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Genome-edited plants in the field

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The application of site directed nucleases (SDN) for Genome Editing (GE) in plant breeding and research increases exponentially in the last few years. The main research so far was on 'proof of concept' studies or improvement of the precision and delivery of the SDN. Nevertheless, a reasonable amount of research is present on market-oriented applications for cash crops such as rice but also for commercially lesser interesting crops and vegetables. Reported field trials involving GE plants are scarce around the world and almost not existing in Europe. This is due to the regulatory landscape for GE plants, which is quite distinct and especially in the European Union very demanding. By far the most field trials involve GE rice varieties in the Asian area, followed up by tomato and other vegetables and crops.

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Introduction

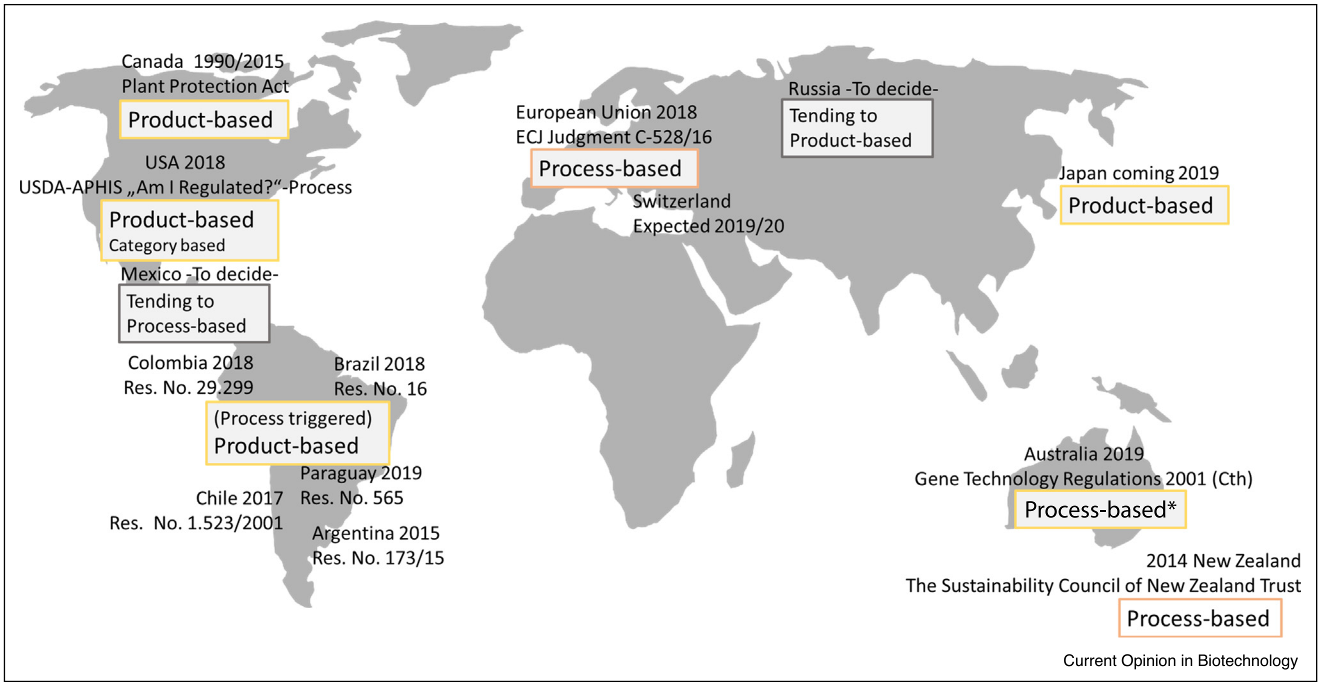
Application of Genome Editing (GE) in plants using site directed nucleases (SDNs) boosted in the last years plant basic research as well as professional plant breeding and commercial applications. Because of the enormous success of CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR associated protein 9)), the genome of at least 40 different plants, including also seldom used niche crops such as Cape gooseberry or watermelon were modified in the last few years [1,2,3••]. The number of publications on directed mutagenesis using SDN is increasing exponentially each year and this should have led to a large number of field trials involving GE plants. However, considering the worldwide diverse legal situation of plants produced by GE this seems not to be the case. For the European Union the European Court of Justice (ECJ) decided in July 2018 that plants produced by new methods of directed mutagenesis are regulated like GMOs (Court of Justice of the EU, case C-258/16). This leads to the situation, that every field trial involving GE plants must been authorized by the competent authorities in the respective member state and in fact only a handful of such field trials have been filed so far. In contradiction to this, in the USA as well as in most South American countries namely Argentina, Brazil, Chile and so on, in Australia and soon in Japan GE plants are under no GMO regulation at all, or deregulated as far as no transgenic sequences persists in the resulting plants (Figure 1) [4]. Therefore, we found not many reports on field trials because it is either too cumbersome to perform them (EU) or it is not mentioned explicitly as a special field trial (South America, USA, China) as the plants are treated like other mutagenized plants. As a logical consequence, there is no special affordance to make risk assessments of GE plants in most countries outside the EU and there is to our knowledge so far no special report at all on this topic. However, there is a number of publications investigating GE plants and their agronomic performance under field conditions (Table 1).

