Engineering Disease-Resistant Cassava

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Manihot esculenta Crantz (cassava) is a food crop originating from South America grown primarily for its starchy storage roots. Today, cassava is grown in the tropics of South America, Africa, and Asia with an estimated 800 million people relying on it as a staple source of calories. In parts of sub-Saharan Africa, cassava is particularly crucial for food security. Cassava root starch also has use in the pharmaceutical, textile, paper, and biofuel industries. Cassava has seen strong demand since 2000 and production has increased consistently year-over-year, but potential yields are hampered by susceptibility to biotic and abiotic stresses. In particular, bacterial and viral diseases can cause severe yield losses. Of note are cassava bacterial blight (CBB), cassava mosaic disease (CMD), and cassava brown streak disease (CBSD), all of which can cause catastrophic losses for growers. In this article, we provide an overview of the major microbial diseases of cassava, discuss current and potential future efforts to engineer new sources of resistance, and conclude with a discussion of the regulatory hurdles that face biotechnology.

OVERVIEW OF MAJOR CASSAVA DISEASES

Cassava Mosaic Disease

Cassava mosaic disease (CMD) was first identified in 1894 in what is now modern-day Tanzania, and by 1950 could be found across almost all of sub-Saharan Africa. The first report of the disease in India was made in 1956, followed by its discovery in Sri Lanka in 1986 (Patil and Fauquet 2009). In 2015, CMD was found in Cambodia, indicating a concerning expansion of the disease into Southeast Asia (Wang et al. 2016). Symptoms of CMD include chlorotic, mosaic patterning of leaves, along with abnormal leaf development that manifests as curling and twisting of leaflets (Fig. 1B). CMD has been reported to cause root yield losses of up to 82% in some studies (Owor et al. 2004). Coupled with

its pervasiveness, this has earned CMD recognition as one of the most economically important plant viral diseases in the world (Scholthof et al. 2011; Food and Agriculture Organization of the United Nations 2013).

The causative agents of CMD are cassava mosaic geminiviruses (CMGs), which are vectored by the whitefly *Bemisia tabaci* (Legg et al. 2015; McCallum et al. 2017; Rey and Vander-schuren 2017). CMGs are single-stranded DNA viruses of the genus *Begomovirus*, family *Geminiviridae*. The genomes of CMGs are small, circular, and bipartite with separately packaged DNA-A and DNA-B molecules (each ~2.7 kb) that together have eight to nine genes encoding multifunctional proteins. Geminiviruses are often found as mixed infections in plants, which when coupled with a propensity for recombina-

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Figure 1. Effects of viral diseases on cassava. (A) Healthy cassava grown in a test plot. (B) Cassava leaves showing developmental defects associated with cassava mosaic disease. (C) Cross-section of a cassava storage root revealing brown necrotic tissue caused by cassava brown streak disease.

tion allows for the emergence of new virus species and variants (Hanley-Bowdoin et al. 2013). To date, nine species account for CMD found in Africa, whereas two other species are present in Asia (Rey and Vanderschuren 2017).

Three types of genetic resistance have been described for CMD, called CMD1, CMD2, and CMD3. None of the underlying genetic determinants have been identified. CMD1 is thought to be polygenic, whereas CMD2 segregates as a dominant single locus. CMD2 and CMD3 both map to the same arm of chromosome 12. (For a more detailed summary of CMD and associated resistance, see Akano et al. 2002; Legg and Fauquet 2004; Wolfe et al. 2016.)

Cassava Brown Streak Disease

Like CMD, cassava brown streak disease (CBSD) has a long history in sub-Saharan Africa and was first reported in 1935 (Patil et al. 2015; Rey and Vanderschuren 2017). Historically, the disease was confined to the coastal lowlands of eastern sub-Saharan Africa; however, during the past two decades, CBSD has spread to East and Central Africa's Great Lakes region (Storey 1936). The virus is spread locally by *B. tabaci*, but distributed over long distances by transport of infected planting materials. The disease is named for brown streaking of cassava stems but also

causes leaf chlorosis, reductions in storage root yield, and necrosis within the storage roots (Fig. 1C). Losses of up to 70% have been reported, highlighting the current impact of CBSD and potential damage should the epidemic continue its spread toward the populous, high cassava production countries of West Africa (Hillocks et al. 2001).

