Genome-edited *Camelina sativa* with a unique fatty acid content and its potential impact on ecosystems

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Abstract

‘Genome editing’ is intended to accelerate modern plant breeding enabling a much faster and more efficient development of crops with improved traits such as increased yield, altered nutritional composition, as well as resistance to factors of biotic and abiotic stress. These traits are often generated by site-directed nuclease-1 (SDN-1) applications that induce small, targeted changes in the plant genomes. These intended alterations can be combined in a way to generate plants with genomes that are altered on a larger scale than it is possible with conventional breeding techniques. The power and the potential of genome editing comes from its highly effective mode of action being able to generate different allelic combinations of genes, creating, at its most efficient, homozygous gene knockouts. Additionally, multiple copies of functional genes can be targeted all at once. This is especially relevant in polyploid plants such as *Camelina sativa* which contain complex genomes with multiple chromosome sets. Intended alterations induced by genome editing have potential to unintentionally alter the composition of a plant and/or interfere with its metabolism, e.g., with the biosynthesis of secondary metabolites such as phytohormones or other biomolecules. This could affect diverse defense mechanisms and inter-/intra-specific communication of plants having a direct impact on associated ecosystems. This review focuses on the intended alterations in crops mediated by SDN-1 applications, the generation of novel genotypes and the ecological effects emerging from these intended alterations. Genome editing applications in *C. sativa* are used to exemplify these issues in a crop with a complex genome. *C. sativa* is mainly altered in its fatty acid biosynthesis and used as an oilseed crop to produce biofuels.

Keywords: Genome editing, CRISPR/Cas, *Camelina sativa*, Environment, Fatty acid composition, Polyploidy, Volatile organic compounds, Plant communication

Background

‘Genome editing’ encompasses techniques such as oligonucleotide-directed mutagenesis (ODM) and site-directed nucleases (SDNs) like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases and clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) techniques. In this paper the terminology ‘genome editing’ is used even though there is some controversy about the term [1, 2]. Recently published literature reviews show that CRISPR/Cas has become one of the most dominant techniques of SDNs applied in plants over the last few years [3, 4]. Therefore, the focus here is on CRISPR/Cas-applications. CRISPR/Cas allows the targeting of an endonuclease (e.g., Cas9 from *Streptococcus pyogenes*) to specific genomic regions using a guide RNA (gRNA) [5, 6]. The gRNA is designed depending on the genomic loci to be altered. Cas9 interacts with the gRNA and upon recognition of the target sequence introduces a DNA double-strand break (DSB) at that part of the genome [7]. DNA DSBs subsequently activate the non-homologous end joining (NHEJ) repair and homology-directed repair (HDR) [8–10]. The NHEJ pathway is
known to be error-prone and frequently results in base insertions or deletions (indels) at the DNA break sites [11]. These indels can generate frameshift mutations or disrupt important functional domains, which, for example, disturb the functions of the target genes [12]. The HDR pathway utilizes exogenous DNA donor templates to introduce nucleotide substitutions and DNA insertions at the target sites [13, 14]. Applications using SDNs are used to either introduce small-sized, undirected (SDN-1) or directed sequence changes (SDN-2 and SDN-3) at specific, predefined genomic loci [15]. SDN-3 approaches aim to insert transgenic constructs at specific, predefined locations [16]. In addition to the intended alterations, CRISPR/Cas causes unintended alterations including off-target effects, on-target effects and chromosomal rearrangements [4, 17–21]. These unintended alterations could potentially lead to a variety of unexpected effects. For example, the integrity of a non-target gene may be compromised if its coding region has been cleaved by CRISPR/Cas. This could lead to changes in the organisms’ metabolism, which could affect its toxicity and allergenicity. Such effects are highly dependent on the genomic context within which such unintended alterations occur [3, 22]. Unintended effects can also be induced by applying first-generation genetic engineering techniques to insert the CRISPR/Cas components into plant cells [23–28]. A detailed and comprehensive description of unintended effects in the genome that correlate with the application of genome editing and older genetic engineering techniques is given elsewhere [3, 22].

Here, the special focus is the potential of SDN-1 techniques to generate novel genotypes and the impact of intended changes in genome-edited plants in relation to the interactions in their respective environments. Numerous applications of genome editing in crops have already demonstrated that SDN-1 techniques can produce plants with novel genotypes resulting in traits unlikely to be achieved by conventional breeding techniques [3, 4, 29–34].

