Sequence-specific nucleases as tools for enhancing disease resistance in crops

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As plant diseases account for massive losses of the agricultural production worldwide and contribute towards malnutrition and economic hardship in many parts of the world, enhancing disease resistance in staple crops (e.g. wheat, rice or maize) has been the focus of multiple breeding programs worldwide over the past decades. However, the conventional breeding process is slow as it usually relies on crosses between two parents and subsequent multiple backcrosses of the selected progeny lines to one of the parents. Acquision of favourable alleles conferring enhanced disese resistance via traditional breeding is often associated with the linkage drag, a phenomenon of introducing deleterious alleles (e.g. from a wild germplasm), which are reducing the agronomic fitness of the cultivar due to them being closely linked to the beneficial allele. Genome editing is a relatively new technology that holds a promise to speed up the process of plant breeding via enabling deployment of a beneficial allele (e.g. conferring enhanced disease resistance) into an eite crop variety of choice. Genome editing relies on applying sequence-specific nucleases (SSNs) as programmable molecular tools for recognition and modification of specific DNA sequences. Using SSNs one can introduce a specific change into a crop genome and recreate a natural beneficial allele present in another variety or a related wild species thus avoiding the lengthy and laborious breeding process as well as a chance of the linkage drag. SSNs include meganucleases, zinc-finger nucleases (ZFNs), TAL effector nucleases (TALENs) with the most recent addition to the list being CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein) RNA-guided nucleases. CRISPR/Cas has become the favourite genome editing tool in various organisms, including plants (Nekrasov et al. 2013), as it is very easy to engineer its specificity towards a desired DNA target by manipulating the guide sequence within the guide RNA.

Using SSNs as molecular scissors, one can delete, replace or insert genomic DNA fragments. Therefore, SSNs make possible deleting or replacing whole or parts of specific genes as well as inserting genes at specified locations within genomes. A targeted gene deletion is a way to inactivate a gene of interest and generate a loss-of-function, or knockout, mutation. It is often performed using two single guide RNAs (sgRNAs) targeting a genomic locus of interest. Upon simultaneous recognition and cutting by CRISPR/Cas at the two target sites, the non-homologous end joining (NHEJ) DNA repair mechanism may reconnect the ends of the cut DNA leaving out the sequence in between the target sites and, as a result, generating a deletion. However, a knockout mutation could also be generated by targeting a gene with a single guide RNA. In this case, instead of a large deletion, small indels could be introduced during the error-prone DNA repair process via NHEJ causing, for example, frame-shift mutations that are likely to result in a loss of gene function. There are multiple reports on SSNs, such as TALENs and CRISPR/Cas, being successfully used for generating loss-of-function mutations within gene coding or regulatory regions, such as promoters, in plants (e.g. (Li et al. 2012; Nekrasov et al. 2017)). On the other hand, there are very few reports on the targeted gene replacement or insertion in plants as such events occur with a very low frequency due to a number of reasons. One of them is a requirement for co-delivery of both SSN and the DNA repair template, encoding a DNA fragment one is trying to integrate, into the plant cell. In addition, integration of the DNA repair template into the genomic DNA is usually achieved via the homology-directed repair (HDR), which is very inefficient in plants. As a result, HDR-based gene editing applications in plants require a selectable (e.g. herbicide tolerance) or visual (e.g. trichomes presence/absence) phenotype conferred by a targeted gene insertion/replacement event (Hummel Aaron W. et al. 2017; Hahn et al. 2018).

So far, enhancing disease resistance in crops via genome editing has only been achieved by generating loss-of-function mutations using SSNs (Li et al. 2012; Blanvillain-Baufumé et al. 2017; Nekrasov et al. 2017). In all cases, edited mutations are recessive and result in compromised function of susceptibility (S) genes. A few examples of successful application of the genome editing technology for the purpose of improving disease resistance in crops are given below.

