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CRISPR/Cas9 based genome editing of *Penicillium chrysogenum*

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CRISPR/Cas9 Based Genome Editing of *Penicillium chrysogenum*C. Pohl,[†] J. A. K. W. Kiel,^{‡,||} A. J. M. Driessen,[†] R. A. L. Bovenberg,^{‡,§} and Y. Nygård^{*,†,§}[†]Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands[‡]Synthetic Biology and Cell Engineering, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands[§]DSM Biotechnology Centre, Alexander Fleminglaan 1, 2613 AX, Delft, The Netherlands

S Supporting Information

ABSTRACT: CRISPR/Cas9 based systems have emerged as versatile platforms for precision genome editing in a wide range of organisms. Here we have developed powerful CRISPR/Cas9 tools for marker-based and marker-free genome modifications in *Penicillium chrysogenum*, a model filamentous fungus and industrially relevant cell factory. The developed CRISPR/Cas9 toolbox is highly flexible and allows editing of new targets with minimal cloning efforts. The Cas9 protein and the sgRNA can be either delivered during transformation, as preassembled CRISPR-Cas9 ribonucleoproteins (RNPs) or expressed from an AMA1 based plasmid within the cell. The direct delivery of the Cas9 protein with *in vitro* synthesized sgRNA to the cells allows for a transient method for genome engineering that may rapidly be applicable for other filamentous fungi. The expression of Cas9 from an AMA1 based vector was shown to be highly efficient for marker-free gene deletions.

KEYWORDS: *Penicillium chrysogenum*, CRISPR/Cas9, genome editing, RNP, marker-free gene deletion

Penicillium chrysogenum is well-known for its capability to produce β -lactam antibiotics in high titers. Additionally, this filamentous fungus naturally produces a wide variety of other secondary metabolites (SMs), several of which are of potential interest for novel applications in pharma, agriculture, food or feed industries. Only a handful of the more than 30 PKS/NRPS gene clusters identified in *P. chrysogenum* have been characterized so far. The exploration of the (silent) SM pool of *P. chrysogenum* is time-consuming and difficult, mainly due to the lack of efficient genetic engineering tools and the complex physiology of filamentous fungi. Industrial β -lactam production strains have been extensively subject to classical strain improvement (CSI) programs, considerably altering metabolic fluxes within the cell.¹ An improved penicillin production strain might therefore be limited in producing alternative non-ribosomal peptide synthetase (NRPS) or polyketide synthase (PKS)-derived SMs. The production of novel SMs could consequently require extensive genome editing to redirect primary metabolism and mend impaired regulation mechanisms. On the other hand, a recent study showed that *P. chrysogenum* could be engineered to produce high amounts of pravastatin, with only a limited set of modifications.² The authors of this study argued that mutations accumulated during the CSI period contributed to the industrial robustness and high flux from glucose to SMs, rendering *P. chrysogenum* a suitable host for production of also heterogeneous SMs. The

number of sequenced filamentous fungi is rapidly increasing,³ leading to a huge untapped reservoir of potentially useful SMs and a great need for efficient genetic tools to explore and utilize this reservoir.

Precision genome editing by clustered regularly interspaced short palindromic repeats (CRISPR)-associated RNA-guided DNA endonucleases (Cas9) has rapidly become a widely used technology due to its high efficiency and straightforward design. In the CRISPR/Cas9 technology, the Cas9 endonuclease is guided by a sgRNA (single chimeric guide RNA) to a specific locus, where it introduces a double strand break (DSB) in the DNA sequence. The protospacer of the sgRNA that defines the target DNA, can be virtually any 17–20 bp nucleotide sequence found adjacent a 5'-NGG DNA motif (the PAM, protospacer-adjacent motif). CRISPR/Cas9 based technologies rely on the cells repair mechanisms for fixing the DSB.⁴ The DSB introduced by the Cas9 nuclease is repaired by the error prone nonhomologous end-joining (NHEJ) mechanism, sometimes leading to insertions or deletions within the target sequence that typically cause a dysfunctional open reading frame (ORF). If a DNA sequence with homology close to the DSB (a so-called donor DNA: dDNA) is available, many organisms prefer fixing the DSB through homologous

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recombination (HR),⁴ referred to as homology directed repair (HDR). Thus, the CRISPR-Cas9 technology can be used both for the creation of gene deletions and insertions. A great advantage of the CRISPR/Cas9 technology is the possibility for marker-free genome editing.

Recently, CRISPR/Cas9 based genome editing has been demonstrated in filamentous fungi, including *Trichoderma reesei*,⁵ *Pyricularia oryzae*,⁶ *Neurospora crassa*,⁷ *Ustilago maydis*⁸ and *Aspergillus* spp.^{9–12} Herein, Cas9 was either integrated in the genome,^{5,9,11,12} or expressed from a plasmid¹⁰ containing the AMA1 (autonomous maintenance in *Aspergillus*) sequence or the *U. maydis* ARS element.⁸ In other studies,^{6,7} Cas9 was transiently expressed from a nonreplicating plasmid introduced to the protoplasts and presumably not integrated in the genome. In the aforementioned studies, different approaches for delivering sgRNA to the cells were taken; addition of *in vitro* transcribed sgRNA⁵ or expression of sgRNA under control of different fungal promoters. Cas9 was shown to increase HDR events up to high efficiencies when a selection marker in the dDNA cassette was selected for.^{5,7,12} Genome editing with preassembled CRISPR-Cas9 ribonucleoproteins (RNPs) has been reported for different animal^{13–18} and plant¹⁹ cells.