Camelina sativa is an allohexaploid plant composed of three sub-genomes which originate from closely related species [35, 36]. Thus, it contains multiple alleles of homologous genes. SDN-1 applications have already been applied in C. sativa, primarily to alter fatty acid composition [37–39], but also modulating the seed meal protein composition by editing factors such as cruciferins [40]. Such alterations are extremely difficult with conventional or mutagenesis breeding as changes to multiple alleles of genes are required. Thus, C. sativa serves as a good example to demonstrate the power of SDN-1 genome editing.

C. sativa is an annual plant in the Brassicaceae family and cultivated mostly in Europe and in North America. Camelina is closely related to the plant model organism Arabidopsis thaliana and the oilseed crop Brassica napus. Unlike other crops of the Brassicaceae family, camelina has historically not been subjected to extensive breeding, and only a small number of cultivars are available for agricultural purposes [41]. However, over the previous decade, C. sativa has become more popular mainly because of its seed oil composition. Camelina oil contains high amounts of polyunsaturated fatty acids (PUFAs) such as linoleic acid and linolenic acid, which are essential omega-6 and omega-3 fatty acids, respectively [42, 43]. The oil is mainly used to produce biofuels, industrial compounds, dietary supplements and human food [44–46]. PUFAs are known for the formation of trans-fatty acids during processing as well as their oxidative instability. Therefore, the genetic material of camelina is being altered to shift the content from linoleic and linolenic acid towards the monounsaturated fatty acid oleic acid which becomes less easily oxidized.

In general, the outcomes of genome editing applications in crops are considered to require assessment on three different levels [3, 22, 47]: in regard to (1) unintended effects resulting from the genetically engineering process, (2) the effects of the intended alteration(s) on the metabolism of the genome-edited organism and its overall composition, and (3) the ecological impact of the genome-edited organism on the receiving environment(s). This paper uses published research on the application of SDN-1 in C. sativa to provide evidence of the extent of genomic changes possible using only SDN-1 applications and how these intended changes have the potential to unintentionally alter secondary metabolism. The intended trait and potential unintentional changes to secondary metabolism are considered in the context of potential ecological consequences following a release to the environment. Finally, the significance in the EU for the regulation of genome-edited crops, developed through the application of SDN-1, is outlined.

Genomic content of C. sativa

Major agricultural relevant crops such as rapeseed, wheat, potato, cotton, apple, sugarcane and camelina are polyploid, i.e. combine more than two paired sets of chromosomes, which either originate from genome doubling within a species (autopolyploids) or interspecies hybridization (allopolyploids) [35]. Hutcheon et al. (2010) suggested that C. sativa is allohexaploid with three single-copy nuclear genes present as three paralogous copies in the genome [48]. Kagale et al. (2014) confirmed the allohexaploidy by publishing a reference genome and showing that camelina contains three sub-genomes of an unknown origin [49]. One of the sub-genomes contains six chromosomes, while the other two contain
seven chromosomes each [49]. Recently, it was proposed that the allohexaploid genome of *C. sativa* (*n* = 20, N⁶, N⁷,H) originated through hybridization between an autotetraploid *Camelina neglecta*-like genome (*n* = 13, N⁶, N⁷) and *Camelina hispida* (*n* = 7, H) [36]. The three sub-genomes remained overall stable since the genomes merged without large translocations between homeologs [36]. Genomic in situ hybridization confirmed that *C. sativa* contains 20 chromosomes (2n = 40) [36] and has a genome size of approximately 785 Mbp [49, 50]. The genome has a high gene density encoding 84,699 genes, the sub-genomes each encoding 28,274, 27,218 and 29,207 genes, respectively [49].

Defining the relationships between the camelina species may help to identify species that are potential novel sources of allelic variation for introgression into *C. sativa* [51]. So far, little genetic diversity exists in currently available *C. sativa* cultivars limiting the effectiveness of traditional breeding programs [52–54].

