Rapid improvement of domestication traits in an orphan crop by genome editing

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Genome editing holds great promise for increasing crop productivity, and there is particular interest in advancing breeding in orphan crops, which are often burdened by undesirable characteristics resembling wild relatives. We developed genomic resources and efficient transformation in the orphan Solanaceae crop ‘groundcherry’ (Physalis pruinosa) and used clustered regularly interspaced short palindromic repeats (CRISPR–CRISPR-associated protein-9 nuclease (Cas9)) to mutate orthologues of tomato domestication and improvement genes that control plant architecture, flower production and fruit size, thereby improving these major productivity traits. Thus, translating knowledge from model crop enables rapid creation of targeted allelic diversity and novel breeding germplasm in distantly related orphan crops.

There has been extensive discussion on leveraging genome-editing technologies to improve staple crops1, yet their application to regionally important plants grown for subsistence purposes is equally exciting, especially in developing countries. Such ‘orphan crops’ are relatively unknown and typically have not experienced intensive selection for domestication and improvement. Thus, orphan crops are less productive, untenable at larger agricultural scales, and benefit less from basic research2. Genome-editing technologies, such as the broadly successful clustered regularly interspaced short palindromic repeats (CRISPR–CRISPR-Cas9) provide opportunities to address these deficiencies, with primary goals to increase quality and yield, improve adaptation and expand geographical ranges of cultivation. The Solanaceae family contains many orphan crops, including the potato (Solanum tuberosum) and tomato (Solanum lycopersicum), that are grown in regions with a growing season of 100–150 days. These crops are not cultivated on a large scale, yet have genetic and genomic resources to make the Solanaceae an excellent platform for translating genome editing to orphan crops.

We focused on the orphan crop Physalis pruinosa (groundcherry), a wild Solanaceae that is more distantly related to the tomato than the pepper, and which is grown in Central and South America for its subtly sweet berries3,4. Barriers to higher productivity and wider cultivation include a wild sprawling growth habit and small ~1 g fruits that drop to the ground due to strong stem abscission (Fig. 1a–g). These undesirable characteristics parallel the wild ancestor of the tomato, Solanum pimpinellifolium, for which selection allowed major improvements in shoot architecture, flower production and fruit size5 (Fig. 1h–m). Although ground-cherry and related Physalis species have the same chromosome number as most Solanaceae (n=12)4, several challenges remain for gene editing, including the absence of reference genomes, limited information on gene content and function, and several architectural and fruit development traits that differ from the tomato (Fig. 1). However, considering its phylogenetic relationship with the tomato, its diploid genome and the fact that key developmental and productivity genes have similar functions across model Solanaceae, we hypothesized that Physalis orthologues of select tomato domestication and improvement genes could be modified through editing for immediate improvements.

A major obstacle for CRISPR–Cas9 plant genome editing is lack of efficient tissue culture and transformation methodologies5. For editing of P. pruinosa to be realized, we developed Agrobacterium tumefaciens-mediated transformation modelled after our tomato methodology (Supplementary Methods), and evaluated editing by targeting the orthologue of the tomato leaf development gene SI-AGO7 (where ‘SI’ relates to S. lycopersicum). This was previously used to test CRISPR–Cas9 in the tomato, because mutations resulted in conspicuous narrowing of the leaves and floral organs6. First-generation (T0) plants were chimeric for Ppr-AGO7 mutations (where ‘Ppr’ relates to P. pruinosa) and, like tomato SI-ago7 mutant T0 plants (where CR indicates CRISPR–Cas9-induced), the leaves and petals were narrower than in the wild type (Supplementary Fig. 1a,b), indicating efficient editing in the groundcherry.

