-scale use of rapeseed oil for foodstuffs is a relatively new invention, as the oil was high in the unpalatable and potentially toxic compounds erucic acid (C22:1) and glucosinolates (GSLs) (Juska et al., 1997). In the 1970s, high-yielding uniform cultivars low in erucic acid and GSLs were developed. The edible oil was marketed as 'Canola' ("Can" from Canada, and "OLA" meaning "oil, low (erucic) acid") to great success, and the production quadrupled in approximately 20 years (Juska et al., 1997). Yield gains and production have however stagnated in recent years (Zheng et al., 2022). Canola was originally a registered trademark name of the Rapeseed Association of Canada but is now used as a generic term for edible rapeseed oil (Mag, 1983; Lin et al., 2013). By definition, canola is high in oleic acid (C18:1) and low in erucic acid (<2%) and GSLs (<30 μmol/g) (Mag, 1983), thus being considered as a healthy oil for human consumption (Lin et al., 2013; Kruse et al., 2015).

1.3 Fatty acid composition in seed oil of oil crops

Triacylglycerols (TAGs) are the principal components of vegetable oils. They are composed of three fatty acids (FAs) esterified to a glycerol backbone. In most oil crops the dominating FAs are 16- and 18-carbon FAs such as palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3), but some species and

cultivars accumulate high proportions of more unusual FAs (Thelen and Ohlrogge, 2002). Seed oil from Brassicaceae species, such as rapeseed and field cress, commonly contain high levels of the monounsaturated very long-chain fatty acid (VLCFA) erucic acid (C22:1) (Guan et al., 2014). The FA profile of different vegetable oils determines their nutritional quality and usefulness for non-food applications. C18:1 is a highly desirable FA for food, fuel, and other chemical industrial applications. It is considered a healthy oil (Lin et al., 2013; Kruse et al., 2015), and due to its high oxidative stability and thermal properties it is suitable for applications such as frying and biodiesel. It can also be converted to derivatives for industrial feedstocks. C18:2 and C18:3 are essential polyunsaturated fatty acids (PUFAs) for the human diet, but are prone to oxidation and have low melting points, making them unsuitable for frying and biodiesel (Durrett et al., 2008; Li et al., 2016; Singh et al., 2019). They are also a source of unhealthy trans-FAs from the processing of many foodstuffs (Wallis et al., 2002; Steinhart et al., 2003). C22:1 is considered toxic, and the content may not exceed 2% in foodstuffs in the European Union (Chain et al., 2016; European Union Commission Regulation, 2019), but it is a valuable industrial FA (Guan et al., 2014).

By modifying the oil composition *in planta* through molecular breeding, it is possible to increase the nutritional value or tailor the oil qualities for other specific purposes, thus increasing the overall value of the oil and reducing the potential need for further processing. To modify the FA composition, it is necessary to understand the complex molecular mechanisms of oil biosynthesis (Napier and Graham, 2010). In most oilseed species, the biosynthesis of the main FAs, C16:0, C18:0, and C18:1, starts with the synthesis of the respective acetyl-CoAs in plastids, which are then exported into the cytosol for further modification and TAG assembly in the endoplasmic reticulum (ER) (Browse and Somerville, 1991; Bates et al., 2013). Elongation of C18:1 to VLCFAs such as C20:1 and C22:1, is catalyzed by the elongase FATTY ACID ELONGASE1 (FAE1) (Millar and Kunst, 1997). TAGs are mainly synthesized via the Kennedy pathway, in which most enzymatic steps have been characterized (Kennedy, 1961; Bates, 2016). Diacylglycerol (DAG) is the final precursor of TAG and also a precursor of phosphatidylcholine (PC) and other phospholipids (Bates, 2016). Conversion of C18:1 to C18:2 is catalyzed by FATTY ACID DESATURASE2 (FAD2) (Okuley et al., 1994), which can be further desaturated to C18:3 by FATTY ACID DESATURASE3 (FAD3) (Arondel

et al., 1992). However, before desaturation, C18:1 must be incorporated into phosphatidylcholine (PC), the only substrate recognized by FAD2 and FAD3 (Lu et al., 2009). Multiple enzymes can incorporate FAs into PC. Notably, PHOSPHATIDYLCHOLINE DIACYLGLYCEROL CHOLINE-PHOSPHOTRANSFERASE (PDCT), encoded by the gene *REDUCED OLEATE DESATURATION1* (*ROD1*), is important for the transfer of C18:1 into PC, and also for the reverse transfer of C18:2 and C18:3 into the TAG synthesis pathway (Lu et al., 2009).

Because C18:1 is desirable for a variety of applications, breeding lines that are high in C18:1 is one of the major breeding goals in oil crops. As it is a substrate for VLCFAs and PUFAs, reducing the content of VLCFAs and PUFAs can result in proportionally higher contents of C18:1 (Ivarson et al., 2016; Li et al., 2016; Okuzaki et al., 2018; Do et al., 2019; Jarvis et al., 2021).

1.4 Seed cake – its nutritional value and anti-nutritional concerns

The rapeseed cake is a by-product of oil extraction. It is predominantly used as a protein source in animal feed, but also as plant fertilizer or compost, and in some processed food products (Kotecka-Majchrzak et al., 2020; Singh et al., 2022). It can contain up to 40% protein (dry weight after oil extraction) and has a favorable amino acid composition (Guo et al., 2010; Liu et al., 2020). Furthermore, it is rich in fiber, carbohydrates, and minerals such as Ca, Mg, and P, and contains vitamins B4 and E (Bell, 1984; Wittkop et al., 2009). However, it also contains high amounts of anti-nutritional compounds such as phenolics, phytate, sinapine, and GSLs (Huang et al., 2008; Arefaine et al., 2019; Singh et al., 2022). One of the main concerns has been the GSL content, as GSL intoxication from consumption of rapeseed cake has been described in all major farm animals (Alexander et al., 2008). Even though modern rapeseed cultivars are very low in GSLs, they are more concentrated in the seed cake, making it unpalatable or even toxic at high levels (Fauduet et al., 1995). For ruminants, the maximum suggested inclusion rates of rapeseed cake in diets is 25%, while for non-ruminants such as pigs, a maximum of 10% inclusion is recommended (Alexander et al., 2008).

As a wild species, field cress has a GSL content that is much higher than modern rapeseed cultivars, but the types of GSLs are entirely different from rapeseed. It also has a good amino acid composition, but a very high dietary

fiber content that can adversely affect digestibility (Andersson et al., 1999; Arefaine et al., 2019). Due to the high GSL content, only 1.6% of the diet for pigs could consist of field cress cake based on the rapeseed GSL guidelines. However, a recent small-scale feeding trial found that the inclusion of up to 12% of field cress cake was palatable for grower pigs, and did not seem to cause any detrimental health effects, indicating that the field cress cake might be more palatable and less toxic than rapeseed cake (Arefaine et al., 2019).

The use of rapeseed protein as human food has been limited to niche applications like flavorings and so-called functional foods (Guo et al., 2010; Kotecka-Majchrzak et al., 2020). However, it could be used in the rapidly growing plant-based meat substitute sector. Plant-based meat products have become increasingly popular during the last decade, especially in Western countries, primarily due to health and environmental concerns (Zhao et al., 2022). Even though Europe is the largest market, the majority of products are made from imported soy, and a much smaller fraction from peas or mushrooms (Zahari et al., 2020). New advances in food processing technologies have been reported to remove anti-nutritional compounds from rapeseed cake, but the processing is costly and the technology is not fully mature (Östbring et al., 2020). If the anti-nutritional compounds in the Brassicaceae seed cakes could be eliminated or substantially reduced by breeding, the improved seed cakes could be incorporated into novel highly nutritious plant-based meat substitute products, thus greatly increasing the value of the seed cake.

1.5 Glucosinolates in oilseed species

GSLs are important plant defense compounds found in most Brassicaceae species, including field cress and rapeseed. They are a diverse and well-studied class of amino acid-derived secondary metabolites (Andersson et al., 1999; Halkier and Gershenzon, 2006). The basic structure of all GSLs consists of a β-thioglucose moiety, a sulfonated oxime moiety, and a variable side chain (Hopkins et al., 2009). Depending on the amino acid precursor of the side chain, most GSLs can be designated to one of the following three major structural groups: (i) indole GSLs derived from tryptophan, (ii) aliphatic GSLs derived from methionine, and (iii) aromatic GSLs derived from phenylalanine or tyrosine. Further structural variation is achieved by oxidation, elongation, or hydroxylation of the side chain (Hopkins et al.,

2009). Plants that accumulate GSLs also contain myrosinases, which are sequestered in vacuoles and thus spatially separated from the GSLs. Upon tissue damage, the myrosinases are released, enzymatic hydrolysis of GSLs is triggered, and herbivore-deterrent catabolites are released (Alexander et al., 2008; Hunziker et al., 2021). GSLs and myrosinases are found in all plant organs at all developmental stages, but vary considerably in concentration (Hopkins et al., 2009). The biosynthesis of GSLs occurs mainly in vegetative tissues, predominantly in leaves, and are translocated via the phloem and xylem to seeds and other sink tissues (Nour-Eldin and Halkier, 2009; Andersen et al., 2013). The highest concentrations are found in tissues decisive for fitness such as reproductive tissues and young leaves (Hopkins et al., 2009; Nour-Eldin et al., 2017; Hunziker et al., 2021; Gershenzon and Ullah, 2022).

GSLs play an important role in plant defense, while their presence in seeds adversely affects the values of the seed oil and seed cake, as they can be toxic and unpalatable for most animals at high levels (Bell, 1984; Fauduet et al., 1995; Alexander et al., 2008; Wittkop et al., 2009). Data on the toxicity of individual GSLs is limited. Still, adverse effects from the ingestion of GSLs described in farm animals include growth retardation, impaired reproductive activity, and impairment of liver and kidney functions (Alexander et al., 2008). Even though modern cultivars of established oilseed crops such as rapeseed contain relatively low amounts of GSLs, the high concentrations found in the seed cake need to be further reduced or eliminated by breeding to better utilize the high-quality protein in the seed cake for feed and food uses (Fauduet et al., 1995; Wittkop et al., 2009). For domestication of novel Brassicaceae oilseed crops such as field cress and pennycress (*Thlaspi arvense*) for food and other applications, reduction in GSL levels is one of the major breeding goals (Andersson et al., 1999; Sedbrook et al., 2014).

Due to its importance in plant defense and the complexity of biosynthesis, molecular strategies for reducing seed GSL content have been mainly focused on targeting GSL transport pathways (Nour-Eldin et al., 2012; Nour-Eldin et al., 2017; Nambiar et al., 2021; Hölzl et al., 2022). The GSL transporter genes *GLUCOSINOLATE TRANSPORTER-1* (*GTR1*) and *GLUCOSINOLATE TRANSPORTER-2* (*GTR2*) from the nitrate/peptide (NTR/PTR) transporter family are essential for GSL transport in several Brassicaceae species, and disruption or down-regulation of these genes

resulted in lines with no or reduced concentration of seed GSLs (Nour-Eldin et al., 2012; Nour-Eldin et al., 2017; Nambiar et al., 2021; Hölzl et al., 2022). Other genes that have been targeted for successful generation of lines that accumulate low amounts of GSLs in seeds are the transcription factors *MYB28* and *MYB29*, which regulate aliphatic GSL biosynthesis (Augustine et al., 2013; Nour-Eldin et al., 2017; Nambiar et al., 2021; Hölzl et al., 2022). In *A. thaliana*, disruption of *GTR1* and *GTR2* was accompanied by a seed yield and seed weight penalty (Nour-Eldin et al., 2012), but not in *Brassica juncea*, *B. rapa* (Nour-Eldin et al., 2017), or *Camelina sativa* (Hölzl et al., 2022). In the *MYB28* and *MYB29* knockout and RNAi lines, no adverse effects were observed, but aliphatic GSLs were reduced or eliminated in the entire plant, which could affect plant defense. As the aromatic sinalbin is the dominant type of GSL in field cress (Andersson et al., 1999), it is improbable that knockout of *MYB28* and/or *MYB29* would affect the GSL content in the same manner in field cress as in other Brassicaceae species.