Camelina displays diploid inheritance in common with most allopolyploid plants, meaning each gene only pairs to its own homolog within its sub-genome [48, 55]. The transcriptome of *C. sativa* has already been published [56–59]. The genome sequence of camelina was also shown to be closely related to the model organism *A. thaliana* with almost 70% of the annotated genes in the camelina genome being syntenically orthologous to *A. thaliana* genes [49]. Both arabidopsis and camelina are classified as members of the tribe Camelineae [60–62] indicating the close phylogenetic relationship. The allohexaploid genome of *C. sativa* with three copies of homologous genes and low efficiency of producing double haploids complicate research and classical breeding attempts [63, 64].

**Alteration of the fatty acid content of *C. sativa***

The fatty acid content of the camelina seed is of major interest for plant breeders as is the nutritional composition of the residual meal after pressing and extracting the oil from seeds. The oil content of camelina seeds is high, often between 32 and 49% of the seed weight depending on the genotype, growth conditions, and fertilizer used [41]. Beside PUFAs, Camelina also contains very-long-chain fatty acids, both are known for low oxidative stability, poor cold flow and a high melting point, making it less utilisable for biofuels and bio-based chemicals applications [65, 66]. Oxidative stability can be increased by reducing the content of highly unsaturated fatty acids and is mainly achieved by an enrichment of oleic acid, which was already done in soybeans [67, 68]. Oleic acid was found to have higher oxidative stability than linoleic acid, resulting in the extension of its shelf life [69]. Oleic acid is desaturated to linoleic acid by the fatty acid desaturase (FAD2) in the endoplasmic reticulum (ER) [70]. Three FAD2 genes (*CsFAD2-1, -2 and -3*) were identified in *C. sativa*, with *CsFAD2-2* and -3 being expressed exclusively in developing seeds, and *CsFAD2-1* in all tissues of the plant [48, 71]. Further desaturation of linoleic acid to linolenic acid is accomplished by the omega-3 fatty acid desaturase (FAD3) also located in the ER [72]. The content of oleic acid was primarily increased by suppression of FAD2 genes, thereby subsequently decreasing the content of PUFAs [48, 57, 71]. Established methods of FAD2 suppression include standard ethyl methane-sulfonate (EMS)-mutagenesis followed by selection leading to plants with oleic acid content increased from 17 to 27% [71]. This effect results from a point mutation in the *CsFAD2-2* gene, whereas the other two homeologous genes *CsFAD2-1* and *CsFAD2-3* were not affected explaining the moderate effect [71]. Thus, even though all *CsFAD2* genes are expressed in the seeds of camelina, the mutation in *CsFAD2-2* cannot be compensated by the other two variants. A transgenic approach to increase the oleic acid content relied on RNA interference (RNAi) leading to a knockdown of FAD2 and an increased oleic acid content of up to 50% [57]. Camelina seeds also contain high amounts of very-long-chain fatty acids, primarily eicosenoic acid and erucic acid. Oleic acid is converted to eicosenoic acid by an enzyme called fatty acid elongase 1 (FAE1) [48]. A knockdown of FAE1 in addition to FAD2 by RNAi lead to an even higher increase of oleic acid [57]. Nevertheless, it is more advantageous to work in a mutant background to obtain a more genetically stable phenotype compared to a gene knockdown by RNAi applications. Therefore, genome editing is now being applied to camelina to generate high oleic acid plants, which is advantageous for scientists and breeders to reach their breeding goals as these techniques are considered faster than conventional or mutation breeding.

**Differences between genome editing and conventional breeding**

Alterations mediated by SDN-1 applications of genome editing are sometimes equated with the outcome of conventional or mutagenic breeding, which underestimate the power of genome editing. SDN-1 applications have potential to penetrate the whole genome and cause profound alterations in the biological characteristics of plants without introducing any additional DNA sequences. Such applications, in many cases, will result in new combinations of genetic information. The risk to unintentionally interfere in the metabolism of a plant with the intended alterations mediated by genome editing increases with its complexity.

Genome editing enables researchers and breeders to alter genomic regions, that were not accessible so far.
Some studies show that the occurrence of (spontaneously occurring) de novo mutations in certain regions of the genome is less likely than in others due to the activity of the DNA mismatch repair correlating with certain cytogenetic factors like H3K36me3 and the GC content [73–75]. These results show that the persistence of mutations in the genome is not only due to their random occurrence and subsequent selection but is also subjected to other cellular mechanisms that protect certain parts of genomes. Genome editing with its highly efficient mode-of-action enables to alter these protected genomic regions. Additionally, genome editing also enables further changes of the genome, that were not feasible until now [29]: CRISPR/Cas can alter all target sequences towards which a gRNA can be directed. Thus, multiple alleles of a gene, all members of a gene family or repetitive DNA sequences can be changed in one CRISPR/Cas application. In addition, it is possible to introduce more than one gRNA at a time to target several different genomic loci [3, 22, 29, 76, 77]. These applications are summarized under the term multiplexing [78, 79]. Multiplexing is increasingly being used to achieve fast and efficient editing of multiple genes in a range of target organisms [31, 33, 34, 80–82].