CBSD can be caused by individual or simultaneous infection with *Cassava brown streak virus* (CBSV) or *Ugandan cassava brown streak virus* (UCBSV) (Hillocks et al. 2001; Tomlinson et al. 2018). These are positive-sense, single-stranded RNA viruses belonging to the genus *Ipomovirus* of the family *Potyviridae*. In general, viruses of *Potyviridae* (potyvirids) have genomes ~10 kb in length that encode a polyprotein that undergoes proteolytic processing to generate 10 mature products (Revers and García 2015). Ribosomal frameshifting allows for the production of an additional movement protein (Rodamilans et al. 2015). Potyvirids account for almost a quarter of all known plant viruses (Wylie et al. 2017). As such, potyvirid-plant pathosystems have been heavily studied.

Cassava Bacterial Blight

Cassava bacterial blight (CBB) is caused by the Gram-negative, bacterial pathogen *Xanthomo-*

nas axonopodis pv. *manihotis* (Xam) and occurs in all cassava growing regions across the globe (López and Bernal 2012). (For an overview of historical incidence and severity of CBB, we refer readers to several research articles, reviews, and book chapters; Hillocks and Wydra 2002; Onyeka et al. 2008; Verdier et al. 2012; Fanou et al. 2018.) The main theme of this literature is that, although CBB is recognized as a common disease of cassava, disease incidence and severity is spatially and temporally variable and difficult to predict. For example, Banito and colleagues attempted to correlate CBB severity to specific agro-ecological zones and/or different cassava varieties but found a high degree of variety x location variability (Banito et al. 2008). The sporadic nature of CBB epidemics has confused scientists for decades. Significant genetic diversity exists among pathogen isolates; however, so far, this diversity has not been tied to disease severity variability in the field (Restrepo et al. 2000; Wydra et al. 2004; Bart et al. 2012). A favored hypothesis is that Xam remains dormant on debris, in the soil, or survives on alternative hosts, such as weeds (Fanou et al. 2017). When environmental conditions are favorable, CBB epidemics will occur. Unfortunately, the parameters that define “conducive environmental conditions” have yet to be discovered. Effect on yield has also been difficult to assess because fields infected with CBB are often also suffering from viral and/or fungal diseases. However, a recent report estimated that CBB could result in a 34% reduction in yield when comparing uninfected versus infected cassava fields (Fanou et al. 2017, 2018). In the same report, cassava variety TMS 30572 was shown to have mild tolerance, but the investigators caution that there is a high genotype x environment (GxE) interaction and that this uneven disease pressure makes screening for tolerance in the field difficult. Regardless, Soto Sedano and colleagues recently report five quantitative trait loci (QTLs) tied to strain-specific resistance derived from TMS 30572. The QTLs were discovered from 117 F1 sibs and with phenotyping performed at two locations and in the greenhouse. Here again, strong GxE interaction was observed (Soto Sedano et al. 2017).

Like many other Xanthomonad pathogens, Xam injects 20 to 30 type 3 effector (T3E) proteins into cassava cells during infection. These proteins are collectively responsible for dampening basal immune responses and inducing susceptibility. The latter is partially accomplished via a specific class of T3Es, the transcription activator-like (TAL) effectors. The vast majority of TAL effector-induced genes have yet to be characterized; however, SWEET sugar transporters and pectate lyases are confirmed to play a role in induced susceptibility (Cohn et al. 2014, 2016).

Other Diseases of Cassava

In addition to CMD, CBSD, and CBB, several fungal diseases affect cassava including *Cercospora* spp. and *Colletotrichum* spp. (Anthracnose) (Hillocks and Wydra 2002). Leaf spot diseases are considered among the most common fungal diseases of cassava with disease symptoms observed as spots on the lower canopy of mature plants. The impact of these diseases on cassava yields has not been extensively evaluated.