The mechanism behind the different responses to the disruption of *GTR1* and *GTR2* in different species remains unclear, but could possibly be attributed to differences in GSL composition and transporter affinity, or differences in hormone transport activity among species. In *A. thaliana*, it has been shown that *GTR* genes are multifunctional and possibly involved in the transport of plant hormones such as jasmonic acid and gibberellin (Saito et al., 2015). It is possible that the *GTR* genes are more important for hormone transport in field cress than in other Brassicaceae species. Furthermore, in *A. thaliana*, perturbation of GSL levels has been shown to potentially affect proteins and metabolites involved in oxidative stress, photosynthesis, hormone metabolism, circadian rhythm regulation, and a variety of abiotic stress response-related genes (Chen et al., 2012; Martinez-Ballesta et al., 2015). As such, careful evaluation of multiple aspects of the agronomic performance of low GSL lines are important.

1.6 Genome editing by CRISPR/Cas – an essential tool for accelerated precision breeding

Traditional plant breeding methods are normally time- and labor-intensive, which is one of the major bottlenecks of plant breeding. Due to the protracted breeding process, it can take decades of crossing, selection, and testing to release a superior cultivar (Voss-Fels et al., 2019). The use of shuttle

breeding, popularized by Norman Borlaug and others, has halved the breeding time in some crops (Borlaug, 2007; Ortiz et al., 2007). Application of traditional biotechnological tools, such as tissue culture to produce doubled-haploids and embryo rescue, has further reduced the breeding process in some species (Bridgen, 1994; Sharma et al., 1996; Maluszynski et al., 2003). More recently, "speed breeding" by extending the photoperiod, optimizing light quality, and application of hormones to achieve rapid plant growth and early flowering have also reduced the breeding cycle time (Ghosh et al., 2018; Li et al., 2018; Watson et al., 2018).

The rapid development in molecular biology and gene technology has made direct genetic modification of elite cultivars, or creating new genetic resources possible, providing a shortcut for direct trait improvement and domestication of new crops. Different transgenic approaches have been developed for many plant species, including rapeseed and field cress. Genetically modified (GM) rapeseed was the first herbicide-tolerant crop commercially released in 1995 (Kim and Kim, 2022). Later, a variety of GM rapeseed varieties that are resistant to various herbicides or have unique oil qualities were developed (Ton et al., 2020). In 2013, the first report on *Agrobacterium*-mediated transformation of field cress was published (Ivarson et al., 2013), which was later used to increase oleic acid content by RNAi silencing of *FAE1* and *FAD2* (Ivarson et al., 2016), and increase seed oil content by overexpression of *WRINKLED1* and hemoglobin genes (Ivarson et al., 2017).

In the last two decades, different applications of site-directed mutagenesis by using specific site-directed nucleases have been developed. These methods include zinc finger nucleases (ZFN) (Wright et al., 2005), transcription activator-like effector nucleases (TALENs) (Christian et al., 2010), and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system (Jinek et al., 2012). The common molecular mechanism of these methods is to create double-stranded breaks (DSBs) at target nucleotide sequences. The DSBs are then repaired by the cell endogenous DNA break repair mechanisms: Most commonly by the error-prone non-homologous end joining (NHEJ) mechanism, which frequently results in small insertion or deletion mutations (indels) that lead to premature stop codons and disruption of the open reading frame, or by homology-directed repair (HDR) (Graham et al., 2020). While the use of ZFNs and TALENs has led to important advances, they are

considerably challenging and costly to apply, which has been a barrier for widespread adoption (Chen et al., 2019). The latest CRISPR/Cas9 system is however cheaper and relatively easy to adopt and use, and has thus revolutionized the entire field of biology since its discovery (Doudna and Charpentier, 2014; Ma et al., 2015; Ledford and Callaway, 2020).

The CRISPR/Cas9 system is derived from the adaptive immune system of *Streptococcus pyogenes*. The system has two components; the Cas9 nuclease and a single guide RNA (sgRNA) that form a Cas9/sgRNA complex. The Cas9 protein is a DNA endonuclease that can be directed to introduce DSBs at target sites by sgRNAs. It is characterized by two nuclease domains, RuvC and HNH, which cleave DNA strands that are complementary and non-complementary, respectively. The sgRNA is an artificial fusion of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) that can form base pairs with a DNA target sequence and direct Cas9 to introduce DSBs in the target DNA. Notably, the crRNA:tracrRNA (sgRNA) duplex contains a spacer sequence of about 20 nucleotides at the 5' end, that can pair with a complementary DNA protospacer sequence adjacent to a 5'-NGG-3' protospacer-adjacent motif (PAM), and a duplex RNA structure at the 3' end that binds to Cas9. In contrast to the complex protein engineering needed for DNA binding using ZFNs and TALENs, the CRISPR/Cas9 system can simply be programmed for new target sites by substituting the spacer sequence of the sgRNA (Jinek et al., 2012). Another major advantage of the CRISPR/Cas9 system is the ease of multiplex editing, which allows knockout of multiple genes, chromosomal deletions, and gene knock-ins (Chen et al., 2019). Furthermore, *in silico* analysis of genome sequences from eight representative plant species revealed that 5 to 12 NGG-PAM-sites are found for every 100 bp of gDNA, enabling targeting of most genes (Xie et al., 2014).

Mutation of catalytic residues in the HNH or RuvC nuclease domains results in a DNA nickase (nCas9), and mutation of both domains results in a deactivated Cas9 (dCas9) that can still target specific sites and function as a scaffold for recruitment of other proteins (Jiang and Doudna, 2017). Cas9 orthologues derived from other bacteria such as *Staphylococcus aureus* (SaCas9), *Streptococcus thermophilus* (StCas9), and *Neisseria meningtides* (NmCas9) with different properties such as protein size, off-target mutation rate, and PAM recognition has been developed (Cebrian-Serrano and Davies, 2017). Moreover, Cas9 variants have been engineered to recognize many

other PAM sites, further expanding the potential target sites (Chen et al., 2019). Furthermore, other Cas proteins have been discovered and developed to serve as genome-editing tools for various purposes. For instance, Cas12a which cleaves DNA in a staggered fashion, and Cas13, that can cleave single-stranded RNA (Zetsche et al., 2015; Cox et al., 2017). These different Cas enzymes enable an immense variety of genome engineering applications, including important novel methods such as base editing (Komor et al., 2016; Gaudelli et al., 2017) and prime editing (Lin et al., 2020).

The most common use of the CRISPR/Cas system for trait improvement in plants has been disruption of genes that confer undesirable traits via the NHEJ repair mechanism, which has been proven to be highly efficient in a number of species. Some important traits that have been improved include quality, yield, and biotic- and abiotic-stress resistance (Chen et al., 2019). For oilseed species, CRISPR/Cas has been used to tailor oil quality for specific uses and to reduce or remove anti-nutritional compounds such as GSLs and phytic acid (Okuzaki et al., 2018; Do et al., 2019; Huang et al., 2020; Sashidhar et al., 2020; Jarvis et al., 2021; Li et al., 2021; Hölzl et al., 2022; Liu et al., 2022). Furthermore, it holds great promise for accelerating the domestication of novel crops, as many of the key domestication traits are regulated by known major effect genes (Chen et al., 2019; Lyzenga et al., 2021). The application of CRISPR/Cas9 in domestication efforts has already been demonstrated in a few species, including the Brassicaceae oilseed species pennycress *T. arvense* (Jarvis et al., 2021).

1.6.1 Delivery of the CRISPR/Cas system

Currently, delivery of the CRISPR/Cas system into plant cells is mainly tissue culture-based. There are in principle two delivery methods: DNA-based delivery, in which plasmid vectors expressing sgRNA and Cas protein are employed, and ribonucleoprotein (RNP)-based, in which synthetic sgRNA and Cas9 protein mixtures are employed. The latter has the advantage of being DNA-free, thus avoiding the chance of foreign DNA insertion. For the DNA-based method, vectors are commonly delivered by *Agrobacterium*-mediated transformation, or by biolistics or polyethylene glycol (PEG)-mediated protoplast transfection. *Agrobacterium*-mediated transformation leads to stable integration of T-DNA in the plant genome. Biolistics and PEG-mediated transfection using RNPs results in transgene-free mutants that are considered as non-GM in some countries outside of

Europe. Biolistics and PEG-mediated transfection using DNA vectors can result in transgene-free mutants. Still, insertion of vector DNA is a possibility, more frequently in the case of biolistics.

The availability of transformation, transfection, and regeneration protocols limits the options of CRISPR/Cas delivery methods for a given species. *Agrobacterium*-mediated transformation is currently the most common delivery method for most species, as robust protocols have been developed for many species. However, the resulting GM mutants limit their commercial applications in many countries and increase the chance of off-target mutations due to continued expression of the CRISPR/Cas system (Chen et al., 2019). Major disadvantages of the transient transfection methods are the lack of selectable markers and lack of efficient transfection and plant regeneration protocols (Li et al., 2021; Sandgrind et al., 2021). Although the absence of selectable markers increase the labor input for screening a large number of putative mutants, the use of high-throughput screening methods such as high-resolution fragment analysis (HRFA) (Andersson et al., 2017) in combination with sequencing have enabled efficient high-throughput screening. Shoot regeneration from protoplasts remains a major bottleneck for many species (Reed and Bargmann, 2021). However, in recent years, application of protoplast approaches in genome editing for trait improvement has been reported in lettuce (Woo et al., 2015), potato (Nicolia et al., 2015; Andersson et al., 2017), cabbage (Park et al., 2019), rapeseed (Li et al., 2021), wild tomato (Lin et al., 2022), etc.

Reports on the mutation efficiency of the CRISPR/Cas system vary significantly depending on CRISPR/Cas delivery method, species, genotype, and sgRNA used. There are many factors that need to be optimized for a given species, and sometimes for different genotypes within a species (Andreasson et al., 2022). These factors range from starting plant material, to the concentration and type of plant growth regulators (PGRs), and other medium compositions, culture duration in various phases, Cas9 codon optimization, sgRNA, etc. The combination of sgRNA and promoter, GC content, presence of specific motifs, sgRNA folding, and possibly many other factors impact the mutation efficiency (Ma et al., 2015; Liang et al., 2016; Ma et al., 2016; Osakabe et al., 2016; Song et al., 2016; Chakrabarti et al., 2018; Concordet and Haeussler, 2018; Aksoy et al., 2022; Riesenberg et al., 2022). It is thus generally recommended to test multiple sgRNAs at the initial phase of a given study. Fortunately, several online and free programs

that consider target specificity and most of the known factors affecting sgRNA efficiency are available, enabling rapid selection of multiple sgRNAs for any given target sequence. These programs include bioinformatic tools such as CRISPOR (Concordet and Haeussler, 2018), CRISPR-P (Liu et al., 2017), CRISPRdirect (Naito et al., 2015), CRISPR-Plant (Minkenberg et al., 2019), and others.