To expand Physalis genomic resources beyond a leaf transcriptome from the related Physalis peruviana, which lacked orthologues of several tomato domestication and improvement genes7,8, we generated Illumina whole-genome and RNA sequencing de novo assemblies from vegetative and reproductive tissues (Supplementary Methods). Analysis9 revealed 93 and 82% complete benchmarking assemblies from vegetative and reproductive tissues (Supplementary Methods). Analysis10 revealed 93 and 82% complete benchmarking assemblies from vegetative and reproductive tissues (Supplementary Methods). Analysis11 revealed 93 and 82% complete benchmarking assemblies from vegetative and reproductive tissues (Supplementary Methods). Analysis12 revealed 93 and 82% complete benchmarking assemblies from vegetative and reproductive tissues (Supplementary Methods). Analysis13 revealed 93 and 82% complete benchmarking assemblies from vegetative and reproductive tissues (Supplementary Methods). 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Analysis49 revealed 93 and 82% complete benchmarking assemblies from vegetative and reproductive tissues (Supplementary Methods). Analysis50 revealed 93 and 82% complete benchmarking assemblies from vegetative and reproductive tissues (Supplementary Methods).

With these tools, our first efforts focused on modifying groundcherry shoot architecture to contain its weedy growth habit. In the tomato, selection for mutations in floriferous flowering pathway genes allowed major improvements in plant architecture and yield8. In particular, a classical missense mutation in the antiflorigen SELF-PRUNING (SP) gene (sp(austr)) provided compact ‘determinate’ growth that translated to a burst of flowers and fruits, thereby enabling large-scale field production9. SP encodes a flowering repressor that modulates sympodial cycling—a hallmark Solanaceae and many other plant species—and mutations in the pepper SP orthologue cause a similar acceleration of sympodial cycling and shoot termination8.

We searched our groundcherry assemblies for SP homologues and related florigen family members, and phylogenetic analysis...
revealed a probable orthologue of SP detected in both genome and transcriptome assemblies (Fig. 2a). We targeted Ppr-SP using two guide RNAs and found several out-of-frame insertion and deletion alleles in multiple chimeric T<sub>0</sub> plants. Notably, these plants were more compact than the wild type, and we validated this phenotype in homozygous and biallelic null Ppr-<i>sp</i><sup>CR</sup> T<sub>2</sub> progeny plants (Fig. 2b,c). Like the tomato <i>sp</i><sup>class<sub>a</sub></sup> mutant, primary shoot flowering time was unaffected in Ppr-<i>sp</i><sup>CR</sup> mutants (Fig. 2d). However, unlike the progressive sympodial termination of tomato <i>sp</i><sup>class<sub>a</sub></sup> plants, sympodial growth in Ppr-<i>sp</i><sup>CR</sup> plants ended immediately after flowering on both primary and axillary shoots, resulting in clusters of three or four fruits per shoot (Fig. 2e–g). Although potentially valuable for specific agricultural conditions, the severity of the Ppr-<i>sp</i><sup>CR</sup> phenotype suggests that null alleles could limit fruit production, similar to null tomato <i>sp</i> alleles that also terminate faster and cause more compact plants compared with the weaker <i>sp</i><sup>class<sub>a</sub></sup> allele<sup>16,17</sup> (not shown).

To identify other targets for plant architecture modification without negative effects on productivity, we used our phylogeny to identify the orthologue of SELF-PRUNING 5G (SP5G) (Ppr-SP5G), another florigen repressor (Fig. 2a).<sup>4</sup> In the tomato, SP5G controls primary and canonical axillary shoot flowering time and is the major contributor to day-length sensitivity in wild tomato species. The groundcherry flowers quickly in long-day conditions, after around five leaves, suggesting little or no day-length sensitivity, which we confirmed by showing that flowering occurred one leaf faster in short-day conditions (Supplementary Fig. 1c). This weak response mirrors the cultivated tomato, where reduced SP5G activity was selected during domestication to mitigate day-length sensitivity. CRISPR–Cas9-induced null <i>sp5g</i> mutations in the tomato eliminate residual day-length sensitivity, causing even faster primary and axillary shoot flowering.<sup>4</sup>