Two additional diseases are worth noting: cassava frogskin disease (CFSD) and cassava witches' broom (CWB). Both are putatively attributed to a unique class of bacterial pathogens known collectively as phytoplasmas (Hogenhout et al. 2008). Although CMD and CBSD are confined to Africa (recently, to Asia for CMD), CFSD has only been reported in Central and South America. CWB has been reported in Asia, Africa, and Latin America (Arocha et al. 2009; Alvarez et al. 2013; Flôres et al. 2013). Relatively little is known about CWB disease, although incidence can be high in commercial fields (Alvarez et al. 2009).

OPPORTUNITIES TO ENGINEER DISEASE RESISTANCE

Engineering Resistance against Viruses through RNA Interference

Historically, biotechnological approaches to engineering virus-resistant plants relied on an approach termed pathogen-derived resistance

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(PDR) (Lindbo and Falk 2017; Rosa et al. 2018). It was observed that transgenic expression of certain viral genes in a plant could confer resistance or attenuated susceptibility (Lindbo and Falk 2017). Certain instances of PDR were found to involve expression of nontranslatable transcripts, suggesting an RNA-mediated silencing mechanism (Lindbo and Dougherty 1992a,b). Coupled with the investigation of transgene-mediated silencing and the discovery of RNA interference (RNAi), it is now understood that RNAi is the likely mechanism underlying many instances of PDR (Lindbo and Falk 2017; Rosa et al. 2018).

In eukaryotes, RNAi is an RNA-mediated mechanism of regulating gene expression and antiviral defense. Double-stranded RNA (dsRNA) is recognized and cleaved by Dicer-like (DCL) proteins into 21-, 22-, and 24-nt small-RNA (sRNA) duplexes that are loaded onto a multiprotein RNA-induced silencing complex (RISC) in which one strand of the duplex guides the RISC complex to a specific sequence-complementary target RNA (Borges and Martienssen 2015). This target is either degraded or subjected to translational repression. dsRNA that induces gene silencing comes from a variety of sources. Depending on the dsRNA source, the resulting sRNA can be classified as either small-interfering RNA (siRNA) or micro-RNA (miRNA). siRNAs are derived from plant-endogenous or exogenous RNA species with extensive base-pair complementarity; examples include dsRNA produced during viral replication, independent transcripts with sequence complementarity, plant RNA-dependent RNA polymerase-derived dsRNA, and individual transcripts with extensive self-complementarity that result in hairpin-loop structures (hpRNA). miRNAs, in contrast, are exclusively produced from nonprotein-coding *MIRNA* genes. *MIRNA* transcripts have stretches of self-complementarity that result in folding into specific structures recognized by DCL proteins.

As viruses are obligate intracellular parasites that proliferate in cellular compartments also populated with RNAi machinery, viral diseases are particularly amenable to control using RNAi. RNAi strategies have been shown to be

effective against potyviruses and geminiviruses infecting squash, papaya, potato, plum, and common bean, suggesting that this technology is a feasible biocontrol strategy against CMD and CBSD (Rosa et al. 2018). siRNA- and miRNA-based strategies exist, and implementation along with the unique advantages and disadvantages of both approaches have been extensively reviewed (Carbonell et al. 2016; Fondong et al. 2016; Fondong 2017). For CBSD, siRNA-based approaches against CBSV and UCBSV, individually, have been shown under greenhouse and field conditions (Yadav et al. 2011; Ogwok et al. 2012; Vanderschuren et al. 2012; Odipio et al. 2014). These efforts used hpRNA encoding coat protein (CP) sequences from CBSV or UCBSV singly. More recently, both viruses have been targeted within the same plant by using hpRNA composed of CP sequences from both viruses fused in tandem within one gene construct. Robust resistance to CBSD in these transgenic cassava plants has been shown in multilocation field trials over successive vegetative cropping cycles within high disease locations in East Africa (Wagaba et al. 2016a; Wagaba and Taylor 2018). A strong positive correlation was shown for levels of CBSD resistance and accumulation of siRNAs derived from the RNAi construct (Beyene et al. 2017). A product development program continues for this technology, with lead plant lines undergoing assessment within regulatory field trials in Kenya and Uganda. In addition, an miRNA approach for CBSD control has been shown in *Nicotiana benthamiana*, an alternative host for CBSV (Wagaba et al. 2016b).

