SUPPLEMENTARY MATERIALS

Foster et al.

ALB1 the native locus at the target sequence

GCCACAAGCTCCCTCACCAAGGCCACGGCCATCCCGGTCTACGGCCCGTACCACGCCCCCCACCTCCACCGCCCTGAGGATGTCGACAACATCC

TGGGCCTCAACAACCCCGAGATGATTGACACCTTCCTCAACACCAAGCCCCGCTCCTCCGTCATGTCC

Alb-(1)top (donor DNA to introduce a stop codon within the genomic target sequence)

CTCCCTCACCAAGGCCACGGCCATCCCGGTCTACGGCCCGTAGCACGCCCCCCACCTCCACCGCCCTGAGGATGTCGAC

Alb-(1)bot (this oligo is annealled to Alb1-(1)top to form a dsDNA donor)

GTCGACATCCTCAGGGCGGTGGAGGTGGGGGGCGTGCTACGGGCCGTAGACCGGGATGGCCGTGGCCTTGGTGAGGGAG

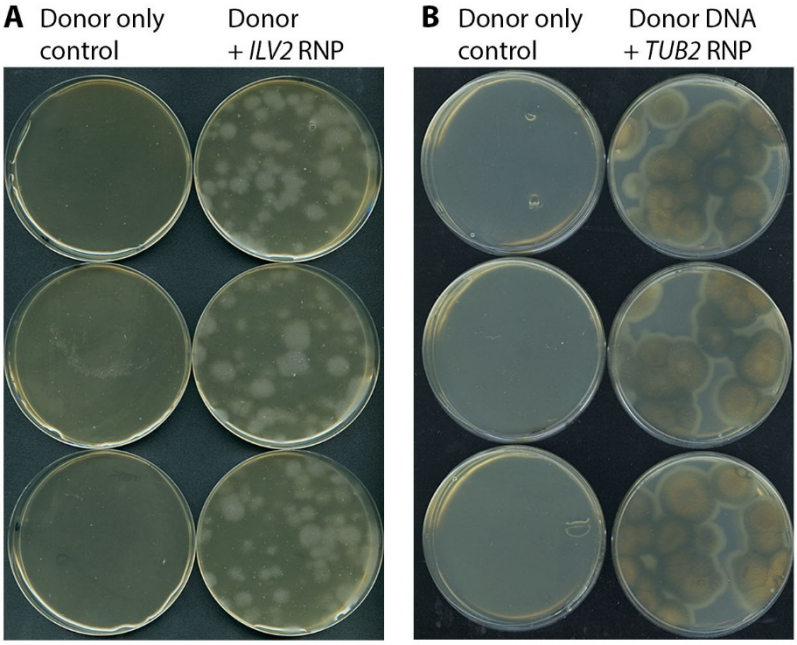
Alb1-(2)top (donor DNA to introduce a stop codon at a distance from the genomic target sequence)

CTCCCTCACCAAGGCCACGGCCATCCCGGTCTACGGCCCGTACCACGCCCCCCACCTCCACCGCCCTTAGGATGTCGAC

Alb-(2)bot (this oligo is annealled to Alb1-(1)top to form a dsDNA donor)

GTCGACATCCTAAGGGCGGTGGAGGTGGGGGGCGTGGTACGGGCCGTAGACCGGGATGGCCGTGGCCTTGGTGAGGGAG

Figure S1. Oligonucleotide donors used in RNP-CRISPR-Cas9 co-editing experiments to introduce a premature stop codon in *ALB1*.
The oligonucleotide donors used to target *ALB1* by introducing a premature stop codon are illustrated together with the genomic target sequence for the *ALB1* targeting RNP-CRISPR-Cas9 complex. In the case of donor 1-1 the edit is within the genomic target sequence whereas in the case of donor 1-2 the edit is located 40 bp away from the PAM site used. DsDNA donors were generated by mix equimolar concentrations of the 'top' and 'bot' oligo together heating at 95oC for 5 mins and then cooling on the bench top. 2 μg of annealed oligo was transformed together with the *ALB1* targeting RNP