To determine whether similar effects could be achieved in the groundcherry, we targeted Ppr-SP5G. Multiple chimeric T<sub>0</sub> individuals showed more compact growth, but not as severe as in Ppr-<i>sp</i><sup>CR</sup>, and these plants were also more floral (Fig. 2b,i). Surprisingly, homozygous null Ppr-<i>sp</i><sup>5G</sup><sup>CR</sup> T<sub>2</sub> progeny carrying identical independently derived deletion alleles were unaffected for primary shoot flowering (Fig. 2j). In contrast, sympodial shoots terminated more rapidly, but to a lesser degree than Ppr-<i>sp</i><sup>CR</sup> plants (Fig. 2c,i,k), explaining the difference in compactness. Importantly, this moderate shoot termination resulted in up to 50% higher concentrations of fruits along each shoot, emulating the burst of fruit production in tomato <i>sp</i><sup>class<sub>a</sub></sup> mutants, and this effect became more pronounced with age (Fig. 2k,l). In addition to Ppr-<i>sp</i><sup>5G</sup><sup>CR</sup> plants showing greater agronomic potential than Ppr-<i>sp</i><sup>CR</sup> plants, phenotypic differences from mutations in orthodox Solanaceae florigen family members reflect how these conserved flowering regulators influence species-specific sympodial growth patterns (Fig. 1).

An additional target trait for improving groundcherry productivity is fruit size. In the tomato, mutations in the classical CLAVATA (CLV)–WUSCHEL meristem size pathway were major contributors to boosting fruit size by increasing the locale number from the bilocular fruits of <i>S. pimpinellifolium</i> (Fig. 1l)<sup>5,12</sup>. In particular, the fasciated (fas) locus—an inversion that partially disrupts the promoter of the <i>SlCLV3</i> signalling peptide gene—was a major fruit size domestication quantitative trait locus (QTL). In contrast, engineered null alleles of <i>SlCLV3</i> are undesirable due to excessive and disorganized production of flower and fruit organs<sup>17</sup>. Rather than creating a range of weak Ppr-CLV3 promoters, as we recently demonstrated for <i>SlCLV3</i><sup>37</sup>, we hypothesized that a subtle increase in the locale number could be achieved by mutating the orthologue of the tomato CLV1 gene, encoding one of several redundant leucine-rich receptors of the CLV3 peptide,
Fig. 2 | CRISPR-Cas9 targeting of domestication and improvement-related genes in *P. pruinosa*. **a**, Phylogenetic tree of CENTRORADIALIS/TERMINAL FLOWER 1/SELF-PRUNING (CETS) family genes in the tomato (*S. lycopersicum*), Arabidopsis and groundcherry (*P. pruinosa*). Contigs from de novo assemblies of both the *Physalis* genome (*Ppr-g*) and transcriptome (*Ppr-t*) were included in the phylogeny. Tomato and *P. pruinosa* SP and SPSG homologues are highlighted by red and orange coloured circles, respectively. Bootstrap values from 100 replicates are indicated on each node. **b**, CRISPR-Cas9 targeting of SP in *P. pruinosa*. *Ppr-sp*<sup>Cr</sup> mutant alleles identified by PCR and sequencing in *T<sub>0</sub>* transgenic plants are shown. **c**, *Ppr-sp<sup>Cr</sup>* stable homozygous null *T<sub>1</sub>* plants (middle and right) showing compact growth compared with the *Ppr-SP<sup>+/+</sup>* control (left). **d**, Quantification of the primary shoot flowering time in *Ppr-SP<sup>+/+</sup>* control and *Ppr-sp<sup>Cr</sup>* *T<sub>1</sub>* plants. Statistical significance was determined by a two-tailed, two-sample *t*-test. *n*, number of biologically independent plants. **e,f**, Photos (e) and schematics (f) of shoot apices of *Ppr-SP<sup>+/+</sup>* control (left) and *Ppr-sp<sup>Cr</sup>* *T<sub>1</sub>* plants (right), showing immediate termination of sympodial shoots into flowers in *Ppr-sp<sup>Cr</sup>*. Vegetative growth continues from axillary shoots. **g**, Formation of fruit clusters on *Ppr-sp<sup>Cr</sup>* *T<sub>1</sub>* plants (right) compared with the *Ppr-SP<sup>+/+</sup>* control (left). Red arrowheads indicate husked fruits. In **c** and **g**, representative images of *Ppr-sp<sup>Cr</sup>* homozygous *T<sub>1</sub>* plants from six repeated independent experiments with similar results are shown. **h**, CRISPR-Cas9 targeting of SPSG in *P. pruinosa*. *Ppr-sp5g<sup>Cr</sup>* mutant alleles identified by PCR and sequencing in *T<sub>0</sub>* transgenic plants are shown. **i**, Homozygous null *Ppr-sp5g<sup>Cr</sup>* *T<sub>1</sub>* plants. Note the more compact growth and fruit clusters in each sympodial unit. **j**, Quantification of primary shoot flowering time in *Ppr-SP5G<sup>+/+</sup>* control and *Ppr-sp5g<sup>Cr</sup>* *T<sub>1</sub>* plants. Statistical significance was determined by a two-tailed, two-sample *t*-test. *n*, number of biologically independent plants. **k**, *Ppr-sp5g<sup>Cr</sup>* *T<sub>1</sub>* plants (right) exhibit shoot units with faster flower and fruit initiation and development compared with *Ppr-SP5G<sup>+/+</sup>* controls (left). Red arrowheads mark fruits. In **i** and **k**, representative images of *Ppr-sp5g<sup>Cr</sup>* *T<sub>1</sub>* plants from two repeated independent experiments with similar results are shown. **l**, Quantification of fruits on five consecutive sympodial shoot nodes in *Ppr-SP5G<sup>+/+</sup>* control and *Ppr-sp5g<sup>Cr</sup>* *T<sub>1</sub>* plants. Significance was determined by a two-tailed, two-sample *t*-test. *n*, number of biologically independent individual shoots. Backgrounds in **e** and **g** were darkened to highlight whole-plant architectures. In **b** and **h**, guide RNA targets and protospacer-adjacent motif sequences are highlighted in red and bold font, respectively. Deletions and insertions are indicated by blue dashes and blue font, respectively. The sequence gap length is shown in parentheses. Scale bars, 1 cm. For all box plots (**d, j** and **l**), the bottom and top of boxes represent the first and third quartile, respectively, the middle line is the median, and the whiskers represent the maximum and minimum values. Hyphenated numbers following mutant allele names in **b** and **h** indicate the individual plant from which the allele was derived.
and for which CRISPR–Cas9 null mutations moderately increase the locule number compared with fss". Phylogenetic analysis revealed a clear orthologue of Ppr-CLV1 (Fig. 3a), which we targeted with two guide RNAs to produce chimeric plants that produced flowers with more organs compared with the wild type (Fig. 3b). Homozygous null Ppr-CLV1 T1 plants for a 7-base pair deletion validated this effect (Fig. 3b–e and Supplementary Fig. 1d–g), and nearly all fruits developed three locules, translating to a 24% increase in mass (Fig. 3g). A similar weak effect on the locule number caused by the tomato locule number (lc) regulatory mutation of the stem cell regulator WUSCHEL was the foundation for the selection of additional QTLs to create a range of larger tomato fruits. Such an approach is now possible in the groundcherry by engineering the coding sequence and regulatory mutations in orthologues of other tomato fruit-size QTLs.