PDR has also been used to address CMD. By constitutively expressing a mutated *AC1* gene from *African cassava mosaic virus* (ACMV), Chellepan and colleagues were able to generate resistance to ACMV and the related species *East African cassava mosaic virus* (EACMV) and *Sri Lankan cassava mosaic virus* (SLCMV) under greenhouse conditions (Chellappan et al. 2004). Although not an hpRNA strategy per se, evidence for production of siRNAs from the transgenic sequence indicated that posttranscriptional gene silencing had been triggered in these plants. Subsequent studies expressing *AC1* sequences from hpRNA constructs in transgenic

cassava plants also report robust resistance to infection with the homologous virus (Vander-schuren et al. 2009; Ntui et al. 2015). Subsequent field trials to assess efficacy of *ACI*-derived hair-pin RNAi approaches for resistance to CMD have taken place in Nigeria and Uganda with significant resistance to EACMV achieved in East Africa (unpubl.). Farmer products have not developed from these initial field trials. Indeed, efforts to engineer resistance to CMD have not been aggressively pursued over the last decade owing to the successful deployment of conventionally bred varieties with high levels of resistance to CMD, and the increased importance of CBSD in sub-Saharan Africa. The emergence of CMD in Asia, where large areas are planted to a single variety such as KU50, may once again provide opportunity and need for development of engineered resistance to CMD.

An interesting phenomenon associated with efforts to develop transgenic cassava was reported by Beyene et al. (2016) and Chauhan et al. (2018), in which plants carrying the CMD2-type resistance became highly susceptible to CMD. Loss of resistance to CMD was found to occur during production of the morphogenic tissues used for transgene integration and not to result from expression of siRNAs or other transgenes. Efforts to exploit this knowledge to understand and utilize CMD2 resistance to CMD are more fully described below.

Gene-Editing Approaches to Cassava Diseases

A common aspect in plant-pathogen interactions is the use of virulence factors by pathogens to subvert host immune responses and co-opt host processes for successful completion of the pathogen life cycle (Mandadi and Scholthof 2013; Toruño et al. 2016). Pathogens may occasionally depend on a singular host-encoded susceptibility gene, or perhaps a small gene family, for progression of disease. An example is the interaction between potyvirus VPg and members of the plant *eIF4E* family of translation initiation factors (Robaglia and Caranta 2006; Bastet et al. 2017). This interaction is conserved in many potyvirus plant pathosystems and is essential for successful virus infection (Robaglia

and Caranta 2006; Revers and García 2015). Natural or artificial mutants in susceptibility genes have been used extensively as sources of resistance but can be difficult to identify or generate (Dangl et al. 2013; Revers and Nicaise 2014). The advent of CRISPR/Cas-mediated gene editing accelerates this process, allowing the transfer of strategies from model plants and genetically tractable crops to orphan crops like cassava (Belhaj et al. 2015; Langner et al. 2018). The most heavily used CRISPR/Cas technology uses Cas nucleases guided by RNA molecules (guide RNAs [gRNAs]) to complementary DNA. In the eukaryotic cell, the Cas nuclease will create a double-stranded break (DSB) at the targeted DNA locus. The DSB is frequently repaired through error-prone nonhomologous end joining (NHEJ), often resulting in insertions or deletions at the repaired locus. By transforming a plant with a construct encoding a Cas nuclease and gRNAs specific for susceptibility genes, the encoded susceptibility factors can be mutated to engineer resistance against certain pathogens (Wang et al. 2014).

CRISPR/Cas editing of susceptibility factors has been demonstrated in *Arabidopsis* and cucumber, where *eIF4E* family genes necessary for potyvirus virulence, but dispensable for normal growth and development, were mutated, resulting in resistant plants (Chandrasekaran et al. 2016; Pyott et al. 2016). In cassava, this strategy has been implemented against CBSD. Cassava encodes five *eIF4E* family genes: one of *eIF4E*, two *eIF(iso)4E* paralogs, and two *nCBP* paralogs. VPg protein of CBSV-Naliende isolate TZ: Nal3-1:07 was found to consistently associate with both cassava *nCBPs* in yeast two-hybrid and co-immunoprecipitation experiments (Gomez et al. 2019). Null *ncbp* double mutants were then generated through CRISPR/Cas editing and graft inoculated with CBSV-Naliende in greenhouse trials. The *ncbp* double mutants were delayed in CBSD aerial symptom development across all experiments and end point root symptom severity was greatly reduced, correlating with mean reductions in storage-root virus titer of 43%–45% (Gomez et al. 2019). The ability of CBSV to still accumulate in *ncbp* double mutants is possibly because of redundancy in