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is an important bacterial pathogen of rice causing the bacterial leaf blight (BLB) disease. *Xoo* virulence is dependent on transcription activator-like effectors (TALEs), which are capable of activating specific S genes, such as *SWEET*, within rice genome via

binding so called effector-binding elements (EBEs) within their promoters (Schornack et al. 2013). A number of *Xoo* TALEs target clade III *SWEET* genes, which include *OsSWEET11*, *OsSWEET13* and *OsSWEET14* (Yang et al. 2006; Streubel et al. 2013; Zhou et al. 2015). *SWEET* genes encode sugar transporters and induction of their expression by TALEs is expected to benefit the pathogen due to increase in sucrose levels in the apoplastic space (Bezrutczyk et al. 2018). Existence of natural rice mutants carrying *SWEET* alleles that cannot be induced by the TALEs due to respective EBEs being disrupted by nucleotide polymorphisms (Chu et al. 2006) suggests that genome editing technologies (e.g. TALENs, CRISPR/Cas) could be used for the purpose of introducing mutations into EBEs in *Xoo*-susceptible rice cultivars and thus generating resistance to the pathogen. By now, both TALENs and CRISPR/Cas technologies have been applied to mutagenise *OsSWEET13* and *OsSWEET14* S genes and, as a result, rice lines resistant to *Xoo* strains carrying TALEs, which target the above-mentioned genes, have been produced (Li et al. 2012; Zhou et al. 2015; Blanvillain-Baufumé et al. 2017). *OsSWEET14* is targeted by four different TALEs (AvrXa7, PthXo3, TalC and Tal5), which are present in geographically distant *Xoo* strains, suggesting importance of this gene for *Xoo* virulence in rice (Streubel et al. 2013). Engineering mutations in AvrXa7, PthXo3 or Tal5 EBEs using TALENs resulted in loss of *OsSWEET14* activation by respective TALEs as well as enhanced resistance to *Xoo* strains carrying these TALEs (Li et al. 2012; Blanvillain-Baufumé et al. 2017). Interestingly, engineered indels within the TalC EBE prevented *OsSWEET14* induction by the *Xoo* strain carrying the cognate TALE, while no enhanced resistance to this strain was observed in this case suggesting presence of additional TalC S gene target(s) within the rice genome (Blanvillain-Baufumé et al. 2017).

Overall, genome editing technologies have a great potential for engineering enhanced resistance to the *Xoo* pathogen in rice and, similarly, other crops, which are hosts to Xanthomonas strains whose virulence is dependent on TALEs. As an example, *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), the causal agent of bacterial blight of cassava, carries TALEs, at least two of which ($TAL20_{Xam668}$ and $TAL14_{Xam668}$) have a virulence function (Cohn et al. 2014). $TAL20_{Xam668}$ specifically induces expression of the *MeSWEET10a* gene in cassava, while $TAL14_{Xam668}$ seems to have multiple gene targets (Cohn et al. 2014, 2016). Therefore, as in the case with rice, it should be possible to exploit genome editing technologies for the purpose of engineering resistance to *Xam* in cassava by mutagenizing EBEs within promoters of S genes (e.g. *MeSWEET10a*) targeted by TALEs. It should be noted that since EBEs often overlap with important promoter elements, such as TATA-boxes, engineering indels in them may result in altered basal expression levels of respective S genes and, consequently, a fitness cost effect for the plant. Such scenario is not surprising as it is common for TALEs to bind promoter sequences that the plant cannot easily lose/mutagenise.

Mildew resistance locus o (*Mlo*) genes are conserved in monocots and dicots and play a role in plant immunity. Loss-of-function mutations in *Mlo* were found to confer recessive resistance to the powdery mildew fungal pathogen in a number of plant species, including barley, wheat, tomato, pea and others making *Mlo* a classic example of an S gene (reviewed in (Kusch and Panstruga 2017)). The *Mlo* locus encodes the MLO protein, which is plasma membrane-localised and carries seven transmembrane domains (Devoto et al. 1999). The molecular function of MLO is unknown. There are various sources of *mlo* mutants, including naturally occurring and artificially induced, using chemical or radiation mutagenesis, as well as genome editing technologies, such as TALENs and CRISPR/Cas (Fig. 1) (Kusch and Panstruga 2017; Nekrasov et al. 2017). The genome editing tools enable rapid and precise targeted mutagenesis of the *Mlo* locus in an elite variety background (Wang et al. 2014; Nekrasov et al. 2017). Unlike the chemical or radiation mutagenesis, genome editing does not generate multiple background mutations in the genome, thus avoiding undesired effects on plant fitness due to their presence. As an example, Nekrasov et al reported that out of 145 putative CRISPR/Cas9 off-targets in tomato, none carried CRISPR/Cas9-induced mutations suggesting that CRISPR/Cas9 is a highly precise tool in tomato (Nekrasov et al. 2017). These findings are consistent with other reports on the CRISPR/Cas system being of high precision in plants (Peterson et al. 2016; Tang et al. 2018).