Camelina is a good example to demonstrate the power of CRISPR/Cas techniques compared to conventional and mutagenesis breeding. As mentioned camelina is allopolyploid, i.e., genes of interest exist in several copies. With conventional breeding as well as classical genetic engineering, it is difficult if not practically impossible to change several copies of a gene in different locations in the genome, especially when they are located in different parts of the genome. Depending on the target sequence and the designed gRNA, it is possible that homoeologous genes in only one or two of the sub-genomes of camelina are edited. Additionally, gene copies on all three sub-genomes can either be edited using one gRNA targeting a DNA sequence of homology to all three genes or through multiplexing approaches using different gRNAs [78, 79]. CRISPR/Cas also allows to investigate and change the gene dosage by, for example, developing different mutant fad2 lines for the identification of a desirable fatty acid profile from different allelic combinations, while simultaneously diminishing unwanted side effects [38]. For that, Morineau et al. (2017) targeted CRISPR/Cas9 to conserved regions in the sub-genomes of C. sativa to alter all CsFAD2 genes [38]. Combinations of different alleles of the three FAD2 target loci were generated, which allowed the evaluation of gene dosage on the accumulation of various levels of PUFAs and the effect thereof on the overall development of the plants. In some mutated fad2 lines, mutations in all three FAD2 homoeologs in the T3 generation were identified and showed drastic developmental defects [38]. The plants showed impaired growth, twisted leaves, and delayed bolting, indicating the importance of a well-balanced fatty acid profile for the development of the plants. These phenotypic defects were even more severe in a recently conducted field trial in the UK of genome-edited camelina containing CsFAD2 double and triple knockouts [83]. In another publication, all gene copies encoding FAE1 were targeted causing an increase of oleic acid content from 13% up to 20% and a reduction of very-long-chain fatty acids from 12 to 1% [39]. No direct effects on the development of the seed and growth of the gene-edited plants were observed. However, effects on metabolism, signalling pathways or further changes in fatty acid biosynthesis were not investigated and can, therefore, not be excluded [39]. These examples of minor changes by SDN-1 applications show that major changes of plant physiology and/or phenotype become possible. In addition, there is evidently potential of disrupting metabolic pathways in the genome-edited plants causing pleiotrophic effects.

**Possible ecological effects of intended alterations induced by genome editing**

Camelina is used in the following as an example to illustrate possible ecological risks that might be associated with a release of genome-edited plants. In addition to generating already existing genetic variants or genetically modified organisms, CRISPR/Cas is frequently used to induce complex alterations in plant genomes using SDN-1 approaches generating novel traits [3, 29, 76]. These novel traits can influence the composition of the genome-edited plants, which can have unintended ecological consequences. Hardly any study using genome editing considers the impact of these novel traits on the respective ecosystem. Thus, there is need for debate on potential ecological risks when genome-edited organisms are released into the environment, also considering the speed of newly developed genome-edited plants and especially combinatorial and accumulating effects upon the release of many different genome-edited organisms.