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the cassava *eIF4E* family of genes. This is supported by CBSV-Naliendele VPg co-immunoprecipitating with not only cassava nCBPs, but also all other cassava *eIF4E* family proteins (Gomez et al. 2019). It is unclear to what degree cassava *eIF4E* and *eIF(iso)4E* proteins contribute to CBSD, but partial resistance observed in the *ncbp* double-mutant background suggests that CBSV cannot use all *eIF4E* family proteins equally well for virulence. Once the full complement of *eIF4E* family susceptibility factors used by CBSV and UCBSV is characterized, targeted gene editing can again be tested as a potential strategy for CBSD control.

CBB is another disease that may be controlled using CRISPR/Cas editing, as pathogenic Xanthomonads secrete TAL effector proteins into host cells to up-regulate expression of susceptibility factors. In particular, the Xam TAL20 effector was found to bind the promoter of cassava *MeSWEET10a* and activate its expression (Cohn et al. 2014). When TAL20 is absent, Xam proliferation and associated water soaking symptoms are reduced. The TAL20-binding site is then a potential target for gene editing to engineer cassava resistance against Xam. A similar strategy was successfully deployed in rice and citrus and may be appropriate for other Xanthomonad-incited diseases (Li et al. 2012; Cox et al. 2017; Jia et al. 2017; Phillips et al. 2017).

In contrast to CBSD and CBB, CMD has no known susceptibility genes that can be targeted by gene editing. However, as CMGs are DNA viruses, the viral genomes themselves can be targeted by CRISPR/Cas. This has been successfully shown against various geminiviruses in *N. benthamiana* and *Arabidopsis thaliana* (Ali et al. 2015, 2016; Baltes et al. 2015; Ji et al. 2015). In these systems, viral load and symptom severity could be drastically reduced through CRISPR/Cas-mediated interference and evidence of viral genome editing was generally observed (Ali et al. 2015, 2016; Baltes et al. 2015; Ji et al. 2015). A number of effective antiviral gRNAs used in these studies targeted geminiviral coding regions. NHEJ-mediated repair could theoretically result in missense or amino acid insertion/deletion events that allow for produc-

tion of functional viral protein, in addition to mutating the gRNA target site so that Cas binding is no longer possible; this would generate viruses resistant to CRISPR/Cas interference. This is supported by the Ali et al. study in which CRISPR/Cas-interference-resistant virus was isolated from infected plants expressing Cas nuclease and gRNA that targeted geminiviral CP gene (Ali et al. 2015, 2016). Similarly, Mehta and colleagues found that a small proportion of viruses isolated from cassava expressing Cas and a gRNA targeting cassava mosaic virus AC2 and AC3 genes had become editing-resistant (Mehta et al. 2019). Furthermore, these editing-resistant viruses were predicted to produce truncated AC2 and AC3 proteins that could potentially be functional. In addition to targeting coding sequences, Ali and colleagues found that targeting a highly conserved nonanucleotide noncoding motif in the geminiviral long intergenic region (LIR), critical for geminiviral replication, was also effective at attenuating geminiviral accumulation (Ali et al. 2016). Targeting these critical noncoding sequences may be preferable if their function is less tolerant of mutations as compared with coding sequences. Furthermore, multiplexing gRNAs to target multiple geminiviral genomic loci simultaneously provided for stronger resistance than only targeting one locus (Ali et al. 2015; Baltes et al. 2015). These findings suggest that future use of CRISPR/Cas-mediated interference against geminiviruses should use simultaneous LIR and coding sequence targeting with multiple gRNAs for effective disease control that does not facilitate the evolution of editing-resistant viruses.