In certain cases (e.g. in barley, wheat), complete loss-of-function mutations in *Mlo* result in a pleiotropic phenotype characterised by premature senescence in addition to powdery mildew resistance (Wolter et al. 1993; Acevedo-Garcia et al. 2017). Also, in barley, *mlo* mutants demonstrate enhanced susceptibility to non-biotrophic pathogens, such as *Magnaporthe oryzae* and *Fusarium graminearum*, suggesting a trade-off between resistance to powdery mildew and susceptibility to the above-mentioned group of pathogens (Jarosch et al. 1999; Jansen et al. 2005). It will therefore be advantageous to exploit genome editing technologies for the purpose of replacing WT *Mlo* alleles with mutant variants, characterised by partial loss-of-function, in order to achieve an optimal balance between powdery mildew resistance and disadvantageous phenotypes affecting plant fitness. Although replacing alleles using the genome editing technology is not straightforward in plants, such

technology applications have been developing (Hahn et al. 2018; Dahan-Meir et al. 2018) with efficiencies reaching 25% in tomato (Dahan-Meir et al. 2018).

In addition to inactivating S genes, there are other ways to apply CRISPR/Cas for the purpose of generating disease resistance in plants e.g. against viruses, such as geminiviruses. Geminiviruses are an important group of single-stranded circular DNA plant viruses, which are insect transmitted and cause a significant amount of crop loss worldwide (Hanley-Bowdoin et al. 2013). During the replication process in the plant cell nucleus, geminiviruses go through the double-stranded DNA stage. Because of this, the CRISPR/Cas system can be exploited for the purpose of generating resistance to geminiviruses via targeting their replicating double-stranded DNA. A few labs have demonstrated feasibility of the above-mentioned strategy for enhancing resistance to geminiviruses in plants using *Nicotiana benthamiana* as a model system (Baltes et al. 2015; Ji et al. 2015; Ali et al. 2015). Although geminiviruses have a potential to evade targeting by CRISPR/Cas via introducing mutations into sgRNA target sites, some regions within geminiviral genomes carry highly conserved elements that the virus cannot easily mutagenise. As an example, the geminiviral origin of replication carries the invariant nanonucleotide sequence, which is conserved in all geminiviruses. As a result, Ali et al were able to generate resistance to three species of geminiviruses at the same time by targeting the invariant sequence with CRISPR/Cas9 (Ali et al. 2015).

Cas13a (formerly known as C2c2) is an RNA-guided RNA-targeting CRISPR/Cas nuclease that can be engineered for the purpose of generating gene knockdowns via specific mRNA degradation in mammals and plants (Abudayyeh et al. 2017). Recently, Aman et al. have reported that Cas13a can be used to target single stranded viral RNA in planta and thus generate resistance against *Turnip mosaic virus* (TuMV), a single stranded RNA virus, in *Nicotiana benthamiana* as a model host (Aman et al. 2018). It is therefore conceivable that Cas13a can be used to enhance resistance against various RNA plant viruses in a way similar to RNAi constructs.