C

MGG_06868 *ILV2* – crispr mediated chlorimuron ethyl resistant allele
(A to D substitution at amino acid)

Genomic target seq GGCGTCGCTTCCAATAGCAGngg

Donor oligos used
SURtop
GTATTCTCAGGACAGGTTGTTACCTCTGaTATTGGAAGCGACGCCTTCCAGGAGGCCGACGTCATAGGCATCTCCCGGTC

SURbot
GACCGGGAGATGCCTATGACGTCGGCCTCCTGGAAGGCGTCGCTTCCAATAtCAGAGGTAACAACCTGTCCTGAGAATAC

D G T P L V V F S G Q V V T S d I G S D A F
CCGACGGTACACCTCTGGTTGTATTCTCAGGACAGGTTGTTACCTCTGaTATTGGAAGCGACGCCTTC

SulphR TPMADALADGTPLVVFSGQVVTSDIGSDAFQEADVIGISRSCTKWNVMVKS
Wt TPMADALADGTPLVVFSGQVVTSAIGSDAFQEADVIGISRSCTKWNVMVKS

D

TUB2 RNP Genomic target GACCAGCTGGTGGACCGAGAGGG

E > A (GAG > GCG) edit at position 198

V V E P Y N A T L S V H Q L V E N S D E T F C I D
GTCGTTGAGCCCTACAACGCTACCCTCTCGGTCCACCAGCTGGTCGAGAACTCTGACGAGACCTTCTGCATTGAC

N E A L Y D I C M R T L K L S N P S Y G D L N Y L
AACGAGGCTCTGTACGACATCTGCATGCGCACCCTGAAGCTGTCGAACCCCTCATACGGTGACCTGAACTACCTG

Edited at 198
GTCGTTGAGCCCTACAACGCTACCCTCTCGGTCCACCAGCTGGTCGAGAACTCTGACGcGACCTTCTGCATTGAC

Donor oligos used
benR-top GTCGTTGAGCCCTACAACGCTACCCTCTCGGTCCACCAGCTGGTCGAGAACTCTGACGcGACCTTCTGCATTGACAACGA

benR-bot TCGTTGTCAATGCAGAAGGTCgCGTCAGAGTTCTCGACCAGCTGGTGGACCGAGAGGGTAGCGTTGTAGGGCTCAACGAC

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34 **Figure S2. Oligonucleotide donors used in RNP-CRISPR-Cas9 co-editing experiments targeting *TUB2***
35 **or *ILV2* and plates showing the selection procedure and the results of the transformation with the**
36 **appropriate RNP complex.**
37 **a.** Transformation plates are shown for the *ILV2*-targeting RNP transformed together with the donor
38 DNAs shown in **c.** **b.** Transformation plates are shown for the *TUB2*-targeting RNP transformed
39 together with the donor DNAs shown in **d**. In **c** and **d** the design of the oligonucleotides conferring
40 resistance to sulfonylurea and benomyl respectively is illustrated with their location relative to the
41 genomic target sequence for the respective RNP complex.
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Table S1. Raw data for the transformation numbers presented in Fig. 1a using constructs expected to stably and constitutively express Cas9

Number of transformants	Empty vector[a]	*ALB1* targeting vector[b]	*PKS1* targeting vector[c]
Experiment 1	67	2	2
Experiment 2	52	1	6
Experiment 3	64	0	1
Experiment 4	83	3	1

Agrobacterium compatible vectors containing the Cas9-NLS encoding gene under the control of the TrpC promoter and terminator were introduced into Guy 11 using *Agrobacterium*-mediated transformation. Transformant numbers were assessed after 7 days on selective medium (transformants were subsequently sub-cultured for assessment of pigmentation after growth on CM). None of the transformants isolated exhibited pigmentation defects. Three different versions (including the version functional in *N. crassa* (Matsu-Ura et al., 2015) shown used here) of the Cas9-NLS-encoding gene codon optimised for different fungi were tested in all every time with the same result that very few transformants were generated and none of these exhibited pigmentation alterations (data not shown).