Engineering Synthetic Resistance Alleles of Susceptibility Factors

Knocking out host susceptibility genes is a simple way of depriving pathogens of necessary tools for carrying out their life cycles. However, this strategy is not always possible as certain susceptibility factors are essential for host viability. This is exemplified in cases in which engineering broad spectrum potyvirus resistance requires knocking out multiple *eIF4E* family members as certain *eIF4E* family mutant com-

binations are nonviable (Bastet et al. 2017). Recently, a novel approach was developed to address this issue. In some potyvirus-plant pathosystems, much is known regarding the amino acid residues involved in VPg-eIF4E interaction (Wang and Krishnaswamy 2012; Bastet et al. 2017). Bastet et al. (2018, 2019) used this knowledge to generate *Arabidopsis* eIF4E1 variants that do not associate with VPg of multiple potyviruses (eIF4E1^R), and then replaced *eIF4E1* with *eIF4E1^R* in an *eif(iso)4e* mutant. *eif(iso)4e* confers resistance to a separate set of potyviruses than *eIF4E1^R*, and both *eIF4E1* and *eIF(iso)4E* are needed simultaneously for viability (Bastet et al. 2017). Bastet and colleagues were also able to use a CRISPR approach in which a modified Cas nickase-cytidine deaminase fusion targeted to *eIF4E1* generated the necessary missense mutation to produce an *eIF4E1* synthetic resistance allele (Bastet et al. 2019). This strategy differs from standard Cas nuclease targeting as cytosines at the targeted region are either directly converted to thymine by cytidine deaminase or are converted to guanine or adenine by error-prone repair. Applying these principles to CBSV should be possible if U/CBSV VPg and cassava eIF4E family interaction domains can be identified. Additionally, this strategy can potentially also be applied to CMD as numerous geminiviral-host protein interactions are known (Hanley-Bowdoin et al. 2013; Castillo-González et al. 2015).

Exploiting Native Resistance Mechanisms to Engineer Disease Resistance

Varying levels of resistance and tolerance to the major diseases affecting cassava exist within the many hundreds of cultivars grown across the tropics. Wild relatives of the crop also provide opportunity for integrating disease resistance into cultivated cassava. Insufficient knowledge of the genes and resistance mechanisms imparting disease resistance presently constrain efforts to exploit these resources. However, increasing quality of genomic platforms and capacity for engineering in cassava is changing this scenario (Wang et al. 2015; Bredeson et al. 2016; Chavarriaga-Aguirre et al. 2016; Wilson et al. 2017). An

early example is efforts to understand *CMD2*. *CMD2* provides resistance to CMD imparted in a dominant manner by a single locus or single gene located on chromosome 12 (Akano et al. 2002). Loss-of-functional *CMD2* by passage through in vitro morphogenesis provides unprecedented opportunity to identify the specific gene(s) responsible for *CMD2*-imparted resistance to geminiviruses (Beyene et al. 2016). One hypothesis is that loss of activity is the result of in vitro morphogenesis-induced differential methylation of regulatory DNA associated with *CMD2*. Once understood, application, as appropriate, of conventional transgenic approaches, gene-editing technology or engineered methylation of the *CMD2* sequence offers potential to enhance its function and/or transfer *CMD2* into presently susceptible varieties. It is considered that such approaches will become increasingly important for future engineering of disease resistance in cassava and other crops.

Immune Receptor Transfer

The plant immune system provides robust defense against microbial pathogens contingent on pathogen perception. Immune responses are initiated by two classes of immune receptors: pattern recognition receptors (PRRs) at the plasma membrane, and intracellular nucleotide-binding domain leucine-rich repeat receptors (NLRs, also known as Nod-like receptors) (Cook et al. 2015). PRRs are typically membrane localized receptor-like kinases or receptor-like proteins (RLPs) that perceive conserved molecular patterns of extracellular pathogens. These patterns can be broadly conserved within classes of pathogens, or more narrowly across genera and species (Ranf 2018). Immunity triggered through this mode is called pattern-triggered immunity (PTI). Nearly all pathogen classes have evolved to secrete effector proteins into host cells that directly interfere with multiple levels of the PTI signaling cascade, via many biochemical modes of action (Toruño et al. 2016). In turn, intracellular NLRs are able to recognize the presence of effectors either through direct NLR-effector interaction or by monitoring for effector-mediated biochemical perturbations made to

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host proteins. NLR activation then results in effector-triggered immunity (ETI) (Thomma et al. 2011; Cook et al. 2015; Cui et al. 2015).