In this study, multiple CRISPR/Cas9 tools have been developed for *P. chrysogenum*. The Cas9 protein could be expressed within the cell or added as preassembled RNPs during the transformation and the sgRNA could be transcribed within the cell or synthesized *in vitro*. Highly efficient gene and gene cluster deletions were obtained *via* HDR through the introduction of dDNAs. In addition, using instable AMA1 vectors for selection, genetic elements could be removed or integrated using dDNAs, without the need for genomic integration of a selection marker. To our knowledge, this is the first time that Cas9-RNP technologies or marker-free gene deletion is reported for *P. chrysogenum*. The methods described are most likely widely applicable also for other filamentous fungi.

■ RESULTS AND DISCUSSION

Providing high flexibility and precision in target selection, CRISPR/Cas9 becomes an attractive and powerful tool that may replace marker-based gene deletions in filamentous fungi in the future. This study describes CRISPR/Cas9 tools for rapid genome engineering of *P. chrysogenum*. This can be expected to significantly advance the research and exploitation of novel natural products like SMs and the development of *P. chrysogenum* as an industrial chassis organism. The genetic toolbox presented opens up the SM chemistry toolbox of filamentous fungi and is expected to be broadly applicable to other filamentous fungi. The CRISPR/Cas9 technology is expected to become a major driver in exploitation of fungal diversity.

Cas9-sgRNA RNPs for Efficient HDR Based Gene Replacements. Deletion of genes using HDR and selection markers such as *amdS* is a well-known, reliable method for genetic engineering of *P. chrysogenum*. In order to study whether the Cas9 protein could be delivered into the protoplasts for cleavage of the targets determined by *in vitro* synthesized sgRNA, the Cas9-sgRNA RNP mixture was cotransformed with an *amdS* cassette having flanks with 1 kb homology to *pks17* (Pc21g16000). This polyketide synthetase encoding gene was selected as a target due to the possibility of a phenotypic screen. The absence of a functional *pks17* will stop

the formation of a green pigment in spores, resulting in white colonies which can be visualized on solid medium.²⁰

First, we describe the use of RNPs together with a dDNA with a selectable marker. The RNP cotransformation of 1.25 μ M of sgRNA (equivalent to 4 μ L of unpurified sgRNA synthesis mixture) and 220 nM of Cas9 (forming a Cas9:sgRNA ratio of 1:5.7) led to a significant, approximately 6-fold, increase in transformants of DS68530 ($\Delta hdfA$), selected for growth on acetamide, compared to traditional HDR based insertion of the *amdS* cassette into the target locus (control). Deletion of *hdfA* renders *P. chrysogenum* impaired in NHEJ and increases the gene targeting success rate by boosting the ratio of correct transformants as the chance that a DSB is repaired by HR is increased.^{21,22} In a $\Delta hdfA$ strain, the ratio of final correct mutants is increased from approximately 1/100 to 1/2 when compared to the wild type.²¹ To confirm that the use of CRISPR/Cas9 indeed led to targeted integration of the dDNA, eight randomly picked colonies were analyzed by colony PCR, for verification of the replacement of *pks17* with the *amdS* cassette (Figure S1b). All the colonies analyzed showed the correct integration of the dDNA. Similarly, previous studies^{5–7,10,12} on Cas9 tools for other filamentous fungi have shown that DSBs created by Cas9 could efficiently be repaired by introduction of a dDNA with homology flanking the cleavage site. For an overview on the influence of the amount of Cas9 protein and sgRNA transformed on the transformation efficiency, see Figure S2.

The simultaneous incorporation of an *amdS* cassette into two loci was also successful. The dDNAs used had ≥ 1 kb flanks homologous to the 5'- and 3'- UTRs of *pks17* or *lovF* (encoding a diketide synthase of the lovastatin biosynthetic gene cluster), both targeted by individual sgRNAs. The *lovF* gene was replaced by the *amdS* cassette in all of the 8 randomly picked colonies analyzed. In 4 of these colonies, also the *pks17* gene was replaced with the *amdS* cassette. (Figure S1c).

DSBs by Cas9 Allows for Efficient HDR Using 60 bp Oligonucleotide Recombination Flanks. Typically, relatively long (at least 700 bp) flanking regions are used for incorporation of novel genetic elements into *P. chrysogenum*. Using the CRISPR/Cas9 technology, we demonstrated correct integration of selection markers with HR flanks as short as 60 bp (Table 1). PCR-amplified dDNA cassettes with various homology lengths to the 5'- and 3'- UTRs of *pks17*, were cotransformed to DS68530 ($\Delta hdfA$) with RNPs targeting *pks17*. When the flank length of the dDNA was reduced to 500 bp or less, colonies were observed on acetamide selection plates only in the presence of sgRNA and the transformation efficiency was decreased (compared to when 1 kb flanks were used, Figure S1d). A flank length of ≤ 100 bp allows the flanks to be added *via* oligonucleotide extension-PCR. Thus, the *amdS* cassette can be easily PCR amplified with oligonucleotides having flanks with homology to any desired target, allowing cloning-free gene deletion with limited experimental effort. In this study, we successfully targeted 8 different loci (with almost all of the colonies examined containing the correct integration, Table 1) and demonstrated the deletion of large genes (>18 kb) and also an entire SM gene cluster, using flanks as short as 60 bp. Only cells that have incorporated the *amdS* marker cassette can grow on acetamide containing medium, and the DSB created by Cas9 increases the transformation efficiency as the DSB is fixed with the *amdS* cassette. Thus, short HR flanks can be used when marker-based genome editing with the CRISPR/Cas9 system is performed. However, when incorpo-