2. Aims and objectives of the thesis

The overall aim of this thesis work was to explore the feasibility of using the CRISPR/Cas9 system to induce targeted mutations for speeding up the domestication process and trait improvement of field cress and rapeseed.

The specific objectives were to:

1) Establish efficient protocols for protoplast regeneration for both species
2) Verify the newly established protoplast protocols for genome editing in both species
3) Develop mutants with improved traits
4) Evaluate the mutants for the target traits

For field cress, successful establishment of the protoplast-based genome editing method would significantly contribute to the domestication process of the species for developing it into a novel oil and cover crop, particularly for the Nordic climate conditions. For rapeseed, a major oilseed crop in Sweden, the availability of an efficient protoplast-based genome editing method would facilitate generation of transgene-free mutants with improved traits, which could be used for further breeding to develop new varieties and to accelerate the breeding process of the species in the long run.

3. Results and discussion

3.1 Development of efficient protoplast regeneration protocols for rapeseed and field cress

In **papers I and II**, we successfully established efficient protocols for protoplast regeneration, including isolation, PEG-mediated transfection, callus formation, and shoot regeneration for both field cress and rapeseed. A schematic figure of the general workflow is shown in Figure 3. In both cases we were able to isolate high-quality protoplasts based on the *A. thaliana* protocol developed by Yoo et al. (2007), with some modifications. Specifically, we increased the macerozyme concentration from 0.4% to 0.6%, reduced the MES concentration from 20 mM to 10 mM, and introduced a 30 min plasmolysis incubation step before incubation in the enzyme solution. Furthermore, we found that 14 – 16 h incubation in the enzyme solution yielded the highest amount of viable protoplasts in both species. The viability and yield of the freshly isolated protoplasts were estimated by counting the proportion of round and compact protoplasts with uniform chloroplast distribution under a light microscope, as shown in Figure 4A.

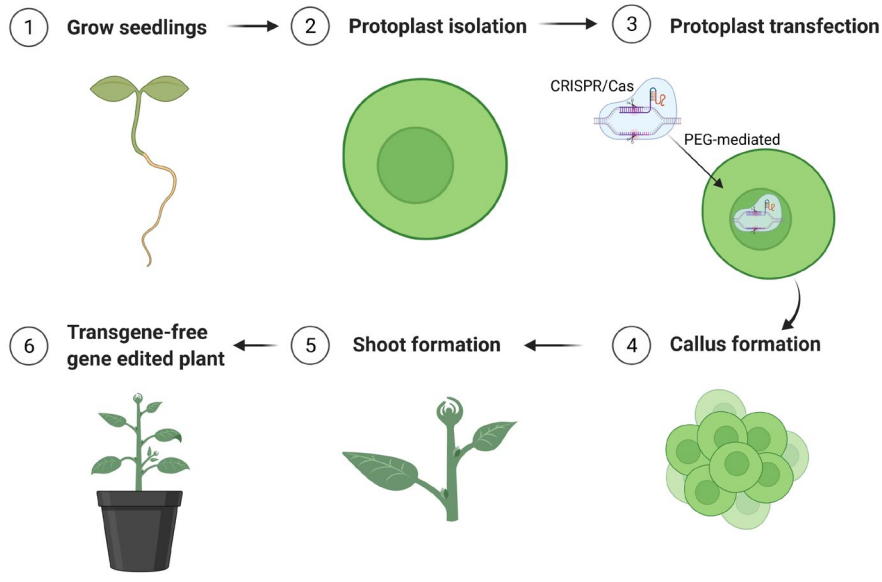

Figure 3. Schematic flowchart for the generation of transgene-free mutant lines from protoplasts using the CRISPR/Cas9 system.

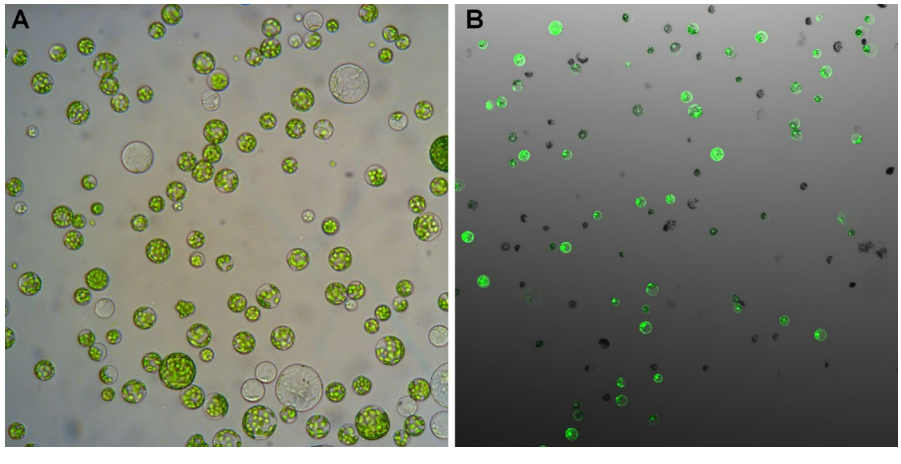

Figure 4. (A) Freshly isolated field cress protoplasts of mostly high quality observed at x20 magnification using a light microscope. (B) Transfected field cress protoplasts expressing GFP fluorescence 48 h after transfection, observed at x10 magnification with CLSM.

To optimize transfection efficiency we used a *GFP* vector, and recorded the transfection results with a confocal laser scanning microscope (CLSM) 48 h after transfection, as shown in Figure 4B. A combination of multiple factors were tested and optimized, such as the PEG concentration, protoplast density, vector DNA concentration, PEG-incubation time, and others. The transfection efficiency was estimated by the number of viable protoplasts exhibiting GFP fluorescence divided by the total number of viable protoplasts, using the viability criteria described above.

Relevant and precise estimation of the transfection efficiency is however not straightforward, as accurate determination of protoplast viability is challenging. In several published protoplast reports, the protoplast viability is determined by staining with fluorescein diacetate (FDA), which accumulates in cells with intact plasma membranes (Widholm, 1972). However, during our preliminary testing, we found that FDA staining results did not correlate with our observed regeneration efficiency, as almost all protoplasts in suspension would typically accumulate FDA, even protoplasts that were clearly in the process of disintegrating. This was especially evident when observing the protoplasts 48 h after transfection, as sub-optimal isolation and transfection conditions would result in a protoplast suspension consisting mostly of enlarged cells devoid of any green chloroplasts that were still accumulating FDA. In our case, we could predict the regeneration efficiency from a given protoplast solution with much higher accuracy by evaluating the protoplast viability as described above. Furthermore, transfection with a *GFP* vector is not directly comparable to transfection with a CRISPR/Cas vector or RNPs. We thus presented the transfection efficiency as a range based on repeated experiments. Eventually we obtained a routine *GFP* transfection efficiency between 50 – 80% in field cress, and 40 – 80% for rapeseed.

In vitro protoplast regeneration is often the most challenging part, limiting the successful establishment of protoplast protocols for the majority of plant species due to the sensitivity of freshly isolated protoplasts to external conditions. A large number of culture conditions need to be optimized, which are often time- and labor-consuming. For field cress and rapeseed, we optimized the density of protoplast plating, type of basal medium, PGRs, type and concentration of carbon source, duration of culture in each type of media for each developmental stage of protoplast regeneration, etc. In general, field cress and rapeseed responded relatively similar to culture

conditions for protoplast regeneration. However, the regeneration rate of rapeseed was much lower (ca. 45%) than that of field cress, where close to 100% regeneration was observed, given sufficient time. Our results indicate that rapeseed protoplasts are more challenging to regenerate than field cress protoplasts. However, 45% regeneration frequency is sufficient to perform genome editing, as a large number of protoplasts can be transfected in a single batch, which can provide an abundance of regenerated shoots for mutant screening. The successful establishment of these protoplast regeneration protocols have enabled genome editing by CRISPR/Cas9 for trait improvement for both species.

3.2 Genome editing of important traits by CRISPR/Cas9

In **paper I**, to reduce GSL levels in the rapeseed seeds, we targeted two GSL transport genes *GTR1* and *GTR2*. Based on the sequences of the homologs in *A. thaliana*, and by BLAST queries in the NCBI database against the rapeseed reference genome cv. ZS11 (Bra_napus_v2.0), we identified and cloned six paralogs of both *BnGTR1* and *BnGTR2* in rapeseed, with the sequence homology between the different paralogs ranging from 86 – 99% for *BnGTR1* and 88 – 98% for *BnGTR2*. To target all 12 paralogs, we designed four sgRNAs (two sgRNAs targeting *BnGTR1* and two sgRNAs targeting *BnGTR2*). From the 16 regenerated shoots, three were found to be mutated. Among these three mutants, we were able to mutate five of the six *BnGTR1* paralogs and four of the six *BnGTR2* paralogs. Furthermore, no foreign DNA could be detected at the cut sites or by PCR using vector-specific primers, indicating that they are most likely transgene-free.

In **paper III**, we explored the possibility of improving the FA composition of field cress by using CRISPR/Cas9 to knockout important genes involved in FA biosynthesis. Improving the oil quality by increasing the oleic acid content and reducing the erucic acid content is one of the major goals for domesticating field cress into a viable food crop. An early study from our group has shown that silencing *LcFAE1* and *LcFAD2* using RNAi resulted in transgenic lines with up to 80% oleic acid (Ivarson et al., 2016). Using the protoplast-based CRISPR/Cas9 system, it is possible to target the same genes for producing high oleic acid transgene-free lines, which would be of interest for the future breeding of field cress. Moreover, we were also interested in investigating if knocking out the *ROD1* gene in field cress

would also promote accumulation of oleic acid, as shown in other Brassicaceae species (Lu et al., 2009; Guan et al., 2015; Bai et al., 2020; Jarvis et al., 2021).

After cloning *LcROD1* and verifying the *LcFAE1* and *LcFAD2* sequences in the accession used in this study, we constructed two CRISPR/Cas9 vectors: one with two sgRNAs targeting *FAE1* and two sgRNAs targeting *FAD2*, and one with four sgRNAs targeting *ROD1*. Four distinct mutant genotypes were generated by targeting *FAE1* and *FAD2*, one of which was a homozygous single knockout *fae1* mutant line, while the others were heterozygous for both genes. One of the heterozygous mutant lines that carried knockout mutations in both genes was selfed for achieving mutants with double knockouts in *FAE1* and *FAD2* or single knockout in *FAD2*. However, single seed analysis of FA profile from the 84 T_1 seeds showed that the segregation did not follow a typical Mendelian pattern, seemingly due to the chimeric nature of the parental line. No homozygous *fae1*/*fad2* or *FAE1*/*fad2* were found by single seed analysis. For *ROD1*, we generated six distinct mutants, which carried knockout mutations in both alleles. Similarly, as also observed in paper I, we detected no foreign DNA at the cut sites or by PCR using vector-specific primers.

As shown in Figure 5A, analysis of the FA profile of the mutant lines revealed that erucic acid could only be detected in trace amounts in the homozygous *fae1* knockout mutant, while the oleic acid content was increased to 29.5%, compared to 13.3% in the wild type. From the best heterozygous T_1 *FAE1*/*fae1 fad2/fad2* lines, the oleic acid content was elevated to 66%, and the PUFA content was reduced to approximately 5%, compared to approximately 50% in the wild type, as shown in Figure 5B. The erucic acid content was decreased to 6 – 14%, compared with 24% in the wild type, apparently due to the presence of a wild type *FAE1* allele in the mutants. In the *rod1* mutant lines, the oleic acid content was significantly increased to 23%, compared to 13.3% in the wild type, and the total PUFA content was reduced to approximately 30%, compared to approximately 50% in the wild type, as shown in Figure 5C.