**Altering the fatty acid biosynthesis can impact the stress response of the genome-edited plant**

Applications of genome editing in C. sativa are currently mainly performed to alter its fatty acid biosynthesis. The intended change of the fatty acid biosynthesis can affect the synthesis and content of additional fatty acids and derived compounds and thereby affect for example stress response of the genome-edited plant. PUFAs are an important component of cellular membranes regulating their fluidity, in particular for adaptation to changing climate conditions. The membrane fluidity can be
considered to be influential to physiological regulation and efficiency of transport processes through the membrane, opening up a wide field of secondary effects that may become apparent under specific external (environmental) conditions only and are difficult to predict or to identify in standardized test situations. In plants, temperature is a major environmental factor that influences fatty acid desaturation. Research on *A. thaliana* has already shown that *fad2* mutant lines were not able to survive in low temperatures [84]. Also, high salt conditions impair the development and survival of mutant *fad2* arabidopsis lines [85]. Compared to the wild type, these *fad2* mutants showed affected root growth, impaired seed germination and a reduced survival rate under high salt conditions. Their abnormal fatty acid profile resulted in an altered composition of membrane lipids and affected the fluidity of their cell membranes. Most likely the integrity of salt ion transporter proteins is disrupted under high salt conditions in *fad2* mutant lines [85]. Furthermore, *fad2* and *fad6* double mutants in arabidopsis indicated that PUFAs are necessary for the composition of cell membrane in chloroplasts to maintain photosynthesis in leaves [86]. Overall, these studies show that the alteration of the fatty acid profile in *fad2* mutated arabidopsis lines can cause severe impairments under abiotic stress conditions. A similar effect can be expected in the closely related *C. sativa*.

It has already been shown that abiotic stress such as salinity changes the gene expression in camelina resulting in altered fatty acid biosynthesis [87], indicating a link between stress response and fatty acid content in the plants. Another structure that can be affected are plant apoplastic barriers, such as the cuticula and suberized tissues, because they comprise polymerized very-long-chain fatty acids as well as non-covalently bound waxes thereof. During cuticular wax biosynthesis, C16- and C18-fatty acids are converted to very-long-chain fatty acids by FAE complex enzymes and subsequently converted to major wax components. Suberin is a glycerolipid-phenolic biopolyester and serves as a protective barrier in the cell wall of different tissue layers such as root endodermis, root and tuber peridermis, and seed coats in plants [88]. Cutinized and suberized barriers control, among others, water and ion transport in these tissues enabling the plants to withstand abiotic stresses, such as drought and salinity, and also biotic stresses acting as anti-microbial barriers [89, 90]. Camelina also contains a wide range of cuticular waxes that mediate the barrier functions and regulate drought tolerance [91], indicating that an extensive intervention in the fatty acid biosynthesis by genome editing techniques can cause unintended effects under abiotic stress. Future studies are needed to understand cuticle metabolic pathways in camelina and the ecological function of specific cuticle lipid profiles, as well as the gene network that regulates their expression properly [91]. In summary, fatty acids and their derivates are part of the composition of many structures in plants, for example, the cell membrane of chloroplasts or the cell wall of roots, that are crucial for the adaptation of plants to stress. Thus, genome-edited changes to fatty acid profiles can affect the plant’s response to stress.

**Intended alterations altering the fatty acid content can influence the synthesis of secondary metabolites in plants**

Intended alterations mediated by genome editing can cause additional changes to the composition of the plant by affecting downstream metabolism (e.g., secondary metabolites). Besides their role in the homeostasis of cell membranes, fatty acids are also essential precursor molecules for several secondary plant compounds, e.g., phytohormones or volatile organic compounds. PUFAs are the starting point for the biosynthesis of oxylipins such as the phytohormone jasmonic acid (JA) and its derivates (such as methyl jasmonate, cis-jasmonate and several other metabolites) or green leaf volatiles [92]. The precursor molecule of JA biosynthesis is linolenic acid, which is released from galactolipids of cell membranes [93]. The initial and rate limiting step in the biosynthesis of JA is the oxygenation of linolenic acid by a lipoxygenase. The complete biosynthesis pathway of jasmonate is reviewed in detail elsewhere [92, 94]. Like other hormones, jasmonates affect a variety of physiological activities, such as growth or leaf senescence, but also have important roles as signalling molecules in plant defence, particularly as a defence against insect herbivores and necrotrophic pathogens [95–97]. Jasmonates are interconnected in a complex network of different signalling pathways (e.g., crosstalk with gibberellin and ethylene signalling) providing plants with regulatory mechanisms to rapidly adapt to environmental changes and stress conditions [98, 99]. One major factor regulating JA biosynthesis is the substrate availability of linolenic acid upon external stimuli such as wounding. If the plant’s ability to synthesize JA is impaired, it is highly likely to become more susceptible to herbivore attacks, diseases and abiotic stress.