Table 1. Integration of the *amdS* Cassette at Various Loci Using Homology Arms of Various Lengths^a

gene	size of ORF (bp)	homology flank (bp)	correct/analyzed transformants
<i>pks17</i>	6659	1000	8/8
	6659	100	8/8
	6659	60	16/16
<i>roqA</i>	7458	100	1/3(8) ^b
<i>lovF</i>	7960	100	8/8
<i>pcbAB</i> (DS54468)	11371	100	8/8
	11371	60	8/8
<i>penDE</i> (DS54468)	1275	100	8/8
<i>hcbA</i>	18536	100	8/8
	18536	60	8/8
Pen-cluster (<i>penDE</i> , <i>pcbC</i> , <i>pcbAB</i> , DS54468)	16155	100	8/8
ΔPen-cluster locus		100	8/8

^aThe *amdS* cassette was integrated into DS68530 (Δ*hdfA*, ΔPen-cluster) or DS54468 (Δ*hdfA*) using RNPs. ^b8 *roqA* colonies were analyzed, but the colony PCR was only successful for 3 of these (actin amplified as control, no PCR products seen for 5 of the colonies).

ration of *amdS* marker cassettes at multiple loci is desired or the strain is *hdfA*⁺, longer flanking regions may be needed. Recently, the replacement of three different target genes in *T. reesei* with *ura5* and flank lengths of 800–1000 bp was reported.⁵ In that study, efficiencies of incorporating the marker varied between different loci, and the authors suggested that regions of lower transcriptional activity may complicate the sgRNA guided direction of Cas9.⁵ In line with findings of other research groups, including the work of Katayama *et al.*¹¹ in *Aspergillus*, we also noted differences in transformation efficiencies, depending on the protospacer sequence chosen for the target (see Figure S3).

Marker-Free Genome Editing with CRISPR/Cas9. The number of efficient markers for genome editing of *P. chrysogenum* is rather limited and recycling of markers is time-demanding. Avoiding the integration of selection markers is thus a desirable setup. The use of an AMA1 plasmid with a selection marker provided an efficient solution for this. In order to select for protoplasts capable of taking up DNA and *in vivo* recombination, the linearized AMA1 containing plasmid pJAK-109, and an *amdS* cassette with 100 bp flanks for *in vivo* recombination into the AMA1 vector were cotransformed into

the protoplasts of DS68530. We hypothesized that this would also select for a high frequency of protoplasts having taken up the dDNA and performed HDR at the genome. This experiment resulted in a much higher transformation efficiency (184 ± 76 cfu/μg dDNA, $n = 4$) compared to transformation of the *amdS* cassette alone (4 ± 1 cfu/μg of dDNA, $n = 3$). This demonstrated that the *in vivo* recombination of the *amdS* cassette to the AMA1 vector was much more efficient than random integration of the cassette into the genome. The AMA1 plasmid that contains the *DsRed-SKL* marker for visualization (pJAK109), could be lost upon growth without selection pressure, see Figure S4.

AMA1 Based Expression of Cas9 and sgRNA. The expression of Cas9 from an AMA1 based plasmid was shown to lead to high frequency genome engineering in *Aspergillus* spp.¹⁰ and the expression of sgRNA under a variety of promoters has been demonstrated for different fungi.^{5–9,11,12} Here, we tested three different RNAP III and one RNAP II type promoter and their corresponding terminators for expression of sgRNA targeting *pks17*. DS68530 (Δ*hdfA*) was transformed with a linearized AMA1 expression plasmid (pYN-2) and two cassettes with ≥1000 bp flanks for *in vivo* recombination of the *amdS* marker cassette and one of the sgRNA cassettes with the Cas9 cassette containing the same sgRNA expression cassette, into pYN-2 (see Figure 6c). The marker-free dDNA cassette with 1 kb homology to the 3′- and 5′- UTRs of *pks17* was simultaneously added to the protoplasts. The expression of Cas9 was induced by plating the protoplasts on acetamide selection medium containing 20 g/L xylose, in which the *xlnA* promoter was shown to lead to high expression (data not shown). After 5 days, the transformants were transferred to R-agar medium for analysis. The results indicate that all the promoters chosen generated functional sgRNA that guided the Cas9 protein to cleave the target sequence (Figure 1). Colony PCR showed that all colonies analyzed (8 colonies for each construct) contained the dDNA cassette. The correct integration of the dDNA cassette was further confirmed for 7/8 of the colonies (data not shown).

RNAP II promoters such as the U6 homologues have typically been used for expression of sgRNA in various organisms,²³ but recent studies have demonstrated that the expression of sgRNA from an RNAP II promoter was also possible, even in filamentous fungi.⁶ Previous studies^{24,25} have shown that the abundance of sgRNA correlated with the

Figure 1. Expression of Cas9 and sgRNA from the AMA1 plasmid. (a) Transformed dDNA for disruption of *pks17* and sgRNA used. (b) Description of promoters and terminators used for driving sgRNA expression from *in vivo* recombined pYN-2-hCas9-sgRNA. (c) Example of PCR analysis of colonies transformed. Expected fragment size: 553 bp.

efficiency of Cas9 mediated genome engineering. The choice of protospacer sequence has also been shown to have a great influence on the success of the genome engineering events with the CRISPR/Cas9 system.^{5,11} It may thus be that the success in targeting when using another protospacer may be very different from the results shown in this example.

AMA1 Based Expression of Cas9 and *In Vitro* Synthesized sgRNA. DS17690 (*hdfA*) was transformed with a linearized AMA1 expression plasmid (pYN-2) and a cassette for *in vivo* recombination of the Cas9 expression cassette, into pYN-2 (see Figures 6b and 2a). The marker-free dDNA

Figure 2. Colonies of DS17690, transformed with pYN-2 and an *amdS* and Cas9 containing expression cassette. The cassette had 1 kb homology for *in vivo* recombination to the pYN-2 vector. *In vitro* synthesized sgRNA and dDNA for disruption of *pks17* was added to the protoplasts. Colonies were picked from transformation plates and grown for 4 days on PPM-Agar to induce sporulation. Spores from white colonies were harvested and inoculated to fresh plates (b), demonstrating the mixed population after the initial transformation.

cassette with 1 kb homology to the 3'- and 5'- UTRs of *pks17* and *in vitro* synthesized sgRNA targeting *pks17* were simultaneously added to the protoplasts selected for on acetamide medium containing 20 g/L xylose. Transformants

were transferred to PPM medium and after 4 days of growth 20 out of the 88 colonies (23%) of the DS17690 strain clearly showed a white phenotype and had grown bigger compared to the green colonies on the solid medium (Figure 2a).