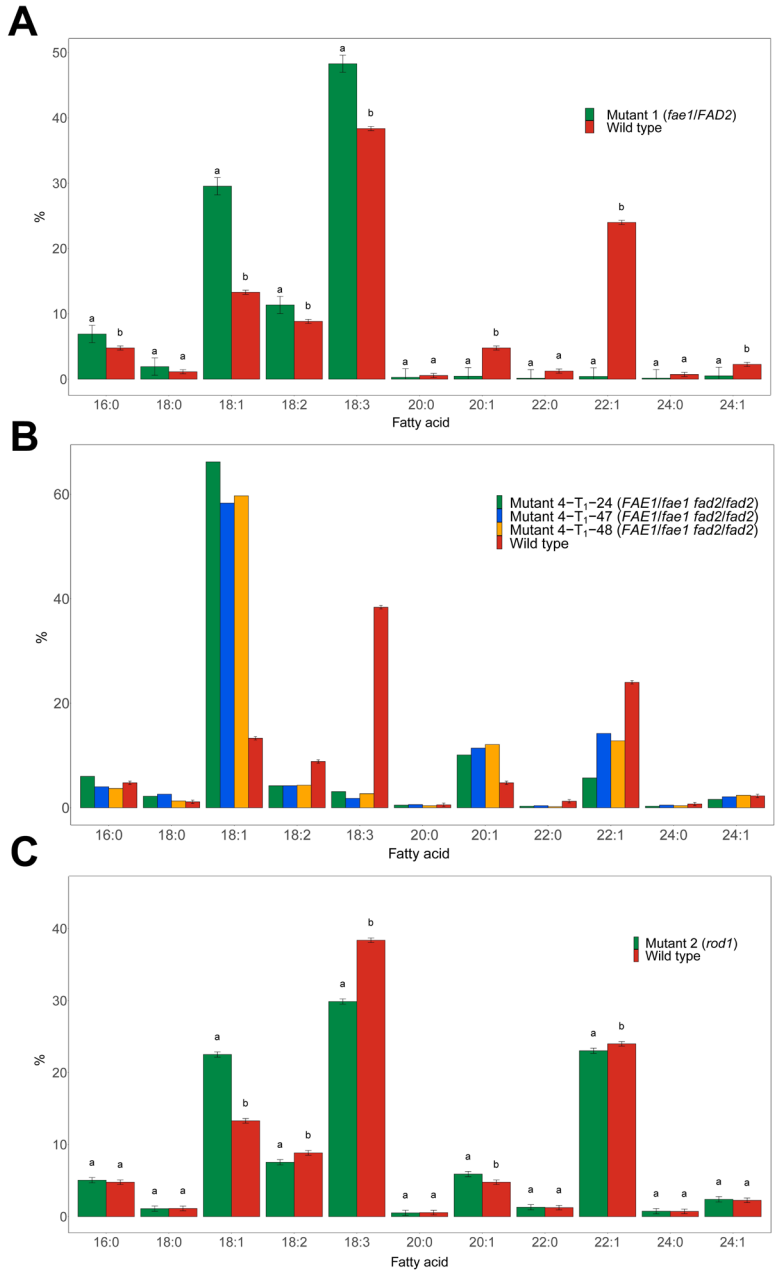

Figure 5. Major FA compositions in seed oil of field cress mutants and wild type. (A) Homozygous *fae1* mutant. (B) Heterozygous *FAE1* and homozygous *fad2* mutants. (C) Homozygous *rod1* mutant. Different letters indicate statistically significant differences at $p < 0.05$ ($n = 3$).

In **paper IV**, to reduce or eliminate the seed GSL content in field cress, we cloned the *GTR1* and *GTR2* genes from field cress based on the gene sequences in *A. thaliana*, and constructed a CRISPR/Cas9 vector harboring two sgRNAs targeting *GTR1* and two sgRNAs targeting *GTR2*. Both stable *Agrobacterium*-mediated transformation and transient protoplast transfection were used to deliver the CRISPR/Cas9 vector. A total of 70 mutant lines were analyzed. The results showed they were all single knockout *gtr2* or double knockout *gtr1*/*gtr2* mutants. No homozygous *gtr1* mutants were obtained, indicating much higher mutation efficiency in *GTR2*: All lines that carried mutations in *GTR1* also had both *GTR2* alleles mutated.

Analysis of the seed GSL content by high-performance liquid chromatography (HPLC) revealed an almost complete elimination of GSLs in seeds in the *gtr1*/*gtr2* and *GTR1*/*gtr2* mutant lines. Evidently, knockout of *GTR2* is sufficient to almost eliminate GSLs from the seeds of field cress. However, we observed that the growth and seed yield were adversely affected in all knockout mutants. The preliminary results showed that the mean seed yield of the *gtr1*/*gtr2* mutants were reduced to 35% of the yield of the wild type, while the yield of *GTR1*/*gtr2* mutant lines were reduced even more, down to 12% of the wild type. We also observed a large decrease in the biomass in the mutants, and the stems needed support to stay erect. A more mild, but significant, yield penalty was also reported for *A. thaliana* *gtr1*/*gtr2* mutants (Nour-Eldin et al., 2012). This is in contrast to the recent study on *gtr1*/*gtr2* knockout *C. sativa* mutant lines, where no adverse effects to yield, seed oil, or protein content were reported (Hölzl et al., 2022).

4. Conclusions and future perspectives

Domestication and selective breeding of plants have led to high-yielding crops adapted to a variety of climate conditions. However, our society faces severe challenges to agricultural systems due to a growing population, climate change, and a reduction of biodiversity and arable land. In combination with traditional breeding methods and more sustainable farming practices, modern breeding tools are needed to improve existing crops and domesticate novel species for tackling issues such as climate change and global food security. The CRISPR/Cas system offers great potential for significantly accelerating the domestication process by reducing breeding cycles, improving important traits of existing crops, and creating desirable genetic diversity.

The protoplast-based tissue culture method is important for delivering CRISPR/Cas complexes into plant cells for producing transgene-free mutants, but the bottleneck of this approach is the difficulty in protoplast regeneration. We have successfully developed efficient protocols for protoplast transfection and regeneration for field cress and rapeseed, and employed them to induce targeted mutations in both species using the CRISPR/Cas9 system. These protocols provide a solid platform for knocking out genes for functional analysis, trait improvement, and creation of genetic variation in these species for both basic research and plant breeding. Furthermore, the protoplast-based CRISPR/Cas gene editing method enables direct generation of transgene-free mutants, which are not classified as GM in some countries.

Apart from the successful knockout of *FAE1* and *FAD2*, and obtaining mutants with improved FA composition in field cress, we have also confirmed the function of the *ROD1* gene in the species for the first time, which showed a similar function as in the few other species studied. This

provides a new pathway for obtaining higher oleic acid levels. The development of high oleic acid and low erucic acid transgene-free field cress lines is an important step towards domestication of the species into an economically viable crop. Further elevation of oleic acid content should be possible by developing homozygous *fae1*/*fad2*/*rod1* lines by crossing the mutant lines obtained in this thesis. Our CRISPR/Cas9-edited lines, even those without any T-DNA insertions, are currently classified as GM in the EU according to current EU legislation (Court of Justice of the European Union, 2018). However, according to scientific opinion, the situation may change in the future due to the arbitrary distinction between *in vitro* and *in vivo* random mutagenesis (Voss-Fels et al., 2019; Eriksson and Zimny, 2020; Turnbull et al., 2021).

Reduction or elimination of seed GSLs in field cress and rapeseed could increase the value of the seed cake as feed and potentially pave the way for food applications. Due to the high ploidy level of rapeseed, it generally takes time to obtain homozygous lines for the target genes, and the *GTR* mutated lines are currently being evaluated. In field cress, knockout of the *GTR1* and *GTR2* genes resulted in mutated lines with only trace amounts of GSLs in seeds. However, the mutants appeared to have poor agronomic performance in the preliminary test in biotron. Other or combined strategies may be needed to develop low seed GSL field cress lines in the future.

Since we have already established an efficient protoplast approach for CRISPR/Cas9 editing for field cress, it would be interesting to improve some other important traits such as pod shatter, oil content, and stress tolerance, to fully domesticate field cress into an economically viable crop. The same applies to rapeseed, where the well-established CRISPR/Cas9 protoplast-based genome editing method would facilitate trait improvement and promote precise and accelerated rapeseed breeding.

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Popular science summary

Our society faces major challenges to meet the 2030 Sustainable Development Goals developed by the United Nations, and the Paris Agreement's goal to keep the rise in mean global temperature to well below 2 °C above pre-industrial levels. As the planet warms and the population grows, global food security will become increasingly endangered. More sustainable and productive agriculture is an essential part of curbing this development.

Vegetable oils are the second most important human food group, and can also be a renewable source of fuel and raw material for products such as bio-plastics, paints, soaps, lubricants, and more. Oil production is restricted by a shortage of arable land and a limited number of existing oil crops. Field cress (*Lepidium campestre*) can be developed into a new oil cover crop that can be grown in cold climates together with cereals. It would as such not require any additional land for cultivation, while simultaneously improving the cereal yield, reducing soil erosion and pollution from excess fertilizer. However, the seed oil of field cress contains compounds that can be toxic at high concentrations. Before it can become an economically viable crop, we must improve the species by breeding varieties that does not contain high concentrations of these potentially toxic compounds, and improve its suitability for food and other applications. Rapeseed is the major oil crop in Sweden, and the third most important worldwide. Like field cress, it used to contain high concentrations of the same potentially toxic compounds, but breeders have been able to develop modern varieties that produce a healthy oil. It does however still contain small amounts of these toxic compounds that become highly concentrated in the seed cake that is left over after extracting the oil, which reduce the value of the otherwise highly nutritious seed cake. Further reduction or elimination of these compounds by breeding

could pave the way for using it in food products such as plant-based meat substitutes, or as high-value animal feed.

Plant breeding is however traditionally a very slow and laborious process. Development of modern breeding tools such as CRISPR/Cas9, for which the discoverers won the Nobel Prize in Chemistry in 2020, can greatly increase the speed and accuracy of plant breeding. By using this tool, it is possible to selectively turn off the genes that are responsible for undesirable traits such as producing toxic compounds, without having to induce thousands of random mutations using radiation or mutagenic chemicals and screen thousands of plants for the desired plant types, as is commonly done in conventional breeding.

In this thesis, we have developed methods for using CRISPR/Cas9 to edit the genes of field cress and rapeseed, without inserting any foreign DNA, as is done when producing traditional genetically modified crops. We used these methods to develop field cress varieties that have improved oil quality and only trace amounts of the toxic compounds by disrupting some of the genes involved. We have also disrupted some of the genes in rapeseed that are responsible for transporting these toxic compounds into the rapeseed seeds.

Populärvetenskaplig sammanfattning

Samhället har besvär för att uppfylla 2030-målen för hållbar utveckling som utvecklats av FN, och Parisavtalets mål att hålla den globala medeltemperaturökningen till långt under 2 °C över förindustriella nivåer. När planeten värms upp och befolkningen växer, kommer den globala livsmedelssäkerheten att bli alltmer hotad. Ett mer hållbart och produktivt jordbruk är en väsentlig del av att bromsa denna utveckling.