Genome-edited plants in the field

Rice

By far the most field trials with GE plants exist for rice and took place in China (Table 1). One of the oldest studies using GE analyzed the four major regulators of yield important traits in rice using CRISPR/Cas9 mediated knockouts [5]. In this study an enhanced grain number, dense erect panicles as well as larger grain size occurred in only two generations vastly accelerating the breeding improvement of elite lines. In another groundbreaking study, knockouts of eight different yield related genes in rice were produced by CRISPR/Cas9 multiplex-transformation and the various phenotypes of single and multiple mutants have been analyzed in field trials [6•]. In a remarkable study by Zhou *et al.*, a CRISPR-knockout of a tetratricopeptide repeat domain protein was introduced to mimic an existing EMS mutant and found that the plants exhibit a disease resistance against *Magnaporthe oryzae* and *Xanthomonas oryzae pv oryzae* without influence on their agronomic performance [7]. Very recently, another group edited three QTLs of grain-related genes in rice elite varieties by CRISPR/Cas9, thereby increasing the yield per panicle by up to 68% in triple mutants. The triple mutant plants did not show any negative agronomic performance in the field trials and performed much better than the respective controls concerning important traits like grain number per panicle, 1000-grain weight and yield per panicle [8]. A reasonable salinity tolerance of

Figure 1



The regulatory system for genome-edited plants in different countries worldwide.
The regulation of GE plants is in most countries based on assessment of the product and the process used is only the trigger. In contradiction to this, in the EU and New Zealand the decision to regulate a plant is justified on the technique used to produce the organism. Several countries decided recently to follow a product-based approach and some will probably follow in near future. *The regulation in Australia is process-based but the decision if a GE organism is under GMO regulation is depending on the fact if it contains foreign DNA or if a nucleic acid template was added to guide the DNA-repair, so in fact product-based.

rice under greenhouse conditions was achieved by GE of OsRR22 [9]. However, in 'follow up field trials' the salinity tolerance was not visible. Very recently the group of Xu modified ten different known heading date genes by GE and could show that most of the mutants exhibit yield loss and other types of poor agronomic traits but not the *se14* mutant which is so far not used in rice elite lines [10••]. This very ingenious work shows clearly the enormous potential of GE in a way that it is possible now to analyze in a short timeframe multiple genes for interesting agronomic traits and transfer them to already existing breeding lines.

Other plants

In maize Shi *et al.* specifically replaced the natural promotor of ARGOS8 a negative regulator of ethylene response by CRISPR/Cas9 directed homologous recombination. The resulting plants exhibited increased grain yield under flowering stress conditions in the field [11]. Using an inspiring experimental setup, Soyk *et al.* produced several wild type and cultivated tomatoes, in which the gene self-pruning 5G (SP5G) is mutated by CRISPR/Cas9. As result of their analyses, they speculate that variation in SP5G expression might be the major reason for the expansion of cultivated tomato beyond its origin in South America [12]. The performance of these tomatoes was better in regard of early flowering but the total yield was lower than in the control plants. Rodriguez-Leal *et al.* tested GE tools to modify promotor sequences of tomato to edit fruit development and size [13]. They were able to modify the size of tomato in both directions, larger fruits as well as smaller ones under field conditions. Also other groups are working on the domestication of wild tomatoes and close related *Solanaceae* but did not progressed to the field so far [2,14,15].