Many of the green colonies were clearly mixed populations, and presumably contained also the white mutant. Notably, the white colonies grew bigger on nonselective medium compared to the green wild type cells. Even though AMA1 vectors are reported to be readily lost without selection,²⁶ some plasmids were clearly kept in cells even after two rounds of sporulation and growth without selection (See Figure S4). After sporulation, no difference in size was seen between the green and white colonies (Figure 2b), indicating that the AMA1 vector or Cas9 was no longer present in the cells. Although carbon catabolite repressed in *A. nidulans*, the *xlnA* promoter was shown to be active also in medium with both xylose and glucose²⁷ and similarly our results with RNPs demonstrate that the Cas9 protein was very stable and presumably only very small amounts of protein are needed for the creation of DSBs. Cas9 expressed from a plasmid was shown to be present for days in mammalian cells.¹⁷ Thus, the differences in colony size may be due to Cas9 being active in the cells that lack a dDNA for extruding the PAM site and protospacer from the genome. The selection on acetamide containing medium allows only cells expressing the *amdS* cassette to propagate, whereas there is no direct selection for cells that take up the dDNA. Previously, it was reported that constitutive expression of Cas9 alone did not alter the growth rate of *A. fumigatus*⁸ or *A. oryzae*.¹¹ Nonetheless, other studies have shown that high levels of Cas9 may have toxic effects on yeast,²⁸ mammalian²⁹ and algal³⁰ cells. PCR analysis of 8 of the white DS17690 colonies showed that all of these contained the deletion cassette. The correct insertion of the dDNA at the *pks17* locus was further investigated through colony PCR, demonstrating that 60% of the colonies showed the expected fragment, but notably also still contained wild type cells. However, since

Figure 3. Removal of an *amdS* expression cassette using marker-free dDNA. (a) Transformation scheme and sgRNA protospacer in the *amdS* gene. The dDNA had 1 kb homology toward the 5'- and 3'- UTRs of *pks17*. (b,c,d) PCR analysis of colonies transformed with a digested AMA1 expression plasmid (pYN-2), a cassette for *in vivo* recombination of the Cas9 expression cassette into the AMA1 plasmid, the marker-free *Pks17* dDNA and *in vitro* synthesized sgRNA targeting *amdS*. Initial screening using primers *pks17_0.25kb_F* and *pks17_0.25kb_R* (b) revealed the presence of the expected dDNA fragment (553 bp). Colonies were further tested (c) with primers *pks17_0.25kb_F* and *pks17_1.5kb_R*, amplifying the entire *pks17* locus where the *pks17* gene was removed (1791 bp). However, also the *amdS* expression cassette (4.9 kb*) was present due to wild type cells in the mixed colonies. (d) After purification through sporulation, the presence of the dDNA was confirmed by colony PCR for 3 of the screened colonies. The colonies were not capable of growing on acetamide any longer, and no band corresponding to the *amdS* cassette (3.7 kb) was seen, demonstrating that the *amdS* cassette was no longer present.

Figure 4. Marker-free deletion of *pks17* using RNPs. Colonies of DS68530, transformed with RNPs, linearized pJAK-109, an *amdS* cassette and dDNA for disruption of *pks17*. The *amdS* cassette had 0.1 kb homology for *in vivo* recombination to the pJAK-109 vector and the dDNA had 1 kb homology to the 5'- and 3'- UTRs of *pks17*.

Figure 5. Deletion of the chrysogine cluster (26,219 kb) using RNPs and a marker-free dDNA. (a) Schematic representation of the dDNA, the chry-cluster and primers used for screening. (b) Colony PCR of single colonies from acetamide plates. Using oligos *chy_clus_F* and *chy_clus_R*, a PCR product of 4047 bp was amplified from 2 of the 22 screened colonies, demonstrating the absence of the entire cluster. (c) After purification through sporulation, no amplification of *chyE* (with oligos *chy_screen_1F* and *chy_screen_1R* resulting in a 2103 bp PCR product in the wt) or *chyA* (with oligos *chy_screen_2F* and *chy_screen_2R* or *chy_screen_3F* and *chy_screen_3R* resulting in a 818 or 213 bp PCR product, *chyA** in the wt) was seen from two of the transformants (colony 17 and colony 21), confirming the absence of *chyA* and *chyE*.

DS17690 is not a NHEJ deficient mutant, the white phenotype may also be caused by NHEJ.

The DS68530 ($\Delta hdfA$) strain is deficient in NHEJ and thus HR should be the preferred means for these cells to repair a DSB. Indeed, in an experiment similar as described above, all analyzed colonies of DS68530 incorporated the marker-free dDNA cassette for replacing *pks17* when Cas9 was expressed from the plasmid and when *in vitro* synthesized sgRNA was added to the protoplasts. When transformants from this experiment were transferred to R-agar plates, where sporulation is slower and less pronounced, it was more difficult to discriminate the colors of the transformants. All colonies showed phenotypes ranging from white to green, indicating that they consisted of mixed populations. Also the positive control, where *pks17* was deleted by the *amdS* deletion cassette with the 3'- and 5'- UTRs of *pks17* (1 kb), clearly consisted of mixed populations (data not shown).

Deletion of an Integrated *amdS* Marker with Transiently Expressed Cas9.