Vegetabiliska oljor är det näst viktigaste livsmedlet för människor och kan också vara en förnybar källa till bränsle och råvara för produkter som bioplast, färg, tvål, smörjmedel med mera. Oljeproduktionen begränsas av brist på odlingsbar mark och ett begränsat antal befintliga oljegrödor. Fältkrassing (*Lepidium campestre*) kan utvecklas till en ny oljetäckgröda som kan odlas i kallt klimat tillsammans med spannmål. Den skulle därmed inte kräva ytterligare mark för odling, samtidigt som den skulle förbättra spannmålsavkastningen, minska jorderosion och föroreningar från överskott av gödselmedel. Fröoljan från fältkrassing innehåller dock föreningar som kan vara giftiga i höga koncentrationer. Innan det kan bli en ekonomiskt lönsam gröda måste vi förbättra arten genom att utveckla sorter som inte innehåller höga koncentrationer av dessa potentiellt giftiga föreningar och förbättra dess lämplighet för livsmedel och andra tillämpningar. Raps är den största oljegrödan i Sverige och den tredje viktigaste i världen. Liksom fältkrassing innehöll den tidigare höga koncentrationer av samma potentiellt giftiga föreningar, men föredlare har kunnat utveckla moderna sorter som ger en hälsosam olja. Den innehåller dock fortfarande små mängder av dessa giftiga föreningar som blir mycket koncentrerade i frökakan som återstår efter att oljan extraherats, vilket minskar värdet på den annars mycket näringsrika frökakan. Ytterligare minskning eller eliminering av dessa föreningar genom förädling skulle kunna bana väg för användning i

livsmedelsprodukter som växtbaserade köttersättningar eller som högvärdigt djurfoder.

Växtförädling är dock traditionellt en mycket långsam och mödosam process. Utveckling av moderna förädlingsverktyg som CRISPR/Cas9, som upptäckarna vann Nobelpriset i kemi för 2020, kan kraftigt öka hastigheten och noggrannheten i växtförädlingen. Genom att använda detta verktyg är det möjligt att selektivt stänga av de gener som är ansvariga för oönskade egenskaper som att producera giftiga föreningar, utan att behöva inducera tusentals slumpmässiga mutationer med strålning eller mutagena kemikalier och screena tusentals växter för de önskade växttyperna, som görs vanligtvis i konventionell förädling.

I detta avhandlingsarbete har vi utvecklat metoder för att använda CRISPR/Cas9 för att redigera generna hos fältkrassing och raps, utan att infoga något främmande DNA, vilket görs när man producerar traditionella genetiskt modifierade grödor. Vi använde dessa metoder för att utveckla fältkrassesorter som har förbättrat oljekvaliteten och endast spårmängder av de giftiga föreningarna genom att stänga av några av de inblandade generna. Vi har också stängt av några av generna i raps som är ansvariga för att transportera dessa giftiga föreningar in i rapsfröna.

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

Xueyuan Li†, Sjur Sandgrind†, Oliver Moss, Rui Guan, Emelie Ivarson, Eu Sheng Wang, Selvaraju Kanagarajan and Li-Hua Zhu**

Department of Plant Breeding, Swedish University of Agricultural Sciences, Lomma, Sweden

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Yiping Qi, University of Maryland, College Park, United States
Zahir Ali, King Abdullah University of Science and Technology, Saudi Arabia

***Correspondence:**
Li-Hua Zhu
li-hua.zhu@slu.se
Selvaraju Kanagarajan
selvaraju.kanagarajan@slu.se

†These authors share first authorship

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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	20.3 b
		Zeatin 1.0 2,4-D 0.5	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.

			Regeneration (%)*				
Duration	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.

			Regeneration (%)*				
Duration	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*.

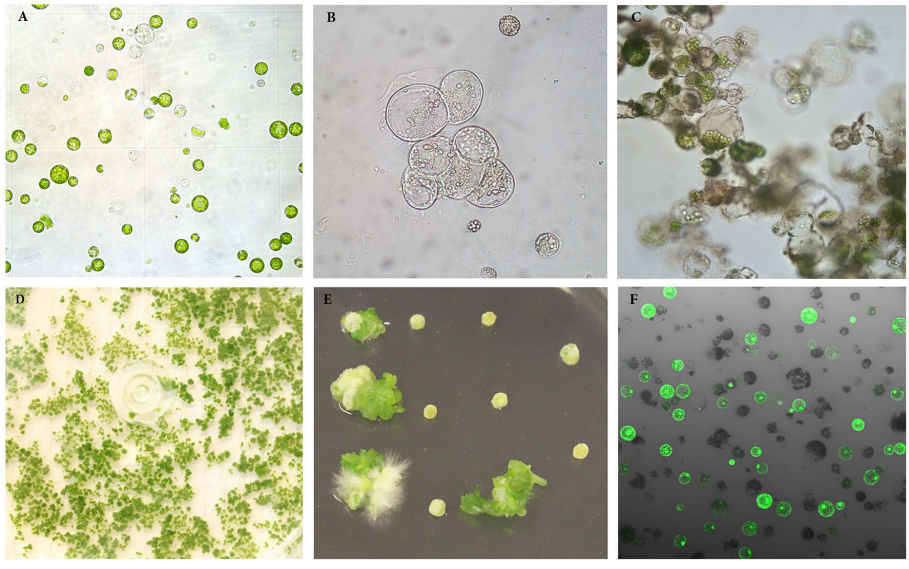

FIGURE 1 | Isolation, regeneration and transfection of protoplasts of rapeseed. **(A)** Freshly isolated protoplasts. **(B, C)** Protoplasts undergoing cell divisions and multiplication. **(D)** Protoplast colonies. **(E)** Shoot regeneration from protoplast colonies. **(F)** Transfected protoplasts 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.

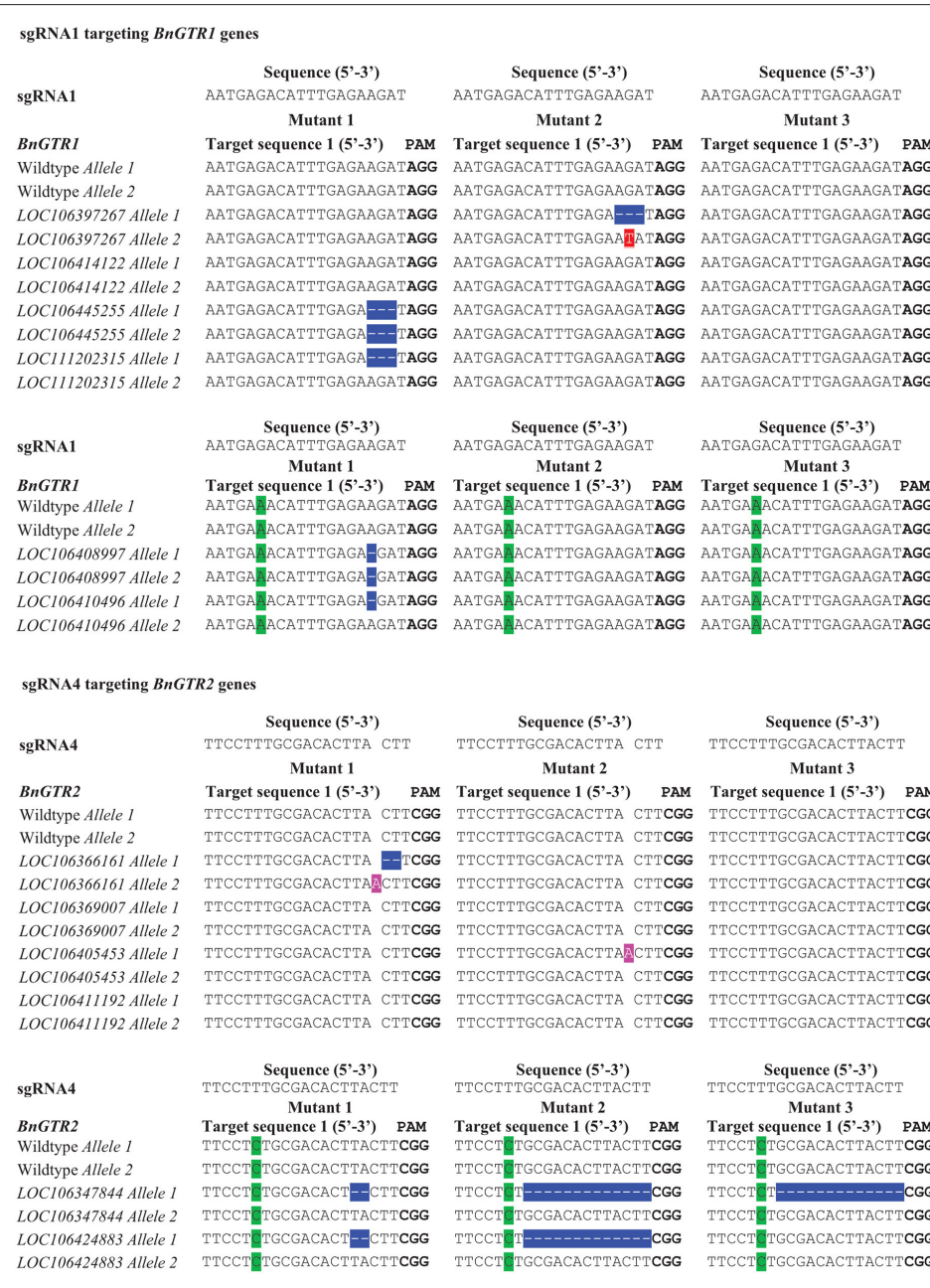

FIGURE 2 | Types of mutations in the *BnGTR1* and *BnGTR2* genes detected in the three mutants in comparison with wild type of rapeseed cv. Kumily, determined by DNA sequencing. PAM sites are highlighted in bold letters. Mismatches with the sgRNAs are highlighted in green. Mutated nucleotides were highlighted 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

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II

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ORIGINAL RESEARCH
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Establishment of an Efficient Protoplast Regeneration and Transfection Protocol for Field Cress (*Lepidium campestre*)

*Sjur Sandgrind†, Xueyuan Li†, Emelie Ivarson, Annelie Ahlman and Li-Hua Zhu‡**

Department of Plant Breeding, Swedish University of Agricultural Sciences, Lomma, Sweden

Field cress (*Lepidium campestre*) is a potential oilseed crop that has been under domestication in recent decades. CRISPR/Cas9 is a powerful tool for rapid trait improvement and gene characterization and for generating transgene-free mutants using protoplast transfection system. However, protoplast regeneration remains challenging for many plant species. Here we report an efficient protoplast regeneration and transfection protocol for field cress. Important factors such as type of basal media, type/combination of plant growth regulators, and culture duration on different media were optimized. Among the basal media tested, Nitsch was the best for protoplast growth in MI and MII media. For cell wall formation during the early stage of protoplast growth, relatively high auxin concentrations (0.5 mg L^{-1} NAA and 2,4-D), without addition of cytokinin was preferred for maintaining protoplast viability. After cell wall formation, 1.1 mg L^{-1} TDZ combined with either 0.05 mg L^{-1} NAA or 2,4-D was found to efficiently promote protoplast growth. On solid shoot induction medium, 1.1 mg L^{-1} TDZ without any auxin resulted in over 80% shoot generation frequency. A longer culture duration in MI medium would inhibit protoplast growth, while a longer culture duration in MII medium significantly delayed shoot formation. Using this optimized protoplast regeneration protocol, we have established an efficient PEG-mediated transfection protocol using a vector harboring the *GFP* gene, with transfection efficiencies of 50–80%. This efficient protoplast protocol would facilitate further genetic improvement of field cress via genome editing, and be beneficial to development of protoplast regeneration protocols for related plant species.