Indications that low levels of linoleic and linolenic acid (e.g. by mutations in FAD2 and FAD3) can raise the susceptibility of plants to pests due to low jasmonate levels come from work on soybeans [100]. The soybean aphid (*Aphis glycines*) is an insect pest which can reduce soybean yield by up to 40% upon infestation [101]. Aphid-infested soybean plants have a reduced level of PUFAs in their leaves and an increase of palmitic acid. PUFAs were also reduced in the seeds associated with an increase of stearic acid and oleic acid [100]. Challenging these aphid
infested plants with other pests did not result in any effective jasmonate-dependent defence reactions. Soybean aphids likely reduce the activity of FAD2, thereby reducing the availability of linolenic acid as a precursor molecule of jasmonate, making these plants more susceptible to other pests [100]. Another effect is a decreased formation of volatile organic compounds, which act as signalling molecules and would attract aphid predators [100]. Under normal conditions, an enhanced production of JA in the soybean leads to the production of methyl salicylate, a volatile organic compound that attracts *Coccinella septempunctata*, a common predator of the soybean aphid [102]. In summary, genetic changes in a genome-edited plant can potentially cause additional changes to secondary metabolism, affecting the genome-edited plant’s ecological interactions. Ultimately, this can impact the respective associated ecosystem in case of a release.

**Altering the lipid content of a plant has an impact on the associated food web**

Lipids, including fatty acids, have essential functions for many biological processes, including energy supply and signalling, and are structural components of cell membranes, both in animals and plants. Animals require linolenic acid as a precursor for many biomolecules for their proper development and the maintenance of health and survival. As animals cannot synthesize PUFAs, they, therefore, have to be part of their diet [103]. There are multiple examples demonstrating that an altered fatty acid content of plants can have an impact on the associated food web (e.g., insects that consume them). Recently, the effect of an omega-3 dietary deficiency on the cognition of honeybees was tested [104]. Bees on a low omega-3 diet had reduced levels of PUFAs in their body, a slightly reduced brain and a reduced hypopharyngeal gland. The omega-3 dietary deficiency also greatly reduced the bee’s performance in both olfactory and tactile associative learning assays [104]. In other, classic transgenic approaches camelina seeds were genetically modified to produce the long-chain PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), that naturally occur in marine fish only and cannot be found in terrestrial animals [105, 106]. The transgenic camelina presents a source of EPA and DHA which is new to the terrestrial environment. It is currently being tested in field trials in the UK [83, 105]. The impact of artificial EPA and DHA added in the diets of a terrestrial crop pest of Brassicaceae plants, larvae of the cabbage white butterfly *Pieris rapae*, was tested [107]. If larvae of *P. rapae* were fed EPA and DHA, the adults were heavier and had smaller wings compared to animals that were fed on normal canola oil [107]. The amount of EPA and DHA added to the artificial diets of *P. rapae* larvae are slight underestimates of those expected by the consumption of the leaves of genetically engineered oilseed crops [107]. This study indicates that an altered fatty acid composition in plants can have an impact on the associated food web, showing the necessity for an adequate assessment of these plants. Colombo et al. (2018) recently discussed ecological and potential evolutionary consequences of EPA- and DHA-producing camelina plants, also highlighting that risks emerging from such transgenic plants need to be critically evaluated [108].

**Additional ecological concerns regarding the release of genome-edited plants**

There are general aspects that need to be considered for a robust environmental risk assessment of genetically engineered plants (including genome-edited plants) [22, 109]. Genetically engineered plants can escape from cultivation and become serious weeds if the traits confer a selective advantage. Feral populations of genetically engineered plants can also become a reservoir for future GMO contamination and this is especially relevant for plants such as camelina that can persist and propagate in the agricultural environment [110]. The genome-edited plants can also hybridize, enter new habitats and infiltrate new phytosociological contexts. Gene flow from genome-edited crops to closely related native or non-native plants can occur potentially providing wild species with a greater capacity in natural selection, especially if the gene confers traits that improve reproduction and survival [111–113]. Camelina is a mainly self-pollinating plant, but some studies indicate that where insects from different taxa visit camelina, most likely attracted by its abundant nectar concentrations and pollen, cross-pollination cannot be excluded [114–116]. Honey bees (*Apis mellifera*), wild bees of the genera *Lasioglossum* (sweat bees), *Hylaeus* (face masked bee) and hoverflies (*Syrphidae*) have been observed as the main flower-visiting taxa of camelina [114, 115]. However, there is no proof yet that insects carrying camelina pollen between plants can cause gene flow. *C. sativa* is sexually compatible with closely related species such as *Camelina microcarpa*, *Camelina rumelica* and *Camelina alyssum*, thus gene flow cannot be excluded [117]. The structure of the genomes of *C. sativa* and *C. microcarpa* appear to be identical indicating a common origin supporting the suggestion that *C. microcarpa* is the wild pre-domesticated hexaploid ancestor of *C. sativa* [36]. Camelina can also hybridize with the species *Capsella bursa-pastoris*, but the reproductive fitness of their hybrids is low, resulting in sterility in the second generation [118, 119]. Nevertheless, hybrids of *C. Sativa* and *C. bursa-pastoris* are
very likely as the latter is an abundant agricultural weed increasing the probability of outcrossing [119].