AmdS resistance is widely used for genetic engineering of *P. chrysogenum* and an efficient removal of the marker would simplify further modification of available mutants. We demonstrated that deletion of the *amdS* marker of the *pks17::amdS* strain using the pYN-2 plasmid and the Cas9 expression cassette (see Figure 6b) that also contained the *amdS* cassette was possible and also remarkably efficient. The plasmid and expression cassette were cotransformed with *in vitro* synthesized sgRNA targeting *amdS* and a dDNA with 1 kb homology to the 5'- and 3'-UTRs of *pks17*. The protoplasts were recovered on xylose containing medium with or without acetamide and the integration of the dDNA at the *pks17* locus was verified with colony PCR. Four out of 6 colonies checked by colony PCR contained the dDNA at the *pks17* locus (Figure 3). These transformants did not grow well on acetamide medium. Nonetheless, the colonies still also contained the *amdS* cassette and had to be purified through two rounds of

Table 2. Comparison on Different Methods for CRISPR/Cas9 Based Genome Editing

	Cas9 <i>in vivo</i> sgRNA <i>in vivo</i>	Cas9 <i>in vivo</i> sgRNA <i>in vitro</i>	Cas9 <i>in vitro</i> sgRNA <i>in vitro</i> (RNPs)
Cloning efforts	Building expression cassettes	No additional cloning needed	No additional cloning needed
Assumed Cas9 expression levels	Higher, may lead to off-target effects	Higher, may lead to off-target effects	Low, transient exposure
Cas9 activity	Only after translation	Only after translation, stability of sgRNA may be an issue	Immediate activity. After forming complex with Cas9, sgRNA may be protected from degradation
Genome targeting success ratio	Selection of plasmid increases ratio	sgRNA uptake and stability may influence ratio	No selection of cells having taken up RNPs possible—lower ratio

sporulation, before the strain containing an empty *pks17* locus was obtained and growth on acetamide medium was abolished. Consistent with our observations, previous studies^{6,7} have shown that transient expression of Cas9 from an introduced (nonreplicating) vector was sufficient for genome editing. The introduction of a Cas9 expression vector that is not maintained in the cells presumably leads to a low level of transcription before the vector is lost. Matsu-ura *et al.*⁷ observed an increase in transformation efficiency when more nonreplicating Cas9-sgRNA plasmid was added to the transformation reaction.

Marker-Free Deletions with RNP's. It is likely that only a very small fraction of the cells take up the RNPs, and the number of protoplasts that have taken up both the RNPs and the dDNA construct might be even smaller. Consequently, the frequency of edited cells is low when using a marker-free dDNA. On the other hand, the RNP approach is very straightforward and has been shown to lead to more rapid genome editing events and less off-target effects compared to when Cas9 and the sgRNA were expressed from a plasmid.^{14,16}

In order to improve genome engineering success with RNPs, by selecting for protoplasts which had taken up dDNA and performed HR, a linearized AMA1 plasmid that *in vivo* would recombine with a provided *amdS* cassette was added to the RNP transformation (see Figure 6a). We hypothesized that in this way, a marker-free dDNA cassette would more frequently be incorporated as the method selects for protoplast that are capable of taking up DNA and *in vivo* recombination. Protoplasts transformed with RNPs, linearized pJAK-109, the *amdS* cassette and the marker-free dDNA (with 1 kb homology toward the *pks17* locus), were after selection on acetamide medium transferred to PPM medium for sporulation (Figure 4). On solid PPM medium, 2 or 5 colonies out of the 144 or 128 colonies picked, cotransformed with 1 or 3 μ g of dDNA respectively, showed a white phenotype. When no sgRNA was added to the Cas9 protein transformed in a similar setup, no white colonies were observed (control).

The marker-free deletion strategy, using the AMA1 plasmid for coselection and RNPs, was also successfully applied for the removal of the putative chrysogene biosynthesis cluster which comprises of the *chyE*, *chyH*, *chyC*, *chyM*, *chyD*, *chyA* and *chyR* genes (Polli *et al.* and Viggiano *et al.*, unpublished). The amount of dDNA added to the protoplasts was increased to 11 μ g and two different sgRNAs (protospacers located at the 3'- or 5'- ends of the chrysogine cluster) were used. Out of the 22 colonies screened, 2 colonies displayed a band which corresponds to a deleted chrysogine cluster (Figure 5). After sporulation, only *DsRed-SKL* expressing colonies were observed on acetamide plates, indicating that the colonies contained the AMA1 plasmid and presumably did not integrate the *amdS* marker into the genome. For final verification, the flanking region of the *chy*-cluster of the purified Δ *chyEHCM*DAR mutant was PCR amplified from gDNA and sequenced.

CONCLUSIONS

This study demonstrates a CRISPR/Cas9 system toolbox for *P. chrysogenum* that is very flexible, fast and efficient (Table 2). The RNP based genome editing has the advantage of transient exposure of the cells to Cas9, after which the RNPs are degraded, minimizing the chance of off-target events. Expressing Cas9 from an AMA1 based plasmid on the other hand allowed for very high ratios of correct transformants, as this setup ensures the presence of Cas9 in all cells upon selection on acetamide medium. The sgRNA can be synthesized *in vitro* or expressed within the cell. A workflow that combines the delivery of *in vitro* synthesized sgRNA together with a plasmid-based Cas9 expression may be the most versatile and rapid option for novel targets, as the sgRNA templates can be ordered as oligonucleotides.

The main focus of this study was to establish marker-free genome editing using the CRISPR/Cas9 technology. The use of a marker-free dDNA was shown to be efficient for gene inactivation and allowed verification of gene editing events by colony PCR. The marker-free dDNA strategy presented is also transferrable to the insertion of additional genetic elements and we believe that the multiplexed genome editing for *P. chrysogenum* can be further developed for increased ratio of correct transformants. The CRISPR/Cas9 tools were demonstrated to work both in NHEJ deficient strains and strains with intact *hdfA*. Moreover, transformation of Cas9 RNPs may be applicable also in fungi that have no previously established molecular biology tools.