Keywords: Braccicaceae, CRISPR/Cas9, oilseed crop, domestication, protoplast regeneration, transfection

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Edited by:
Leena Tripathi,
International Institute of Tropical Agriculture (IITA), Kenya

Reviewed by:
Kutubuddin A. Molla,
National Rice Research Institute (ICAR), India
Yutaka Tabei,
National Agriculture and Food Research Organization (NARO), Japan

****Correspondence:***
Li-Hua Zhu
Li-Hua.Zhu@slu.se

†These authors share first authorship

‡ORCID:
Li-Hua Zhu,
orchid.org/0000-0002-9325-3845

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INTRODUCTION

Domestication of new plant species has the potential to increase food security by increasing crop diversity and utilize marginal arable land. It is however a tedious and slow process using traditional breeding methods for domestication, as wild species typically carry many undesirable traits for agriculture. Molecular mechanisms underlying important domestication-related traits such as pod shattering, yield, flowering time, seed dormancy etc. have been identified in both model and crop species (Doebley et al., 2006). As such, employing modern breeding technologies holds great potential for speeding up the domestication process of wild species. The latest gene editing technique

CRISPR/Cas9 is an efficient and powerful tool for functional analysis of important genes, and can be used to drastically increase the domestication speed. This technique has been used successfully in domestication efforts of plant species such as pennycress (McGinn et al., 2019), wild tomato (Li et al., 2018; Zsögön et al., 2018), and groundcherry (Lemmon et al., 2018).

The delivery of CRISPR/Cas9 vectors is commonly performed by *Agrobacterium*-mediated stable transformation, resulting in transgenic plants that are strictly regulated in some countries. Furthermore, the integration of CRISPR/Cas9 DNA into the plant genomes can cause insertional gene disruption, and increase the likelihood of off-target mutations due to constant expression of the CRISPR/Cas9 system (Zhang et al., 2019). Polyethylene glycol (PEG)-mediated transfection is an alternative and effective approach to deliver CRISPR/Cas9 vectors or ribonucleoprotein complexes into protoplasts, which enables generation of transgene-free mutated lines (Woo et al., 2015). The CRISPR/Cas9 protoplast transfection system has been used successfully to edit genes in several plant species (Kim et al., 2017; Liang et al., 2017; Lin et al., 2018; González et al., 2020; Li et al., 2021), and the protoplast transfection system has also been successfully used for gene editing in plants by base editors (Molla et al., 2020) or prime editing (Lin et al., 2020). However, as protoplast regeneration remains a major obstacle for obtaining mutated lines for most plant species, the method has mainly been used to evaluate mutation efficiencies of sgRNAs of target genes, not for trait improvement in general. An efficient and reliable protoplast regeneration method is thus a prerequisite for crop improvement by CRISPR/Cas9 using the protoplast system.

Field cress (*Lepidium campestre*) belongs to the Brassicaceae family and has a great potential to become a new crop for plant oil production. It is very cold-hardy and can thus be grown in regions where other winter oilseed crops cannot be cultivated, greatly expanding the possible planting region for oilseed crops. Furthermore, it has a high yield potential and some good agronomic traits such as an upright stature, synchronous seed maturity, and resistance to the pollen beetle (Merker and Nilsson, 1995; Bertholdsson, 2017). Due to its biennial nature, field cress has also shown its potential as a catch crop with a positive effect on the yield of barley when it was undersown (Merker et al., 2010). This cropping system could reduce nutrient leaching and tillage, providing valuable ecosystem services and reducing on-farm energy-consumption. Field cress has been under domestication in the last few decades, and has been improved via genetic transformation (Ivarson et al., 2013, 2016, 2017a, b) and marker assisted breeding (Gustafsson et al., 2018; Geleta et al., 2020). Development of an efficient protoplast regeneration and transfection method could facilitate the use of CRISPR/Cas9 for rapid trait improvement, and thus further speed up the domestication process of the species.

In this study, we have studied some important factors affecting protoplast regeneration and transfection, and have successfully established an efficient protocol for protoplast regeneration and transfection of field cress. This method is now routinely used in our lab for trait improvement of the species through genome editing by CRISPR/Cas9.

MATERIALS AND METHODS

Plant Material and *in vitro* Growth Conditions

Seeds from field cress (*L. campestre* L.), accession no. 94–7, were used in this study. This accession was initially collected in Öland, Sweden, and further multiplied in greenhouse. All *in vitro* cultures were maintained in a climate chamber with 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 in 15% (v/v) calcium hypochlorite ($Ca(ClO)_2$) for 20 min, followed by rinsing with sterile water. Surface sterilized seeds were planted on germination medium (half strength MS, 10 g L^{-1} sucrose, 7 g L^{-1} Bacto agar, pH 5.7) in sterile plastic containers, which were placed in the climate chamber as stated above.

Protoplast Isolation and Culture

Protoplast isolation was based on the Arabidopsis protocol developed by Yoo et al. (2007), with some modifications. About 40–50 fully opened true leaves of 3–4 week old field cress seedlings were finely sliced and incubated in plasmolysis solution (0.4 M mannitol, pH 5.7) for 30 min in the dark at room temperature (RT). After removing the plasmolysis solution, 10 ml enzyme solution (1.5% (w/v) cellulase Onozuka R-10 (Yakult Pharmaceutical Co., LTD, Tokyo, Japan), 0.6% (w/v) Macerozyme R-10 (Yakult Pharmaceutical Co., LTD.), 0.4 M mannitol, 10 mM MES, 0.1% (w/v) BSA, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol, pH 5.7) were added and incubated in the dark at RT for 14–16 h with gentle shaking.

The enzyme solution was then diluted with 30 ml W5 solution without glucose (Menczel et al., 1981), filtered through a 40 μm nylon cell strainer, and the protoplasts were collected by centrifugation at 100 *g* for 10 min. After removing the supernatant, the pellet was gently resuspended in 10 ml W5, and centrifuged at 100 *g* for 5 min using a swing-bucket rotor. This washing step was repeated once. Afterwards, the pellet was resuspended in 5 ml W5 and incubated on ice for 30 min in the dark to allow intact protoplasts to sink naturally. The supernatant was removed and the protoplasts were resuspended in 10 ml W5. A sample of the protoplast solution was loaded on a Hemocytometer and observed under a light microscope at ×20 magnification to estimate the amount of intact viable protoplasts isolated. The solution was then centrifuged for 3 min at 100 *g*. After removing the supernatant, the protoplast density was adjusted to 0.4 to 0.6 million protoplasts per ml with 0.5 M mannitol. Sodium alginate solution (2.6% (w/v) sodium alginate, 0.4 M mannitol) was added to the protoplast solution in a 1:1 ratio, and, after gentle mixing, 500 μL aliquots of the suspension were pipetted onto calcium-agar plates (0.4 M mannitol, 2.2 g L^{-1} $CaCl_2$, 10 g L^{-1} Phyto agar) for making alginate disks and incubated at RT for 30 min. Thereafter, approximately 2 ml

TABLE 1 | Effect of basal medium on protoplast viability of field cress.

Basal medium	Protoplast viability in MI medium (%)[a]	Protoplast viability in MII medium (%)[a]	Regeneration (%)[b]
MS	0.0 c	0.0 c	0.0 b
½ MS	0.0 c	0.0 c	0.0 b
Kao	49.7 b	0.0 c	0.0 b
B5	49.7 b	10.0 b	0.0 b
Nitsch	80.0 a	80.0 a	75.0 a

MI medium composition: Basal medium, 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-D, 0.5 mg L^{-1} NAA, pH 5.7. MII medium composition: Basal 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, pH 5.7. SIM medium composition; MS, sucrose 15 g L^{-1}, 1.1 mg L^{-1} TDZ, 0.5 mg L^{-1} $AgNO_3$, 2.5 g L^{-1} Gelrite, pH 5.7.

[a]Protoplast viability was indicated by being round, compact in form, and green in color, observed under light microscope. The results were recorded after 7 days in MI, and 14 days in MII.

[b]The results were recorded after 4 months. Values followed by the same letter were not statistically different at p=0.05 (n = 3).

TABLE 2 | Effect of PGRs in MI medium on protoplast viability of field cress.

PGR in conc. (mg L^{-1})	Protoplast viability (%)[a]	PGR in conc. (mg L^{-1})	Protoplast viability (%)[a]
TDZ 1.1 2,4-D 1.0	0.0 c	BAP 0.5 NAA 0.5 2,4-D 0.5	19.7 b
TDZ 1.1 2,4-D 0.5	0.0 c	NAA 0.5 2,4-D 0.5	80.0 a
TDZ 1.1 2,4-D 0.25	0.0 c	BAP 2.0 NAA 0.5	0.0 c

MI medium composition: 2.18 g L^{-1} Nitsch, PGRs, 10 g L^{-1} sucrose, 10 g L^{-1} glucose, 100 g L^{-1} mannitol, 100 mg L^{-1} casein, pH 5.7.

[a]Protoplast viability was indicated by being round, compact in form, and green in color, observed under light microscope after 7 days. Values followed by the same letter were not statistically different at p=0.05 (n = 3).

TABLE 3 | Effect of PGRs in MII medium on protoplast callus formation of field cress.

PGR in conc. (mg L^{-1})	Callus formation (%)[a]	PGR in conc. (mg L^{-1})	Callus formation (%)[a]
BAP 1.0 Zeatin 0.6 NAA 0.5	0.0 b	TDZ 1.1 NAA 0.1	0.0 b
BAP 1.0 Zeatin 0.6 NAA 0.1	0.0 b	TDZ 1.1 NAA 0.05	75.7 a
TDZ 1.1 Zeatin 0.6 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

MII medium composition: 2.18 g L^{-1} Nitsch, PGRs, 10 g L^{-1} sucrose, 10 g L^{-1} glucose, 100 g L^{-1} mannitol, 100 mg L^{-1} casein, pH 5.7.

[a]Protoplast colonies formed with a size of ≥0.1 mm in diameter after 30 days. Values followed by the same letter were not statistically different at p=0.05 (n = 3).

TABLE 4 | Effect of PGRs in SIM medium on shoot regeneration of field cress.

PGR in conc. (mg L^{-1})	Appearance of calli by visual observation	Regeneration (%)[a]
TDZ 1.1	Normal and green	82.0 a
TDZ 1.1, NAA0.1	Big and hard	0.0 c
TDZ 1.1, NAA 0.01, GA_3 0.1	Small, yellow, and hard	5.0 b
TDZ 2.2, NAA0.1	Big and hard	0.0 c
Zeatin 2.0, NAA0.1	Yellow and hard	0.0 c
Zeatin 1.0, NAA0.1	Yellow and hard	0.0 c
Zeatin 2.0, NAA 0.01, GA_3 0.1	Small, yellow, and hard	0.0 c
BAP 2.0, NAA 0.1	Small and yellow	1.0 c

SIM medium composition: MS, PGRs, 15 g L^{-1} sucrose, 0.5 mg L^{-1} $AgNO_3$, 2.5 g L^{-1} Gelrite, pH 5.7.

[a]The results were recorded after 4 months. Values followed by the same letter were not statistically different at p=0.05 (n = 3).

calcium-solution (50 mM $CaCl_2$, 0.4 M mannitol) was added onto each disk, and incubated for 1 h at RT to complete the polymerization. The disks were finally transferred to MI medium, which consisted of different basal media and PGRs (**Tables 1**, **2**), in 6-well sterile tissue culture plates. The plates were covered with aluminum foil and transferred to the climate chamber with conditions as stated above. After 24 h, the foil was replaced with fiber cloth to provide a dim lighting for ensuring callus formation. After 3–20 days, the MI medium was replaced with MII medium, which consisted of different PGRs (**Table 3**). The MII medium was renewed every 5–7 days until protoplast colonies reached a size of approximately 0.1–0.2 mm in diameter.