Table S2. Summary of transformation experiments targeting *ALB1* and *RSY1* using the RNP-Crispr method and donor only controls

RNPs donors used	Number of transformants	Number that are albino/rosy	Efficiency (%)
ALB1 Donor DNA + *ALB1* -RNP	4	4	100 %
Control: *ALB1* Donor DNA only	2	0	0 %
ALB1 Donor DNA + *ALB1* RNP	112	96	86 %
ALB1 Donor DNA only	64	3	5%
ALB1 Donor DNA + *ALB1* RNP	16	8	50 %
ALB1 Donor DNA only	11	1	9 %
ALB1 Donor DNA + *ALB1* RNP	76	54	71 %
ALB1 Donor DNA only	43	5	12 %
ALB1 Donor DNA + *ALB1* RNP	86	69	80 %
Donor DNA only	48	7	14 %
RSY1 Donor DNA + *RSY1* RNP	20	20	100 %
Control: *RSY1* Donor DNA only	3	0	0 %
RSY1 Donor DNA + *RSY1* RNP	38	37	97.5 %
Control: *RSY1* Donor DNA only	8	1	12.5 %
RSY1 Donor DNA + *RSY1* RNP	12	12	100 %
Control: *RSY1* Donor DNA only	13	1	8 %
RSY1 Donor DNA + *RSY1* RNP	61	60	98 %
Control: *RSY1* Donor DNA only	47	5	11 %

The number of transformants for individual transformation experiments using *ALB1*-trageting-RNP-CRISPR-Cas9 or *RSY1*-targeting RNP-CRISPR-Cas9 complexes are shown. Also shown are donor only controls which show the efficiency obtained in the absence of the RNP complex and illustrate the efficiency of gene targeting which would be obtained in a 'traditional' gene deletion experiment.

Table S3. Summary of transformation experiments targeting *ALB1* using the RNP-Crispr method and PCR amplified donor DNAs with 40 bp or 30 bp regions of homology to *ALB1* at either end

RNPs	Donor	Number of transformants	No. of albino mutants	Efficiency (%)
ALB1 -RNP	40 bp flanks	11	8	73 %
None	40 bp flanks	5	0	0 %
ALB1 RNP	40 bp flanks	48	27	56 %
None	40 bp flanks	37	0	0%
ALB1 RNP	40 bp flanks	16	10	63 %
None	40 bp flanks	22	0	0 %
ALB1-RNP	30 bp flanks	29	17	59 %
None	30 bp flanks	12	0	0 %
ALB1-RNP	30 bp flanks	50	28	56 %
None	30 bp flanks	59	0	0 %
ALB1-RNP	30 bp flanks	24	11	46 %
None	30 bp flanks	21	0	0 %

The number of transformants for individual transformation experiments using *ALB1*-targeting-RNP-CRISPR-Cas9 complexes in combination with the PCR generated donor DNA where a repair template with either 30 bp or 40 bp *ALB1* homologous regions flank the *BAR* gene conferring glufosinate-ammonium (basta) resistance. Also shown are donor only controls which show the efficiency obtained in the absence of the RNP complex.

Table S4. Co-editing experiments based on selection for carboxin, sulfonylurea or benomyl resistance