Implementation of the CRISPR/Cas9 toolbox presented is expected to lead to an acceleration in the discovery and activation of novel SMs. The prospect of bringing these tools to nondomesticated fungal species further opens novel paths for natural product discovery and exploitation.

METHODS

Fungal Strains and Genetic Targets. *P. chrysogenum* strains were kindly provided by DSM Sinochem Pharmaceuticals Netherlands B.V. All strains^{20,21,31,32} used in this study and all genetic targets are listed in Tables S1 and S2.

Selection of Protospacers for sgRNA and Construction of sgRNA Templates. CasOT³³ was used for selection of sgRNA protospacers (Table S2), using the following parameters: 0 mismatches allowed in seed or nonseed, 20 bp protospacer length, G at 3'-end for T7 polymerase and possibly enhanced pol-III polymerase activity. Contigs of *P. chrysogenum* Wisc54–1255³⁴ were searched via the NCBI nucleotide database and 49 contigs (assembly GCA_000226395.1) were assembled in ascending order as a reference for sgRNA off-target searches. Protospacer candidates identified by CasOT were individually examined using the criteria of Doench *et al.*³⁵ sgRNA-templates were constructed as DNA oligos by fusing the 20 bp protospacer to a T7-promoter sequence (ATG-

Figure 6. (a) The AMA1 vector and the *in vivo* repair cassette used in RNP transformations. (b) The AMA1 vector and the *in vivo* repair cassette containing Cas9 used when *in vitro* synthesized sgRNA was added to transformations. (c) The AMA1 vector and *in vivo* repair cassettes containing Cas9 and sgRNA expression cassettes. (d) SBOL presentation³⁶ of *in vivo* recombined plasmids presented in a–c.

TAATACGACTCACTATA, for *amdS* and *pks17*, or ATG-TAATACGACTCACTATAGG, for the other targets, adding a GG dinucleotide as suggested by Kim *et al.*³⁷) and a 77 bp sgRNA tail²⁴ (GTTTTAGAGCTAGAAATAGCAAGT-TAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT-GGCACCGAGTCGGTGCT). The final 117 or 119 bp sequence used as template for *in vitro* sgRNA synthesis, was ordered as oligonucleotides (all oligos purchased from Sigma-Aldrich, UK) that were annealed by mixing equal amounts of forward and reverse oligos in ligase buffer (Thermo Fisher Scientific, USA). The mixture was incubated at 100 °C for 5 min followed by cooling to 25 °C by a gradual decrease of 1 °C for 30 s in 75 cycles.

Construction of Deletion Plasmids and dDNA. pDest-final-*pks17*²⁰ that contained the acetamidase gene, *amdS*, under the control of the *gpdA* promoter, and the terminator of *amdS* (all genetic elements from *Aspergillus nidulans*), was used for deletion of *pks17*. In this vector, the *amdS* marker cassette was combined with 1.5 kb flanks with homology to the 5'- or 3'-region of *pks17* (Pc21g16000). The *pks17* deletion cassette was PCR amplified with flanks of 1 kb, 0.5 kb, 0.25 kb or 0.1 kb. The pDest-final-*lovF* plasmid (kindly provided by O. Salo,

unpublished) used for deletion of the *lovF* gene (Pc16g11480), contained the same *amdS* expression cassette as the pDest-final-*pks17* vector, but only 1 kb flanks with homology toward the 5'- and 3'-region of the *lovF*.

For deletion of *roqA*, *pcbAB*, *penDE*, *hcrA*, *lovF* or integration of a dDNA into the empty *pen*-locus of DS68S30, the *amdS* cassette from vector pDest-final-*pks17* was PCR-amplified with primers containing a 100 bp sequence of the 5'- and 3'-region of each target gene. Likewise, primers that only added a 60 homologous sequence to the *amdS* cassette, were used for generating dDNAs for deletion of *hcrA*, *pcbAB* or *pks17*.

Marker-free deletion cassettes for *pks17* and the putative chrysogene SM cluster were constructed using the MoClo modular cloning system.³⁸ PCR-amplified fragments of 1–1.7 kb of the 5'- and 3'-UTRs of the targets were assembled into pICH41331, resulting in plasmids pCPO-1-*pks17* and pCPO-2-ChyCluster. The dDNA added to the transformation was PCR-amplified using these vectors as templates. For generation of a 2 kb marker-free dDNA for *pks17*, primers *pks17_1kb_F* and *pks17_1kb_R* were used. The sequences of all the primers used in this study can be found in the [Supporting Information](#), Tables S3–S7. *Escherichia coli* DH5 α strain, restriction enzymes

and T4 DNA ligase used in this study were purchased from NEB or Thermo Fisher Scientific, USA. Phusion HF (Thermo Fisher Scientific, USA), or KAPA HiFi HotStart (Kapa Biosystems, USA) polymerase were used for amplification of ddNAs and all genetic elements to be incorporated in plasmids. Phire Hot Start II (Thermo Fisher Scientific, USA) was used for diagnostic PCRs.