In case genome-edited plants (e.g. the high-oleic, genome-edited camelina) can persist and propagate outside of the agricultural environment in addition to producing viable offspring, next-generation effects can occur in subsequent generations [110]. Next-generation effects emerging from spontaneous propagation and gene flow can be influenced by heterologous genetic backgrounds and unexpected effects can be triggered in interaction with environmental conditions [110]. Thus, if the plants can persist in the environment and/or if gene flow with domesticated and/or wild relative plants can be established, leading to viable offspring, then hazard identification and characterization must include several and complex scenarios with hazards that cannot be predicted from the data of the original events. Therefore, even when changes as introduced by genome editing in camelina might not increase their fitness, hybrids in future generations might show increased survival rates. Such effects may cause irreducible uncertainty in the risk assessment [110].

Significance for the regulation status of genome-edited crops

In 2018, the European Court of Justice ruled that genome-edited organisms are regulated under the full provisions of the Directive 2001/18/EC for the deliberate release of GMOs [120]. Thus, in the EU, all genetically engineered organisms, including genome-edited plants, need to undergo an environmental risk assessment [120]. Risk assessment guidelines for products of first-generation genetic engineering technology have been developed by the European Food Safety Authority (EFSA) for the environment [109] and for food and feed [121]. The regulatory situation in Europe in regard to GMOs stands in contrast to some other countries like the U.S., where many plants derived from processes of genome editing are exempted from any oversight [122–124]. In Europe, there is an ongoing debate whether certain genome-edited organisms that were altered by SDN-1 (and possibly also by SDN-2) applications should be exempted from the EU GMO regulation [125, 126]. The argument for that is, that the results, i.e. the mutations, of classical breeding, traditional mutagenesis and naturally occurring mutations are of the same type (i.e., point mutations, small indels) as the outcomes of SDN-1 applications of genome editing. As shown in this paper, the scale and the possibilities to induce far reaching changes in the genome by SDN-1 applications are different from classical and mutagenic breeding techniques. Genome Editing allows the generation of novel genotypes in these crops. The resulting intended biological characteristics of the genome-edited plants may pose substantial new challenges for the comparative approach as currently applied in the EU [109]. Another additional challenge for the risk assessment is the identification of adequate comparators or their absence. Therefore, additional approaches, technologies and concepts that include and (where appropriate) go beyond the current regulatory regime need to be developed to adequately assess the risks of these plants [3, 22, 47].

Conclusions

SDN-1 and SDN-2 applications of CRISPR/Cas induce small-sized changes of the DNA sequence such as small insertions or point mutations at targeted genomic regions. These alterations are often considered comparable to naturally occurring genetic variants in crops. However, many genome-edited plants contain traits or complex genetic combinations that so far have not been established using conventional approaches and must be considered novel. This novel genetic variability can cause unwanted effects in the plants during their development or under stress conditions, and potentially disturb signalling pathways and ultimately plant-environmental interactions in case of a release.