Kannan *et al.* used the TALEN (Transcription Activator-Like Effector Nuclease) system to knockout multiple copies of a large gene family in sugarcane [16••]. They chose a conserved region in the Caffeic Acid *O*-methyltransferase (COMT) as target for specific TALEN-mediated GE and could successfully co-edit almost all of the COMT genes (107 out of 109 copies). This experiment is so far the largest success in multiplexing GE in plants. For the first time the mutant plants have been assessed in the field in replicated trials and they exhibited no major difference to wild type plants regarding good agronomic performance but an altered cell wall composition drastically (up to 44%) improving the saccharification behavior of sugarcane during production of biofuel [16••].

Table 1

Published field trials investigating agronomic traits of plants obtained by Genome Editing

Plant/country	Trait	Agronomic performance	Reference
Rice - nd	Storage tolerance	nd	[17]
Rice - China	Enhanced grain size and number, more dense and erected panicles;	Better as control	[5]
Rice - China	Enhanced blast resistance	Normal	[18]
Rice - China	Early flowering/maturing	Growth inhibition in multiple knockouts	[19]
Rice - Japan	Low Cs + plants	nd	[20]
Rice - China	Multiplexing of quantitative traits	Diverse	[6•]
Rice - China	Low Cd-accumulating plants	Normal	[21]
Rice - China	Immune response	Dwarfed plants	[22]
Rice - China	Grain size	Normal	[23]
Rice - China	Grain yield	Better as control	[24]
Rice - China	Grain weight and altered protein content	Better as control	[25]
Rice - China	Waxy rice	Normal	[26]
Rice - China	Disease resistance	Normal	[7,27,28]
Rice - China	Knockout amino acid transport ('proof of concept')	Growth inhibition, yield reduction	[29]
Rice - China	Negative effect on grain architecture	Thinner and lighter grains	[30]
Rice - China	Salt tolerance in greenhouse, no salt tolerance in field observed	Normal	[9]
Rice - China	Higher grain number, higher 1000-grain weight;	Better as control	[8]
Rice - China	Early heading but yield loss	Better in *se14*-mutant	[10••]
Tomato - USA	Self pruning, early flowering,	lower total yield as control	[12]
Tomato - China	Shelf life	Normal	[31]
Tomato - USA	Control of fruit size	nd	[13]
Rapeseed - China	Multilocular siliques	nd	[32]
Camelina - England	Altered oil composition	Severe dwarfing in FAD2 triple knockout	[33,34]
Maize - USA	Increased grain yield under drought stress	Better as control	[11]
Peanut - China	Altered oil composition	Slight decreased pod number	[35]
Sugarcane - USA	Saccharification behavior	Normal	[16••]
Wheat - Belgium		nd	VIB

Table 1 displays all published field trials using GE plants and if available their agronomic performance and altered traits. The vast majority of GE plants in the field are rice and most of them showed an unaltered agronomic performance, except if the knockout of the edited gene itself was disadvantageous.

Agronomic performance and environmental risk

A number of field releases exist for several traits modified by GE not only in rice but also some other plants. In none of these field trials, any harmful effect on the environment occurred [7–9,10••,11,17–23]. In other field trials gene knockouts were analyzed to investigate the gene function, some of these knockouts were neutral and some deleterious for the plants but not for the environment (Table 1). In example, one group disrupted the amino acid transporter LHT1 in rice and not surprisingly, it became clear that its disruption led to growth inhibition and therefore yield reduction [29]. Summarizing these examples of field tests using GE plants, in none of these experiments specific risks could be predicted and no harmful outcome for the environment occurred. A good agronomic performance in several plant varieties was shown but also unexpected yield reduction was observed (Table 1) [12,34]. Table 1 displays a summary of field releases of plants modified by GE.

To our best knowledge, in Europe only a few field trials involving plants altered by GE were performed in the last three years. One field trial in Belgium using GE maize (at the Vlamisch Institute of Biology, VIB), one in Sweden from Lyckeby and one in the UK (at the Rothamsted Institute). The latter one comprises the only published field trial and it was performed with GE *Camelina sativa* exhibiting an altered oil composition [34]. During this field trial, the Camelina plants harboring a triple knockout of FAD2 were severely dwarfed and their phenotype much more disadvantageously pronounced as it was observed in greenhouse studies previously [33]. Besides the already published reports on GE plants tested under field conditions, additional trials are displayed on the webpages of the individual institutes performing them. As mentioned, in Europe the VIB is performing field trials on GE maize, which are registered under EU-law (website VIB (http://www.vib.be/en/news/Pages/Permit-for-CRISPR-field-trial.aspx).