Construction of AMA1 Based Vectors. The AMA1-containing replicating plasmids are based on plasmid pAMPF21*, a derivative of plasmid pAMPF21³⁹ that was modified by removing specific restriction sites in the AMA1 region. These modifications did not affect replication in *P. chrysogenum*. To create an AMA1-containing control plasmid with a constitutively expressed *DsRed-SKL* gene, a 2108 bp DNA fragment containing the *gpdA* promoter of *A. nidulans*, *DsRed-SKL* and the *P. chrysogenum* terminator of *penDE*, isolated from pBBK-007,⁴⁰ was digested with EcoRV + NotI and cloned between the BglII (blunted by Klenow treatment) and NotI sites of plasmid pAMPF21*, yielding plasmid pJAK-109 (Figure 6a). The plasmid was transformed in circular form into *P. chrysogenum* DSS4465 protoplasts and selected for using phleomycin resistance. *DsRed-SKL* expression resulted in strongly red fluorescent colonies that could even be visualized with normal light. pJAK-109 was later recovered from the *P. chrysogenum* cells and transformed to *E. coli*, where plasmids were selected for with chloramphenicol. pYN-2 was modified from pJAK-109 isolated from *E. coli* cultures. In pYN-2, the *DsRed-SKL* marker was switched to *mKate* and put under the *A. nidulans* 40S promoter (Figure 6b).

The pJAK-109 vector was also used as a selection marker carrier, for marker-free genome editing with RNPs. In order to select for cells that were able to take up the plasmid and successfully perform HR for repairing the plasmid, the *ble* expression cassette in pJAK-109 was switched to an *amdS*-cassette using *in vivo* recombination in *P. chrysogenum* (Figure 6a). The *amdS* expression cassette was PCR amplified from pDest-final-*pks17* with primers that add 100 bp of homology toward the 5'- and 3'- regions flanking the *ble* expression cassette in pJAK-109.

Construction of Cas9 and sgRNA Expression Vectors. The MoClo modular cloning system³⁸ was employed for construction of Cas9 and sgRNA expression cassettes, that were inserted to an AMA1 based expression plasmid (pYN-2) through *in vivo* recombination (Figures 6b and c).

The MoClo scheme allows for construction of multigene constructs in three rounds using the Golden Gate technology.⁴¹ We included 5' and 3' flanking regions of 1 kb for incorporation of the expression cassettes to the AMA1 vector as separate level 1 modules that were combined with the level 1 modules for Cas9, sgRNA or *amdS* expression (Figures 6b and c). The level 1 modules were combined in one or two expression cassettes that were inserted in pYN-2 by *in vivo* HR in *P. chrysogenum*.

The endogenous elements for the expression cassettes constructed in this study, were amplified by PCR from genomic DNA (gDNA) of *P. chrysogenum* DS68530, whereas the *gpdA* promoter and *amdS* gene were amplified from the vector pDONR221 (used for creation of pDest-final-*pks17*).²⁰ The xylose inducible promoter and its terminator from *xlnA* of *A. nidulans* were amplified from gBlocks (IDT, USA). The sgRNA constructs were ordered as oligos that were annealed before assembly to level 0 vectors. Internal BsaI and BpiI recognition sites of the DNA elements were removed during

the cloning. hCas9, originating from *Staphylococcus pyogenes* was kindly provided by Sophien Kamoun⁴² via Addgene (Plasmid #49770).

Three different RNAP (RNA polymerase) III type promoters and terminator constructs were tested for transcription of the sgRNA (Figure 1b). The hypothetical U6 of *P. chrysogenum* was selected based on homology to the *Trichophyton rubrum* U6 gene.⁴³ U6 and other RNAP III promoters have commonly been used for expression of sgRNA in various organisms. In addition, we tested two tRNA promoters from *P. chrysogenum* for transcription of sgRNA. 145 tRNA encoding genes have been identified in *P. chrysogenum* by sequence annotation.³⁴ The tRNA promoters tested were chosen based on sequence suitability (e.g., no overlapping hypothetical promoter/terminator regions, large amount of BsaI/BpiI recognition sites or repetitive sequences). Furthermore, the promoter of *utp25* was tested for transcription of sgRNA. *Utp25* is presumably a RNAP II type promoter; *utp25* was annotated to encode a U3 small nuclear RNA-associated protein.³⁴

Cas9 Purification. The isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible Cas9-NLS expression vector, pET28(a)-Cas9-Cys¹⁵ was kindly provided by Hyongbum Kim via Addgene (Plasmid #53261). *E. coli* T7 Express lysY (New England Biolabs, UK) was used for Cas9 overexpression in 2xTY (16 g/L Tryptone, 10 g/L Yeast Extract, 5.0 g/L NaCl) medium with 25 μ g/mL kanamycin. Expression was induced at OD₆₀₀ of 0.4 by adding IPTG to a final concentration of 4 mM. Cultures were grown at 250 rpm at 18 °C for 16 h in a rotary incubator. Cas9 was purified as described previously,¹⁵ using Ni-NTA resin (Qiagen, Germany) for affinity-chromatography of His-tagged Cas9. After purification, the protein concentration was determined by measuring the absorption at 280 nm using NanoDrop ND1000 (Thermo Fisher Scientific, USA). Cas9 protein samples were analyzed for purity using SDS-PAGE and the ImageJ⁴⁴ software was used to quantify band intensities.

In Vitro sgRNA Synthesis and In Vitro Activity of Cas9.

The sgRNA was synthesized using the Ambion MegaScript RNA synthesis Kit (Thermo Fisher Scientific, USA), with 0.75 μ g of sgRNA-template (see Results) and 0.25 μ L SUPERase In RNase Inhibitor (20 U/ μ L, Thermo Fisher Scientific, USA) added to the 10 μ L sgRNA synthesis reaction that was incubated at 37 °C for a minimum of 6 h. For synthesis control, 0.5 μ L of sgRNA synthesis mix was analyzed by electrophoresis on 2% agarose gels and the sgRNA was directly used for transformation experiments without any purification. For sgRNA synthesis quantification, five different RNA constructs were amplified during 12 h incubation at 37 °C. The template DNA was digested with Turbo DNase (Thermo Fisher Scientific, USA) and the sgRNA was purified by phenol-chloroform-extraction. The sgRNA concentrations were determined using NanoDrop ND1000.