RNPs	Donors	Strain	No. examined	No. of *alb1* mutants	No with correct edit	Efficiency (%)
SDI1 + *ALB1*	*SDI1R* (80 bp oligos) + *ALB1 STOP inside target*	Guy 11	191	5	3	1.6%
SDI1 + *ALB1*	As above	Guy 11	276	3	3	1.1 %
SDI1 + *ALB1*	As above	Guy 11	339	4	3	0.9 %
SDI1 + *ALB1*	As above	Guy 11	369	11	6	1.6 %
SDI1 + *ALB1*	As above	Δ*Ku70*	37	3	3	12.3%
SDI1 + *ALB1*	As above	Δ*Ku70*	23	0	0	0 %
SDI1 + *ALB1*	As above	Δ*Ku70*	29	1	1	3.4 %
SDI1 + *ALB1*	As above	Δ*Ku70*	45	3	3	6.7 %
SDI1 + *ALB1*	SDI1 carboxinR(80 bp oligos) *ALB1* STOP outside target	Guy 11	275	5	3	1.1%
SDI1 + *ALB1*	As above	Guy 11	241	2	2	0.8 %
SDI1 + *ALB1*	As above	Guy 11	298	2	2	0.7 %
SDI1 + *ALB1*	As above	Guy 11	351	4	2	0.5 %
TUB2 + *ILV2*	SulfonylureaR+ BenomylR	Guy 11	259	2	2	0.7 %
TUB2 + *ILV2*	SulfonylureaR + BenomylR	Guy 11	384	4	4	1.0 %
TUB2 + *ILV2*	SulfonylureaR + BenomylR	Guy 11	256	4	4	1.5 %

The number of transformants generated in co-editing experiments using Guy 11 or the *Ku70*$^{-}$ strain (23) are shown. Transformants were picked before any pigmentation was apparent. Co-editing-based mutation of *ALB1* used the oligonucleotides shown in Fig. S1 where the edit is either within the genomic target sequence (donor 1-1) of 40 bp distant to the PAM site (donor 1-2). Because *ALB1* mutants might also contain indels rather than integrations of the donors all albino strains were also checked by PCR amplification of DNA surrounding the

target site and the efficiency is defined as the total number of transformants from those picked that have the correct integration of the donor DNA confirm by sequencing. In the case of co-editing with the *TUB2* and *ILV2* targeting CRISPR-Cas9-RNPs sequencing was only performed to check that the desired edits were present. In the case of *TUB2* indels can be expect to lead to loss of viability so that the efficiency was assessed directly as the proportion of the sulfonylurea resistant transformants that were also benomyl resistant. *TUB2* is MGG_00604 and *ILV2* is MGG_06868

Table S5. Summary of genome reads and assemblies used in the current study

Strain	Number of reads[a]	Size of assembly (bp)[b]	Number of scaffolds[b]	N50 contig length (bp)[b]	Mean Coverage[c]
Alb1_3	25,418,446	38,054,642	3,676	49,277	132.1x
Alb1_6	10,549,692	38,356,752	1,900	78,092	52.9x
R1	30,222,537	38,313,674	3,985	53,480	158.7x
R2	26,471,457	38,078,197	3,727	53,653	139.8x

[a]125 base paired-end

[b] Genome assembled 'de novo' using SPAdes 3.11.0 (46)

[c] mapped against reference genome 70-15 (47) using BWA (48)

To determine whether off target mutations had occurred in the sequenced mutants, the two *alb1* mutants generated by CRISPR-Cas9-RNP and the two strains that had been through the transformation procedure and regenerated but not exposed to RNP complexes were sequenced. Sequences were generated using an Illumina HiSeq 2500 with standard reagents and protocols producing 125 base paired-end reads. Reads were filtered using the fastq-mcf program from the ea-utils package, ea-utils (45): "Command-line tools for processing biological sequencing data"; https://github.com/ExpressionAnalysis/ea-utils). Genomes were assembled 'de novo' using SPAdes 3.11.0 (46).