The relatively broad spectrum of resistance afforded by PRRs makes PRR transfer between plant species a particularly attractive disease control strategy. Intra- and interfamily PRR transfer has been demonstrated in numerous instances, as well as between monocots and dicots, underscoring a conservation of signaling pathways downstream from PRR-ligand binding events (Holton et al. 2015; Boutrot and Zipfel 2017; Rodriguez-Moreno et al. 2017; Ranf 2018). In cassava, the main use of PRR transfer would be for engineering resistance against extracellular pathogens like fungi, oomycetes, and bacteria. In particular, PRRs that recognize *Xanthomonads* have been identified and could be of use against CBB. Xa21, an RLK-type PRR from rice, perceives the sulfonated RaxX protein from *Xanthomonads*, and RaxX as well as the enzyme responsible for its sulfonation are found in at least one *Xam* strain (da Silva et al. 2004; Pruitt et al. 2015). The elongation factor receptor (EFR) is a PRR from *Brassicaceae* that perceives bacterial elongation factor Tu (EF-Tu); EF-Tu is highly conserved among all bacteria and transfer of *Arabidopsis* EFR to tomato confers immune responsiveness to *Xanthomonas perforans* (Lacombe et al. 2010). Another PRR, the RLP ReMAX from *Arabidopsis*, can initiate immune responses when exposed to *Xanthomonas axonopodis* pv. *citri* extract; this extract contains a suspected proteinaceous ligand, eMax, which is yet to be identified (Jehle et al. 2013). Interestingly, the widely conserved PRR FLS2 has slightly varying specificities and sensitivities between species, and interspecific transfer can confer recognition of additional pathogens (Chinchilla et al. 2006; Sun et al. 2006; Hao et al. 2016). There is good evidence that many of these receptors will be readily functional after transfer into cassava, whereas a subset may require additional engineering (Mendes et al. 2010; Afroz et al. 2011; Jehle et al. 2013; Tripathi et al. 2014; Holton et al. 2015).

NLR transfer to confer resistance to CBB and CBD is also a possibility as a number of NLRs that recognize *Xanthomonads* and poty-

viruses have been identified (de Ronde et al. 2014; Kapos et al. 2019). In some instances, NLR transfer may be even more useful than PRR deployment as certain adapted pathogens can disrupt PRR signaling cascades (Toruño et al. 2016). However, in contrast to PRRs, inter-specific transfer of NLRs has rarely been successful, likely caused by the diverse mechanisms of NLR activation (Toruño et al. 2016; Rodriguez-Moreno et al. 2017). Some NLRs perceive effectors through direct association, but many characterized NLRs are activated when an effector exerts its enzymatic activity on an NLR-associated host protein (Monteiro and Nishimura 2018). This host protein can be the actual effector target or a homologous protein and is then described as either a guard or decoy, respectively, and must be present for proper NLR activity. Of the five known NLRs that confer resistance to *Xanthomonads*, all have unknown modes of effector recognition (Kapos et al. 2019). One of these, Roq1 from *Nicotiana*, directly associates with its cognate effector avr-Roq1 (conserved in many *Xam* strains), but it is not clear whether this is sufficient for Roq1 activation (Schultink et al. 2017). There are also a handful of NLRs known to confer resistance to potyviruses, but their cognate viral proteins or modes of recognition are unknown (de Ronde et al. 2014). Further complicating matters is the revelation that NLRs directly involved in effector perception, “sensor” NLRs, may also require the presence of additional “helper” NLRs (Rodriguez-Moreno et al. 2017; Białas et al. 2018). These helper NLRs can be either genetically linked or unlinked with the sensor NLR and their necessity may be difficult to predict a priori. Given this current understanding of NLR biology, successful NLR transfer to cassava for CBB and CBD resistance will likely require significant effort.