For *in vitro* activity tests, four different sgRNAs targeting *pks17* were designed and synthesized as described above. The DNA sequence to cleave was amplified from gDNA of DS68530, isolated with the E.Z.N.A Fungal DNA Kit (Omega Bio-Tek, USA). Approximately 600 ng of unpurified PCR product was pretreated with ExoSAP-IT (Affymetrix, USA) and assayed in a 30 μ L Cas9 cleavage reaction containing 1 μ L sgRNA and 2 μ L Cas9 protein in 1x Cas9 activity buffer (20 mM HEPES pH 7.5, 150 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 10 mM MgCl₂). After 90 min incubation at 37 °C, the reaction was quenched by adding 3 μ L 0.25 M EDTA and 4 μ L

Purple gel loading dye (New England Biolabs, UK). Samples were incubated for 10 min at 60 °C to release Cas9 from the DNA, after which the cleavage of the target sequences was analyzed by electrophoresis (Figure S5).

Fungal Transformations and Analysis of Transformants. Transformations of *P. chrysogenum* were done as described previously.⁴⁵ Protoplasts were diluted to approximately 1×10^7 protoplasts per ml and 100–200 μL of protoplast solution was used per transformation. For initial tests of integrating an *amdS* dDNA into the *pks17* locus of DS68530 ($\Delta hdaA$, Figure S1) a slightly modified transformation protocol, including a sorbitol gradient centrifugation was employed. Using this protocol higher transformant numbers were obtained, when compared to the more rapid method by Kovalchuk *et al.*,⁴⁵ that was used in all proceeding experiments.

For the experiments with Cas9 expressed from the AMA1 plasmid, about 1 μg of each DNA component was added to the transformation. pYN-2 was linearized using SnaBI and AgeI, whereas the Cas9 and sgRNA expression constructs were linearized using MreI that cuts twice in the backbone of the MoClo vectors. The dDNA amplified by PCR was purified with the PCR cleanup Kit (Sigma-Aldrich, UK) prior to addition to the transformation mixture.

For the RNP experiments, 220 nM of Cas9 protein (10 μL) was mixed with 30 μL 10xCas9 activity buffer, 35 μL 2xSTC buffer (2.4 M sorbitol, 100 mM CaCl_2 , 20 mM Tris-HCl at pH 7.5) and 4 μL sgRNA. For control experiments, 5 mM Tris-HCl, pH 8.5, was used instead of sgRNA. Before addition to the protoplasts, the mixture was incubated at 37 °C for 15 min in order to form RNP complexes. Depending on the experiment, 1–11 μg of dDNA was cotransformed with the RNPs. Aurintricarboxylic acid (ATA) was omitted in RNP experiments, as this was shown to lead to inactivation of Cas9 (data not shown).

For marker-free genome editing using RNPs, pJAK-109 was linearized with *KpnI* and *HindIII* to remove the phleomycin expression cassette. One μg of the linearized vector was cotransformed with 1 μg of PCR-amplified *amdS*-expression cassette (amplified from pDest-final-*pks17*) having 0.1 kb homologous flanks for HR into pJAK-109 (Figure 6a).

After transformation, protoplasts were incubated at 25 °C and increased humidity for 4–6 days on recovery plates containing 1 M sucrose and in some cases 0.1% acetamide.⁴⁵ Colonies from the transformation plates were transferred to minimal selection (S-agar), rich sporulation (R-agar) or penicillin production (PPM) medium solidified with 1% agar.⁴⁵ To confirm the integration of a dDNA at the correct locus, colony PCR was performed using the Phire Plant Direct PCR Kit (Thermo Fisher Scientific, USA) or with standard PCR reactions using gDNA extracted from the cells using Lysing Enzymes from *Trichoderma harzianum* (Sigma-Aldrich, UK). In some cases, the genome editing event was verified by amplifying gDNA extracted from the colonies using the E.Z.N.A Fungal DNA Kit (Omega Bio-Tek, USA). In addition, PCR products of selected transformants were sequenced (by Macrogen, UK).

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00082.

Strain and target information as well as sequences of oligonucleotides used in this study together with additional data on the function of the CRISPR/Cas9 technology. Figure S1. PCR verification of transformants and transformation efficiencies with dDNAs having different homology lengths. Figure S2. Influence of the amount of Cas9 protein and sgRNA on the transformation efficiency. Figure S3. Influence of use of different protospacers on transformation efficiency. Figure S4. Demonstration of AMA1 plasmid instability in *P. chrysogenum*. Figure S5. *In vitro* cleavage of *pks17* and *amdS* by Cas9. Table S1. Fungal strains used. Table S2. Genetic targets and strains used. Table S3. Primers for preparation of dDNA-plasmids using MoClo. Table S4. Primers for amplification of dDNA. Table S5. Primers with long flanks, for preparation of dDNA. Table S6. Primers for preparation of MoClo and AMA1 vectors. Table S7. Primers for colony PCR. (PDF)

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Author Contributions

Y.N., A.J.M.D. and R.A.L.B. designed the experiments; C.P. and Y.N. performed the experiments with the help of J.A.K.W.K.; Y.N. and C.P. wrote the manuscript with the improvements from A.J.M.D. and R.A.L.B.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas, CRISPR-associated RNA-guided DNA endonucleases; RNP, ribonucleoprotein; SM, Secondary metabolite; CSI, classical strain improvement; PKS, Polyketide synthase; NRPS, Non-ribosomal peptide synthase; DSB, Double strand break; sgRNA, single chimeric guide RNA; PAM, protospacer adjacent motif; NHEJ, Nonhomologous end-joining; ORF, open reading frame; dDNA, donor DNA; HR, Homologous recombination; HDR, Homology directed repair; AMA1, Autonomous maintenance in *Aspergillus*; UTR, untranslated region

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