Protoplast Regeneration

Microcalli from the alginate disks were spread directly onto shoot induction medium (SIM) (**Tables 4–6**). The microcalli were subcultured to fresh medium every 3–4 weeks until shoots had appeared. Shoots were transferred to shoot elongation medium (SEM) (MS, 20 g L^{-1} sucrose, 0.05 mg L^{-1} 6-benzyladenine (BAP), 0.03 mg L^{-1} gibberellic acid (GA_3), 7 g L^{-1} Bacto agar, pH 5.7).

TABLE 5 | Effect of C-source in SIM medium on shoot regeneration of field cress.

C-source in conc. (g L^{-1})	Regeneration (%)[a]
Sucrose 15	80.0 a
Sucrose 30	46.7 c
Glucose 10	67.0 b
Glucose 20	45.0 c

SIM medium composition; MS, sugar, 1.1 mg L^{-1} TDZ, 0.5 mg L^{-1} $AgNO_3$, 2.5 g L^{-1} Gelrite, pH 5.7.
[a]The results were recorded after 4 months. Values followed by the same letter were not statistically different at p*=0.05 (n = 3).*

TABLE 6 | Effect of cytokinin in SIM medium on shoot regeneration of field cress.

Cytokinin conc. (mg L^{-1})	Regeneration (%)[a]
TDZ 0.5	13.3 c
TDZ 1.1	88.8 a
TDZ 2.2	40.0 b
BAP 2.0	0.0 c

SIM Medium composition: MS, PGR, 15 g L^{-1} sucrose, 0.5 mg L^{-1} $AgNO_3$, 2.5 g L^{-1} Gelrite, pH 5.7.
[a]The results were recorded after 4 months. Values followed by the same letter were not statistically different at p*=0.05 (n = 3).*

For optimizing protoplast regeneration efficiency, various MI, MII, and SIM medium compositions and culture durations in MI and MII medium (**Table 7** in the result section) were tested.

The detailed information about the medium compositions is given in **Tables 1–7**.

Statistical Analysis

For evaluating the protoplast viability, protoplast solution was loaded on a Hemocytometer, and five 1 mm^2 squares were observed under a light microscope seven or 14 days after isolation, with three biological replicates. For the callus and shoot regeneration tests, a single treatment consisted of 50 calli with three biological replicates. Results were recorded at different time points depending on experiment, and the detailed information is presented in each corresponding table in the result section. The data were analyzed with ANOVA and Tukey's test using Minitab (LLC) version 19.2020.1.

Protoplast Transfection and GFP Detection

To optimize transfection efficiency for field cress, protoplasts were transfected with a vector harboring a gene encoding for green fluorescent protein (GFP) (pCW498-35S-GFiP-OcsT, 14 743 bp (Wood et al., 2009)).

Approximately 150 000 to 200 000 washed protoplasts were mixed with 20–40 µg vector DNA in a 2 ml Eppendorf tube containing 200 µL freshly prepared MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES). Freshly prepared PEG-calcium solution (25% (w/v) PEG4000, 0.4 M mannitol, 0.1 M $CaCl_2$) was carefully added to the tube in a 1:1 ratio, and after 5 min the reaction was stopped by addition of 1.5 ml W5 and gentle mixing. The suspension was subsequently centrifuged at 100 *g* for 3 min, and the supernatant was carefully removed. The protoplasts were then re-suspended in 1 ml of MI medium and transferred to 12-well sterile tissue culture plates, wrapped in aluminum foil, and kept in the growth chamber. After 48 h, the protoplasts were observed with a Zeiss LSM 880 Airyscan confocal laser scanning microscope using an EC-Plan-Neofluar 10x/0.30 M27 objective for estimating transfection efficiency. Excitation wavelength was set to 488 nm and detection wavelength was set to 490–585 nm. To ensure that no auto-fluorescence could be observed, non-transfected protoplasts were used as a control. The transfected protoplasts were cultured on the culture media for shoot induction using the optimized regeneration protocol as described above to further verify the protocol.

RESULTS

Effect of Basal Medium on Protoplast Regeneration

In this study, we have tested widely used media in plant tissue culture, including Murashige and Skoog (MS) (Murashige and Skoog, 1962), Kao (Kao and Michayluk, 1975), B5 (Gamborg et al., 1968) and Nitsch (Nitsch and Nitsch, 1969) for their effects on protoplast regeneration of field cress. The results showed that during the early stage of protoplast culture, Nitsch medium gave the best result in maintaining a higher percentage of viable protoplasts, followed by Kao and B5, while the protoplasts grown in the MS medium become shrunken and the color faded. The protoplast viability was judged by protoplast appearance under a light microscope, which remained green in

TABLE 7 | Effect of culture duration in MI or MII medium on shoot regeneration of field cress.

Medium	Regeneration (%)[a]							
	3 days	5 days	10 days	15 days	20 days	30 days	40 days	50 days
MI[b]	53.3 a	50.0 b	9.0 c	0.0 days	0.0 days	-	-	-
MII[c]	-	-	0.0 e	9.7 days	40.0 b	55.0 a	19.3 c	5.0 de

MI medium composition: 2.18 g L^{-1} Nitsch, 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, pH 5.7. MII medium composition: 2.18 g L^{-1} Nitsch, 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, pH 5.7. SIM medium composition; MS, 15 g L^{-1} sucrose, 1.1 mg L^{-1} TDZ, 0.5 mg L^{-1} $AgNO_3$, 2.5 g L^{-1} Gelrite, pH 5.7.
[a]The results were recorded after 2 months. Values followed by the same letter were not statistically different at p*=0.05 (n = 3).*
[b]Protoplasts were cultured 25 days in MII medium prior to transfer to SIM medium.
[c]Protoplasts were cultured 3–5 days in MI.

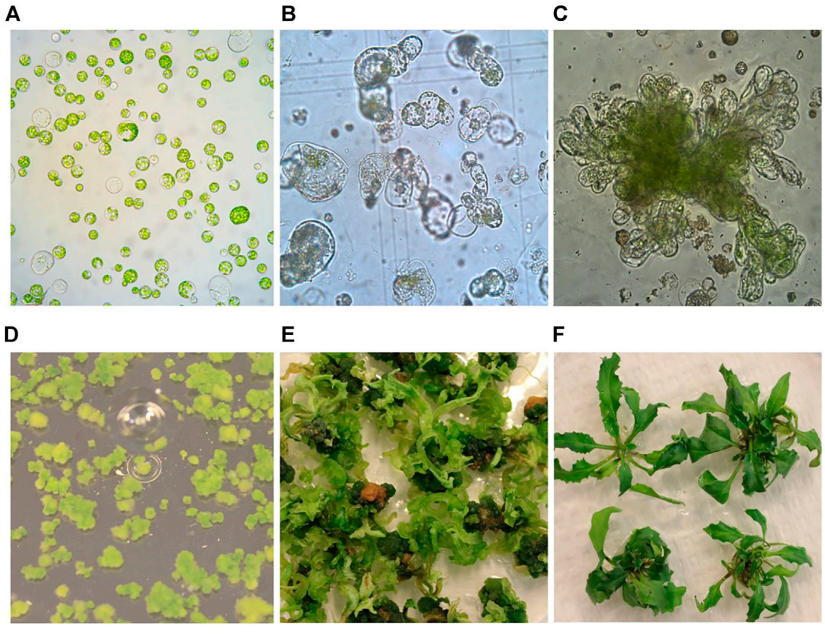

FIGURE 1 | Protoplast isolation, callus formation, and shoot regeneration of field cress. **(A)** Freshly isolated protoplasts (1 day). **(B)** Protoplasts undergoing cell divisions and multiplication (0.5 month). **(C)** Protoplast callus formation (1 month). **(D)** Protoplast colonies (2 months). **(E)** Shoot regeneration from protoplast colonies (4 months). **(F)** Regenerated shoots grown on rooting medium (6 months).

color and more round in form (**Figure 1A**). However, the protoplasts did not grow well after 14 days in the Kao medium, while only about 10% of the protoplasts from the B5 medium seemed to be viable. No shoots could be regenerated from the protoplasts initially grown on either MS, Kao, or B5 medium after 4 months. In the Nitsch medium, approximately 80% of the protoplasts grew well after 14 days, significantly higher than in Kao and B5 medium, and 75% of these protoplasts gave rise to shoots after 4 months (**Table 1**).

EFFECT OF PLANT GROWTH REGULATORS (PGRS) IN MI MEDIUM ON PROTOPLAST VIABILITY

Protoplasts are vulnerable to culture conditions, especially during the early stage of development. The first step in protoplast culture is cell wall formation, during which PGRs play a crucial role. The results of the PGR tests on protoplast growth in this study showed that 0.5 mg L^{-1} 2,4-D combined with 0.5 mg L^{-1} NAA in MI medium was essential to ensure high protoplast viability during the early culture phase. All the other types of PGRs tested would lead to inviable protoplasts, namely being faded in color and shrunken in form (**Table 2**).

EFFECT OF PGRS IN MII MEDIUM ON PROTOPLAST GROWTH

After the cell wall had formed, the protoplasts would undergo rapid cell division and callus formation, given appropriate growth conditions (**Figures 1B, C**). We investigated MII media supplemented with different PGRs to determine the most suitable PGR combinations for callus formation. The results showed that the PGR combination of 1.1 mg L^{-1} TDZ with either 0.05 mg L^{-1} 2,4-D or 0.05 mg L^{-1} NAA resulted in the highest percentage of protoplasts with callus formation (**Table 3**). This result indicates that a relatively lower concentration of auxin was necessary for protoplast development during this stage for field cress.

PROTOPLAST REGENERATION

Difficulty in protoplast regeneration is the major obstacle for the protoplast method to be used for research and crop improvement for most plant species. In order to obtain a high regeneration frequency for field cress, we have investigated the effects of type and concentration of sugars, PGR combinations, and the culture duration in MI, MII, and SIM media on callus formation and subsequent shoot regeneration (**Figures 1D–F**). The detailed results are presented below.

Effect of PGR Combinations in SIM Medium on Shoot Regeneration

PGRs is an important factor affecting *in vitro* shoot regeneration. We have thus tested different types and combinations of PGRs to find the best combination for protoplast regeneration of field cress. The results showed that 1.1 mg L^{-1} TDZ alone resulted in a high regeneration frequency (82%), while the combinations of 1.1 mg L^{-1} TDZ with 0.01 mg L^{-1} NAA and 0.1 mg l^{-1} GA_3, or 2.0 mg L^{-1} BAP with 0.1 mg L^{-1} NAA resulted in very poor regeneration frequencies (5 and 1%, respectively). All other treatments tested did not result in any regeneration. Apart from the 1.1 mg L^{-1} TDZ treatment, all other treatments resulted in hard and/or yellow calli (**Table 4**).

Effect of Carbon Source in SIM Medium on Shoot Regeneration

The results showed sugar type and concentration could significantly affect protoplast regeneration frequency (**Table 5**). Among all the treatments tested, 15 g L^{-1} sucrose gave the best regeneration frequency (80%), followed by 10 g L^{-1} glucose (67%), while 30 g L^{-1} sucrose and 20 g L^{-1} glucose resulted in relatively lower regeneration frequencies (47 and 45%, respectively), suggesting relatively lower concentrations of sugar were more effective in promoting shoot regeneration.