133 **Table S6. Sequences of the oligonucleotides used in the current study**

Name	Sequence
	Primers used in construction of vectors
ALB1-for-EcoRI	ctaatcaatcgaattcGGATTCCTCGCCGAGTTCTAC
ALB1-rev-SpeI	ctatacaactagtTCACGCCAGTCATCGGAAGTC
ALB1-for	GGATTCCTCGCCGAGTTCTAC
ALB1-rev	CATGCCAGGCTTCTCAATCCAG
RSY-donor-f	gccgactttgagccaagaagc
RSY-donor-r	cccggtgcaagccttacctag
Cas9-recom-f	GGATAATTCAATAGGCCAACAACACGTTGACGGAGAAGATGATATTGAAGGAGCATTTTTGG
Cas9-recom-r	GGTTCAATTGCGATTCATGTGGTGCGAGCAGTCTTTGTCCTCTAGAAAGAAGGATTACCTCTAAAC
PKS1gRNA-f	TCTTTGAAAAGATAATGTATGATTATGCTTTCACTC
PKS1gRNA-r	CTTATTTTAACTTGCTATTTCTAGCTCTAAAACCTCGTTTAGTTGTTGCAGTGGATCATTTATCTTTCACTGCGGAGAAG TTTCGAACGCC
SDH1-sg-r	CTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGCTTGTCGAGGAAGGAGCGATCATTTATCTTTCACTGCGGAGAA GTTTCGAACGCC
gRNAto315Cas-R	GTTCAATTGCGATTCATGTGGTGCGAGCAGTCTTTGTCTCTTTGAAAAGATAATGTATGATTATGCTTTCACTC
sgRNA-rec-R	GAGCAGTCTTTGTCCTCTAGAAAGAAGGATTACCTCTAAACAGACATAAAAAACAAAAAAAGCACCACCGACTCGG
	Primers used as templates for sgRNA synthesis
sgSDI1	TTCTAATACGACTCACTATAGAATGGTGTGGCAACGGTACGTTTTAGAGCTAGA
SgTUB2	TTCTAATACGACTCACTATAGACCAGCTGGTGGACCGAGAGTTTTAGAGCTAGA
sgILV2	TTCTAATACGACTCACTATAGGCGTCGCTTCCAATAGCAGGTTTTAGAGCTAGA
Sg-SEP5-n-gfp	TTCTAATACGACTCACTATAGAACTTGAAAGGAATTTGGTGTTTTAGAGCTAGA
sgSEP5ts	TTCTAATACGACTCACTATAGTCTGTGGAGCCTCAGGAACGTTTTAGAGCTAGA
sgSDH1	TTCTAATACGACTCACTATAGCTCCTTCCTCGACAAGCTCGTTTTAGAGCTAGA
sgPKS-2	TTCTAATACGACTCACTATAGCGTGGTACGGGCCGTAGACGTTTTAGAGCTAGA
	Primers used for PCR amplification of donor DNAs with BAR or GFP
ALB-BARkoF	GCCGCCACAAGCTCCCTCACCAAGGCCACGGCCATCCCGGTCGTCGACAGAAGATGATATTGAAGGAGC
ALB-BARkoR	GCAGAGCCTCTTGCATGAAACCTTGAAGCTGCTTGGCCTGGTCGACCTAAATCTCGGTGACGG