In summary, there is a number of ways in which genome editing technologies, such as CRISPR/Cas, can be harnessed for the purpose of enhancing disease resistance in plants. One strategy in this case is S gene inactivation, either full or partial, as demonstrated for *SWEET* and *Mlo* loci, while the other is ectopic in planta expression of CRISPR/Cas constructs (e.g. Cas9 or Cas13a) targeting DNA or RNA plant viruses. In the former case, the genetically edited plant carries a mutation in a respective S gene and no transgenic DNA, thus qualifying for the non-GM status under the product-based legislation (e.g. in the USA), while in the latter case plants are transgenic as the virus resistance is conferred by a transgenic cassette expressing CRISPR/Cas components. Both non-GM and GM strategies described above have a significant potential for improving resistance to important bacterial, fungal and viral pathogens in a variety of crops and should be applied in agriculture subject to a regulation, which is based on scientific evidence. The recent European Court of Justice (ECJ) decision to consider transgene-free genetically edited plants as GMOs is clearly not based on such evidence. Without any doubt, the decision is a backward step for the EU and will inevitably have a damaging impact on the European plant science and agbiotech sectors. There is, therefore, an urgent need for the ECJ ruling to be reversed and the EU GMO legislation to be reformed in order to save EU plant science and agriculture from falling behind counties like the USA, which are far more supportive towards genome editing and GM technologies.

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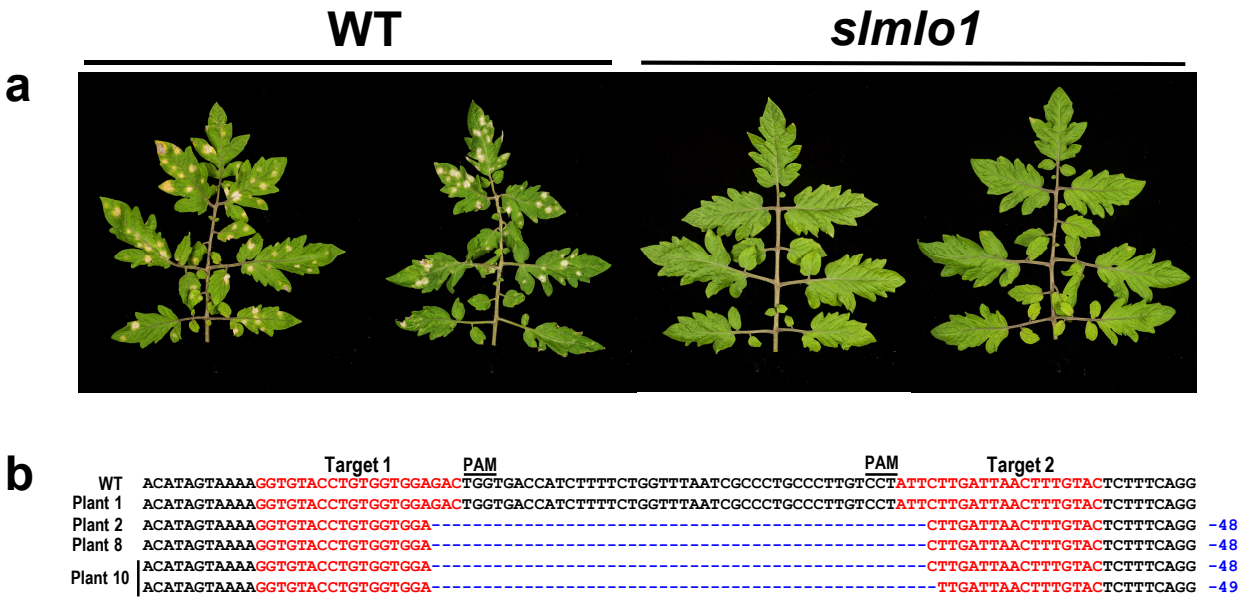

Fig. 1
Knocking out the tomato *Mlo* gene (*SlMlo1*) using CRISPR/Cas results in resistance to powdery mildew.
a The CRISPR/Cas-mutagenized *slmlo1* line is resistant to the powdery mildew pathogen *Oidium neolycopersici.* **b** Knockout mutations in the *SlMlo1* gene were generated by targeting with two sgRNAs at the specified sites. The figure is modified with permission from (Nekrasov et al. 2017) under the CC BY 4.0 license (https://creativecommons.org/licenses/by/4.0/).