Many plant species have complex genomes exhibiting considerable diversity in both size and structure [127]. Challenges to plant breeding include polyploidy, a large number of orthologous genes, heterozygosity, repetitive DNA and the genetic linkage of multiple genes [22]. Genome-edited C. sativa was used here as an example to illustrate how far biotechnology has come to generate novel, genetic combinations in an agriculturally relevant crop, and the likely and potential ecological impacts. Since C. sativa is an allohexaploid plant composed of three sub-genomes homozygous mutations of homoeologous genes require a lengthy breeding process. Genome editing is supposed to induce changes in complex genomes of, for example, camelina, wheat or sugarcane [31, 34, 38] generating novel genotypes in plants. These intended alterations can interfere with the metabolism of the plants, which might be undetected in risk assessment. Alterations at multiple target sites of the genome are also possible having the potential to fundamentally intervene in the metabolism of a plant. Attractive molecular targets in camelina are genes that are involved in fatty acid biosynthesis to change the oil composition of seeds that serve different needs: human consumption, generation of bio-products and biofuels. As yet, it has mostly been ignored that crops altered in their fatty acid content might also be impaired in their ability to produce biomolecules essential for a proper signalling of the plants in their respective environments. High-oleic camelina could be impaired, for example, in its fatty acid
biosynthesis causing altered defense mechanisms and stress responses. A still underestimated and less well understood part of communication between plants or plants and animals (i.e., insects) relies on the production of volatile organic compounds produced by plants. These volatiles can, for example, attract insect species, or might act as warning signals for other plants in case of an herbivore attack. There is a need for more research on how these volatiles work in plants communication to assess the impact of complex and novel biological characteristics of genome-edited plants on other species in their respective environment.

In its most effective way, CRISPR/Cas might change all alleles of a gene leading, as was also the case for studies in *C. sativa*, to the generation of weaker plants as compared to its wildtype counterparts. CRISPR/Cas enables the identification of the best allelic combinations maintaining the best attainable fitness of the plants and obtaining plants with the desired, novel characteristics. Thus, it is unlikely that researchers will commercialize genome-edited crops that show a poor performance in the field. However, if cultivated it has also to be considered that, due to gene flow, hybrid effects may occur in next generations causing enhanced or lowered fitness that cannot be predicted from the original event or varieties as commercialized.

Beside camelina, also in other crops complex, genomic alterations are generated by genome editing efficiently fast giving rise to many novel traits [4]. These traits include, for example, alteration of agronomic value [128, 129] or nutritional quality [130–134] showing a need for a proper environmental risk assessment and documentation of these organisms in adequate databases [135].

In summary, in regard to environmental risk assessment, there are additional challenges concerning genome-edited plants that may go beyond current experiences with transgenic plants. These include changes in the composition of plants that may impact the weediness, the food web and their invasiveness. Genome-edited plants containing complex alterations of their biological characteristics causing larger metabolic changes also challenge the comparative approach in the EU, because it may be difficult to identify adequate comparators [76].

There are also special concerns regarding interventions in well-balanced signalling pathways that regulate communication and interactions between plants, animals, associated microbiomes, beneficial predators and pollinators potentially affecting ecoservices. In addition, next-generation effects can occur in case genome-edited plants have the potential to persist and propagate in the environment. Risk assessment related to novel traits will require additional knowledge of their consequences for the organism and the ecological impacts when released into the environment. This is particularly necessary for biological characteristics where experience with either current GM plants or conventional plants are lacking. In addition, genomic irregularities may be important in terms of gene x environment interactions and could be combinatorial and/or cumulative. This aspect could magnify uncertainties and unknowns in regard to environmental risk assessment of genome-edited organisms and the potential of the occurrence of next-generation effects [22, 110].

### Abbreviations

- A. thaliana: Arabidopsis thaliana
- C. sativa: Camelina sativa
- CRISPR/Cas: Clustered regularly interspaced palindromic repeats/Clustered regularly interspaced palindromic repeats-associated
- DHA: Docosahexaenoic acid
- EFSA: European food safety authority
- EMS: Ethyl methanesulfonate
- EPA: Polyunsaturated fatty acids
- FAE1: Fatty acid elongase 1
- GC-content: Guanine-cytosine content
- GMO: Genetically modified organism
- gRNA: Guide ribonucleic acid
- HDR: Homology-directed repair
- JAS: Jasmonic acid
- NHEJ: Non-homologous end joining
- NMD: Nutritional quality
- P. rapae: Pieris rapae
- PUFA: Polyunsaturated fatty acids
- RNAi: RNA interference
- SDN-1: Site-directed nuclease-1
- SDN-2: Site-directed nuclease-2
- SDN-3: Site-directed nuclease-3
- TALEN: Transcription activator-like effector nuclease
- TILLING: Targeting induced local lesions in genomes

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### Authors’ contributions

KK planned and wrote the manuscript. The author read and approved the final manuscript.

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### Declarations

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### Competing interests

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

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