Engineering New Immune Receptor Specificity

Despite predicted difficulties in using NLRs for potyvirus resistance, it is worth noting that an NLR that recognizes the bacterial pathogen *Pseudomonas syringae* has been co-opted for

potyvirus resistance. The particular NLR, RPS5 of *Arabidopsis*, is activated on proteolytic cleavage of the PBS1 kinase by the *P. syringae* effector AvrPphB (Shao et al. 2003; Qi et al. 2014). When the AvrPphB cleavage site within PBS1 was replaced with the cleavage site of Turnip mosaic virus (TuMV) NIa protease, RPS5 was able to drive an immune response against TuMV (Kim et al. 2016). Similar results were observed by modifying the soybean PBS1 homolog to be cleaved by NIa of soybean mosaic virus (Helm et al. 2019). NIa cleavage sites of different potyvirids are easily identifiable, and this artificial mechanism of potyviral resistance should be readily transferable to any plant species with PBS1, including cassava, as long as a suitable NLR is also present for perceiving PBS1 cleavage (Adams et al. 2005). CBSVs are an obvious target for this strategy, which could also be applied toward any cassava pathogen that secretes protease-type effectors with known cleavage sites. In the future, perhaps NLR surveillance systems that can detect other biochemical activities, such as phosphorylation or ADP-ribosylation, can also be engineered to expand the scope of effectors that can be recognized.

A PROMISING FUTURE FOR ENGINEERING DISEASE-RESISTANT PLANTS

Many mechanisms underpinning gene regulation and the plant immune system have become better understood in recent years. These developments are accompanied with novel biotechnological strategies and enable accelerated engineering of resistance against diseases that in the past would have required laborious germplasm screening and breeding efforts. The strategies discussed in this review are extremely promising for deploying resistance to CMD, CBSD, and CBB, but also may be applied to newly emerging cassava diseases. For example, plants have recently been found to secrete sRNAs in extracellular vesicles that can then be taken up by fungal pathogens, resulting in cross-kingdom RNAi and subsequent silencing of fungal pathogenicity genes (Cai et al. 2018). Such an approach could be applied to cassava anthracnose. Alternatively, susceptibility factor

editing and engineering is broadly applicable as effector-mediated modulation of host processes is universal among all pathogen classes. PTI and ETI have also been shown to be effective against insects, indicating that cassava may even be engineered to be resistant to the CMD and CBSD whitefly vector (Douglas 2018; Erb and Raymond 2019). The successful translation of these approaches will undoubtedly require much work as many questions still exist about underlying principles, but the future of engineering disease-resistant plants is particularly bright.

Multiple strategies are described above for engineering disease resistance in cassava. In a few cases, performance has been shown at the field level and, in the example of RNAi-imparted resistance to CBSD, is being pursued within a product development program to seek regulatory approval and deployment to farmers in Kenya and Uganda (Wagaba et al. 2016a). Other strategies remain unproven in cassava. All such research efforts are valid. However, focus must also remain on the goal of such investments, which is to improve livelihoods and opportunities for cassava farmers, processors, and consumers in the tropics. Many of the strategies described require a transgenic approach. In such cases, a significant program of field performance, food feed, and environmental assessment must be performed to comply with regulatory requirements in each country where deployment is sought. Dossiers are compiled and submitted for review to seek approval by regulatory authorities in each country. If successful, national performance trials are required before variety registration and release to farmers. This multiyear process presents challenges and is complicated by the fact that many cassava-producing countries do not have functional regulatory or legal structures in place to facilitate development and approval of transgenic crops. In some cases, important progress is being made, for example, in Nigeria, the world's largest cassava-producing country, which recently approved Bt cowpea for cultivation by farmers. In other countries, inclusion of restrictive language in their biosafety laws effectively prevent deployment of transgenic crops, ensuring that their farmers cannot benefit from cassava or other

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crops enhanced through the use of biotechnology. Of concern is how cassava-producing countries will regulate the products of gene-editing technologies. It is critically important that they do not follow the path of GMOs but instead seek processes that will allow cassava farmers to benefit from the important opportunities to develop disease resistance described above.

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