Additional to these, companies perform field experiments with GE plants. One of the first field experiments have been done in the US by the company CIBUS. CIBUS is performing field trials with plants modified by Oligonucleotide directed mutagenesis (ODM). Mainly ODM modified canola was tested in the field and a first variety is launched already. CIBUS is also working on additional canola lines as well as line seed and potato, but it is unclear if such plants are already in field trials in the US or in South America (https://www.cibus.com/crops.php).

Another company performing field trials with GE-edited plants, using TALEN transcription activator like effector nucleases (TALEN) is CALYXT. CALYXT has performed field trials with GE soybean in Argentina since 2015 and launched its first commercial soybean variety edited by GE in 2018. CALYXT is also working on additional plants and is performing field trials on high fiber wheat that is planned to be launched in 2022 (https://calyxt.com/news/page/2/).

Regulatory landscape of GE

The regulatory landscape on GE today is a rag rug on a worldwide scale (Figure 1). The global landscape divides in mainly process as well as predominantly product-based regulations with different mixed regulations in place as well. The Americas as well as Australia are in favor of a mainly product-based regulation whereas Europe and New Zealand have a predominantly process or technique-based regulation in place. The changing regulatory landscape will result in an increasing number of GE plants in the field. Lately Russia announced to promote research on GE plants and animals and aims to produce up to 30 varieties of GE animals and plants in the next 10 years. Russia is also planning to deregulate and commercialize some of these varieties after they showed their performance in field trails [37]. This year Switzerland and Japan will amend or renew their regulations on GE plants and Paraguay already did so [38] (Figure 1). It is to expect that these countries will test GE plants in field trials in the near future.

Discussion

Double strand breaks appear constantly and naturally during any lifetime of a plant and are repaired by the same protein machinery that processes a DSB induced by SDN [39]. A difference between the risk for the environment by genome-edited plants with mutations by site directed double strand break (or breaks, using multiplexing) and unspecific mutations during conventional or mutation breeding is hard to imagine. The question if unintended effects in GE plants might give rise to hazard that is immanent of the technique has been analyzed very recently [40••]. The authors concluded that most of the unintended effects arising by GE could be addressed early in the development process. Furthermore, they made the very clear statement that 'unintended effect is not necessarily synonymous of hazard' [39]. Under consideration of the precautionary principle, there must be a scientific base for a risk assumption and that can only be the case if there is a described hazard provoked by the process and the likelihood that the damage occurs (Court of Justice of the EU case C-111/16; [40••,41].

In conventional breeding, mutation enhancement is a common method to increase the genetic variability of plants, usually by methods such as gamma-irradiation or application of ethyl-methyl-sulfonate. Breeders then select the population for surviving offspring with desired phenotypes. These mutagenizing techniques and products thereof have been declared safe due to a history of safe use by law (Directive 2001/18EC) but in contradiction to this, new techniques like GE using SDN, that show much less unintended effects have to be treated like GMOs (Court of Justice of the EU case C-258/16).

Considering that around two DSBs per hour occur in each cell, the likelihood that any of all possible mutations provoked by DSB-repair lead to a harmful superior plant is negligible. If not so, such plants would arise on each field and harness the environment without human intervention. Given a large genome as it is present in wheat this means on any single field, will be no two plants whose genome is identical. Therefore, we cannot discriminate if a mutation was provoked by technical means or by nature. This scenario is valid for all nature-like repair processes using DSB induced by SDNs. If a donor sequence is used as a template to initiate a specifically defined repair that introduces a foreign gene or DNA sequence that could not arise by natural recombination or crossing, then and only then there is a slight chance that this new sequence might have unexpected negative consequences for the environment or human health. Therefore, the most interesting point in field trials of GE plants will be if their benefits which they exhibit in the greenhouse, are stable under real life conditions in the field. The case of *C. sativa* with improved oil composition but poor growth shows that this might not ever be the case [34].

The changing regulations on a worldwide scale, aiming mainly at low or no regulation of GE plants, that are only mutagenized without integration of foreign DNA and therefore could occur also by natural mutation or conventional mutagenesis (commonly named as SDN category 1) will foster the application and commercialization of such plants. This is already observable in a systematic map, which identified much more than 100 studies with potential marked relevant traits developed until May 2018 [3••]. The positive regulation outside the EU will further boost development of GE plants and they will appear in the fields. In many cases, we are and will be unable to identify and follow such field releases, as the plants be deregulated under the local regulations and thus are not specifically declared.

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