ALB-BARkoS-f	AAGCTCCCTCACCAAGGCCACGGCCATCCCGGTCGTCGACAGAAGATGATATTGAAGGAGC
ALB-BARkos-r	TTGCATGAAACCTTGAAGCTGCTTGGCCTGGTCGACCTAAATCTCGGTGACGG
SEP5-N-GFP-f	CAACATCGATCTAACCAACCAAATTCCTTTCAAGTTCAACACATGGTGAGCAAGGGCGAG
SEP5-N-GFP-r	gaaacggtccagaaggttgcggtcaacgtacGGCGGGAAACGACATCTTGTACAGCTCGTCCATGCC
	Primers used to anneal together to form dsDNA donor DNAs
Alb-(1)top	CTCCCTCACCAAGGCCACGGCCATCCCGGTCTACGGCCCGTAGCACGCCCCCCACCTCCACCGCCCTGAGGATGTCGAC
Alb-(1)bot	GTCGACATCCTCAGGGCGGTGGAGGTGGGGGGCGTGCTACGGGCCGTAGACCGGGATGGCCGTGGCCTTGGTGAGGGAG
Alb1-(2)top	CTCCCTCACCAAGGCCACGGCCATCCCGGTCTACGGCCCGTACCACGCCCCCCACCTCCACCGCCCTTAGGATGTCGAC
Alb-(2)bot	GTCGACATCCTAAGGGCGGTGGAGGTGGGGGGCGTGGTACGGGCCGTAGACCGGGATGGCCGTGGCCTTGGTGAGGGAG
SDI1-80bp-top	CGCCTGGCCCTTGACAACTCCATGAGCCTGTACCGTTGCCTCACCATTCTTAACTGCACAAGGACCTGCCCGAAGGGCCT
SDI1-80bp-bot	AGGCCCTTCGGGCAGGTCCTTGTGCAGTTAAGAATGGTGAGGCAACGGTACAGGCTCATGGAGTTGTCAAGGGCCAGGCG
SDI1-60bp-top	TTGACAACTCCATGAGCCTGTACCGTTGCCTCACCATTCTTAACTGCACAAGGACCTGCC
SDI1-60bp-bot	GGCAGGTCCTTGTGCAGTTAAGAATGGTGAGGCAACGGTACAGGCTCATGGAGTTGTCAA
SDI1-50bp-top	TTGACAACTCCATGAGCCTGTACCGTTGCCTCACCATTCTTAACTGCACA
SDI1-50bp-bot	TGTGCAGTTAAGAATGGTGAGGCAACGGTACAGGCTCATGGAGTTGTCAA
SDI1-40bp-top	TTGACAACTCCATGAGCCTGTACCGTTGCCTCACCATTCT
SDI1-40bp-bot	AGAATGGTGAGGCAACGGTACAGGCTCATGGAGTTGTCAA
SDI1-30bp-top	AACTCCATGAGCCTGTACCGTTGCCTCACC
SDI1-30bp-bot	GGTGAGGCAACGGTACAGGCTCATGGAGTT
SDI1-20bp-top	GAGCCTGTACCGTTGCCTCA
SDI1-20bp-bot	TGAGGCAACGGTACAGGCTC
benR-top	GTCGTTGAGCCCTACAACGCTACCCTCTCGGTCCACCAGCTGGTCGAGAACTCTGACGcGACCTTCTGCATTGACAACGA

benR-bot	TCGTTGTCAATGCAGAAGGTCgCGTCAGAGTTCTCGACCAGCTGGTGGACCGAGAGGGTAGCGTTGTAGGGCTCAAC
	GAC
TUB2-wt-top	GTCGTTGAGCCCTACAACGCTACCCTCTCGGTCCACCAGCTGGTCGAGAACTCTGACGAGACCTTCTGCATTGACAAC
	GA
TUB2-wt-bot	TCGTTGTCAATGCAGAAGGTCTCGTCAGAGTTCTCGACCAGCTGGTGGACCGAGAGGGTAGCGTTGTAGGGCTCAAC
	GAC
SURtop	GTATTCTCAGGACAGGTTGTTACCTCTGaTATTGGAAGCGACGCCTTCCAGGAGGCCGACGTCATAGGCATCTCCCGG
	TC
SURbot	GACCGGGAGATGCCTATGACGTCGGCCTCCTGGAAGGCGTCGCTTCCAATAtCAGAGGTAACAACCTGTCCTGAGAA
	TAC
Sep5-ts-donor-top	GAAGGGGATTCAGTTCTGTCTGATGGTCTGTgagGCCTCAGGAACTGgtgagttGCAGGACTACCTTCGTCAACACTCTT
Sep5-ts-donor-bot	AAGAGTGTTGACGAAGGTAGTCCTGCaactcacCAGTTCCTGAGGCctcACAGACCATCAGACAGAACTGAATCCCCTTC
	Primers used for generating PCR products to confirm mutations
PKS-ck-f	GGATTCCTCGCCGAGTTCTAC
PKS-ck-r	CAATCATCTCGGGGTTGTTGAGG
SEP6ts?-f	CACACCCTGAAGCCCCTTGATATC
SEP6ts?-R	CTCCTCGGTTGTGTGGATGAG