Effect of Cytokinin in SIM Medium on Shoot Regeneration

As our results showed that TDZ without any auxin was sufficient and efficient in promoting shoot regeneration, we investigated the effect of different concentrations of TDZ alone on shoot regeneration. The results showed that 1.1 mg L^{-1} TDZ was the best concentration tested for shoot regeneration, while higher or lower TDZ concentrations decreased the shoot regeneration frequency. Furthermore, we found that BAP was not effective for shoot regeneration (**Table 6**).

Effect of Culture Duration in MI and MII Media on Shoot Regeneration

In this study, we found that the culture duration in MI and MII media played an important role in protoplast regeneration. The results in **Table 7** showed that 3 and 5 days culture durations in MI medium gave the best regeneration results, while a culture duration longer than 10 days would inhibit protoplast growth. The culture duration in MII seemed not to have such a critical influence on regeneration frequency, but shoot regeneration was significantly delayed if the culture duration was too long. Given enough time, most of the calli derived from the cultures with an extended period of time in MII medium would eventually develop shoots, with some delay. Approximately 1 month of culture duration in MII medium resulted in the most rapid growth and highest regeneration frequency.

PROTOPLAST TRANSFECTION EFFICIENCY

The parameters affecting transfection efficiency can be species dependent, and it is thus necessary to optimize the transfection protocol for each species. We tested DNA concentrations and PEG/DNA incubation time for field cress in this study. The results showed that a transfection efficiency of 50–80% could be obtained using 25% (w/v) PEG4000, 20–40 μg plasmid DNA, and 5 min incubation time, in which no obvious variation in the GFP protein expression was found between 20 and 40 μg DNA (**Figure 2**). Furthermore, we were able to use the regeneration protocol described above to regenerate shoots from transfected protoplasts with normal regeneration efficiency. A flowchart from protoplast isolation to generation of edited transgene-free plants is presented in **Figure 3** to facilitate readers' understanding of protoplast-based gene editing. This protocol will be very valuable for our ongoing work on genetic modification of important traits in field cress by CRISPR/Cas9.

DISCUSSION

The interest in applying protoplast culture technique for plant research and crop improvement has increased alongside the increased application of gene editing by CRISPR/Cas9, as it can generate transgene-free mutant lines. However, due to the major obstacle in protoplast regeneration, application of the protoplast method for gene editing is still very limited for trait improvement for most important crops. It is very challenging to develop an efficient protoplast regeneration protocol for the majority of crop species. Protoplasts from different species, genotypes, and different tissues, may require different culture conditions for successful regeneration. Some of the critical parameters include protoplast isolation method, medium composition, culture duration, and callus development phase suitable for shoot induction, which has been shown to be critical for successful protoplast regeneration in our earlier report on rapeseed (Li et al., 2021). To obtain a high regeneration frequency it is often necessary to optimize the abovementioned important parameters and other culture conditions, which is very time- and labor-intensive. There is so far, to the best of our knowledge, no published report available on protoplast culture for field cress. In this study, we have systemically investigated some important factors affecting protoplast culture and regeneration, and have established a highly efficient and relatively simple protocol for protoplast regeneration and transfection for the species. This protocol would provide a solid foundation for further improvement of this potential novel oilseed crop through gene editing technologies, and also provide important information for developing protoplast regeneration protocols for other plant species.

Protoplasts can be isolated from various tissues and organs of plants, such as leaves, roots, petioles, cotyledons, hypocotyls, embryos, and microspores. The use of some types of tissues, such as roots and hypocotyls, usually requires a large amount of materials to obtain satisfactory protoplast yields, which makes them unpractical to use (Klimaszewska and Keller, 1987; Eeckhaut et al., 2013). Leaf tissues often provide satisfactory protoplast yields, and protoplast isolation from leaves is thus

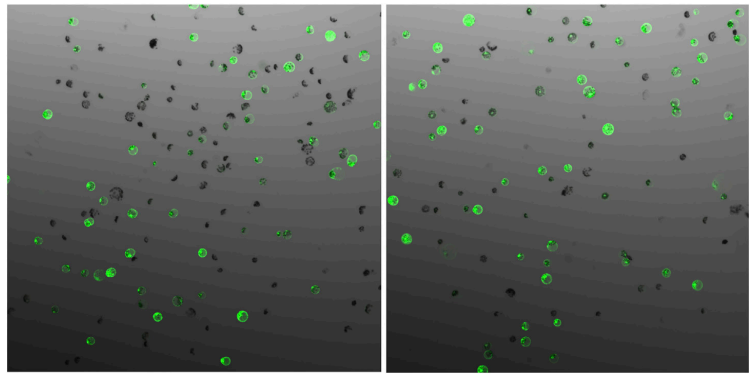

FIGURE 2 | GFP expression 48 h after protoplast transfection of field cress, showing no obvious difference when different vector DNA concentrations (**(A)**, 20 μg and **(B)**, 40 μg) were used for transfection.

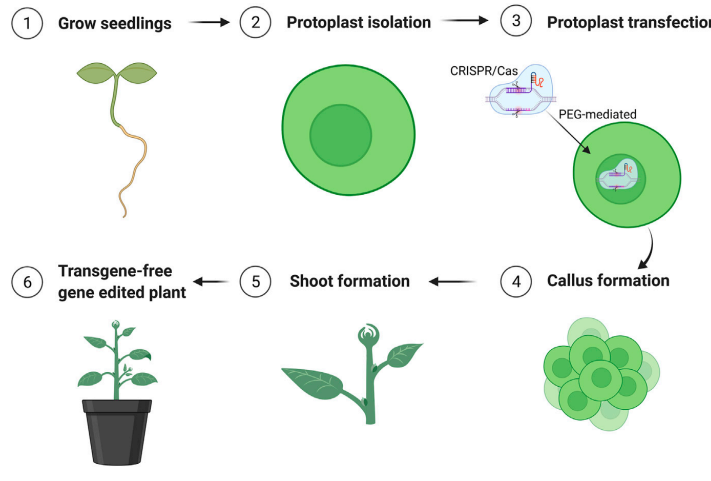

FIGURE 3 | Schematic flowchart of generation of transgene-free mutation lines edited by CRISPR/Cas9 using protoplast approach. The figure was created with BioRender.com.

preferred by most researchers (Yoo et al., 2007; Nicolia et al., 2015; Lin et al., 2020; Molla et al., 2020). In our preliminary studies, we tested both leaves and hypocotyls for protoplast isolation and culture for field cress. The protoplasts from hypocotyls could be isolated and developed into protoplast colonies, but it required several fold more plant materials to yield a satisfactory quantity of protoplasts compared with leaves. We thus only used leaves for protoplast isolation in further studies. Although not tested systematically, it seemed that fully opened true leaves from 3–4 week old seedlings was the most suitable material for protoplast isolation and subsequent regeneration. Leaves from more than 4-week old seedlings could also be used successfully, but the regeneration frequency might be compromised.

Protoplasts are naked cells without cell wall, which are very vulnerable to certain culture conditions. The first step in protoplast culture is to promote cell wall formation, and then rapid cell division and callus formation. The cell wall formation starts within a few hours after protoplast isolation and it may take several days to complete the process (Zaban et al., 2013). Protoplast cell necrosis usually occurs during this period if culture conditions are unfavorable. We found that both the MI medium composition and the culture duration in the medium are crucial for protoplast viability, growth, and

subsequent regeneration. It has been reported that for successful culture of *Brassica* protoplasts, both 2,4-D and NAA are necessary at the early culture stages to sustain protoplast survival and induce cell division, and that the appropriate ratio of NAA to 2,4-D is genotype-dependent (Glimelius, 1984). In this study, our results showed that equal amounts of 2,4-D and NAA (0.5 mg L^{-1} of each) was the most suitable for maintaining protoplast viability and subsequent regeneration of field cress. Moreover, the culture duration in MI medium also appears to be crucial for successful protoplast regeneration. In this study, 3–5 days gave the best results, as longer culture duration would cause the cells to stop growing. The culture duration in MII appeared not to be so critical compared to MI for protoplast regeneration in field cress, as a prolonged culture duration would mainly delay shoot regeneration.

Low regeneration frequency is the main obstacle affecting application of the protoplast approach in research and trait improvement for most economically important crops. Under suitable culture conditions, protoplasts would undergo a series of differentiation stages and finally form shoots. Among the factors affecting protoplast regeneration, PGRs are of critical importance. Although a high cytokinin/auxin ratio is required for shoot regeneration, this ratio often varies from genotype to genotype (Kao and Seguin-Swartz, 1987), apparently due to differencen in concentrations of both endogenous hormones. In this study, we found that 1.1 mg L^{-1} TDZ alone in the SIM medium was sufficient to give the highest regeneration frequency among all treatments tested. This is in agreement with our previous study, in which 1.1 mg L^{-1} TDZ without auxin resulted in a high regeneration frequency when hypocotyls were used as explants for genetic transformation (Ivarson et al., 2013). For sugar tests, we observed that sucrose gave a significantly higher regeneration frequency than glucose. Sucrose is often efficiently used in most of the crop species in tissue culture, likely because it is the most common carbohydrate synthesized and transported in the phloem sap of many plants. In case of the protoplasts, it is also likely that sucrose may better facilitate growth and development due to its impact on cell osmolarity (Yaseen et al., 2013).

The density of protoplasts in MI and MII media appears to be an important factor affecting the protoplast viability. Some studies suggested that relatively high protoplast culture densities would promote cell growth and division (Chuong et al., 1985; Kiełkowska and Adamus, 2012). It could be that growing protoplasts stimulate growth and mitotic division of adjacent cells by releasing growth factors into the medium (Davey et al., 2005). In this study, we also observed that a low protoplast density would usually result in poor protoplast viability during the early stage of cultures. However, a too high protoplast density would results in brownish protoplast colonies. This is probably due to competition for limited available nutrients in the medium, resulting in a large number of protoplasts failing to undergo cell divisions. In the case of field cress, we found in this study that the suitable protoplast plating density was 0.4–0.6 million protoplasts per ml. When performing transfections it was necessary to increase the initial protoplast density to 0.75–1.0 million per ml for better regeneration, as the PEG-incubation would result in a loss of protoplasts.

In conclusion, through optimizing various important culture conditions, we have developed a highly efficient protoplast regeneration and transfection protocol for field cress. This protocol will provide a solid foundation for using the protoplast approach for molecular studies and developing CRISPR/Cas9-edited transgene-free mutant lines of field cress. The protocol would also be helpful in establishing protoplast regeneration protocols for other related plant species.

DATA AVAILABILITY STATEMENT

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

AUTHORS CONTRIBUTIONS

L-HZ led the research and, together with XL, EI, and SS designed the studies. XL, SS, EI, and AA performed the experiments. XL, SS, and L-HZ wrote the manuscript. All authors read the manuscript and approved the submitted version.

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We have developed protoplast-based methods for genome editing of rapeseed and field cress by CRISPR/Cas9, and subsequent generation of mutated lines. Using these methods we have mutated multiple glucosinolate transporter genes in rapeseed and field cress, to improve the value of the seed cake. Furthermore, we have generated field cress lines with improved oil quality, which constitutes major progress towards domestication of this novel winter-hardy oilseed species.

Sjur Sandgrind received his doctoral education at the Department of Plant Breeding, Swedish University of Agricultural Sciences, Alnarp. He obtained his MSc in Biology at the Norwegian University of Life Sciences.

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