Our study represents the first steps towards improving the groundcherry orphan crop, and we have already extended this work by targeting additional genes that we anticipate will benefit a range of agronomic and consumer quality traits, such as the homologue of the fruit abscission gene JOINTLESS-2 (ref. 19) (Supplementary Fig. 1h,i). Beyond these first targets, we are applying multiplex CRISPR–Cas9 of coding and regulatory sequences to simultaneously create qualitative and quantitative variation for multiple traits to rapidly enhance breeding germplasm. While an immediate goal is to elevate the uniquely flavoured fruits of several Physalis species alongside the strawberry, blueberry, blackberry and raspberry as the elusive ‘fifth berry’ crop in large-scale agriculture, we also aim to demonstrate the speed at which other important orphan crops, such as the grass teff, pseudocereal amaranth and legume cowpea, could be improved for regional production and potentially catapulted into mainstream agriculture by exploiting the large knowledge base of related model crops. Success will not be limited by gene-editing technologies per se, which continue to rapidly improve and expand 14, but by the availability of efficient transformation methodologies. Alleviating bottlenecks will require open access to genome-editing technologies for both public and private entities, as well as highly efficient, genotype-independent gene-delivery approaches 15,20.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Raw data from this study have been submitted to the National Center for Biotechnology Information Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession number SRP142654. Transcriptome and genome assemblies have been deposited at the Sol Genomics Network (ftp://ftp.solgenomics.net).

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References
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**Author contributions**
Z.H.L., N.T.R., J.D., J.V.E. and Z.B.L. designed and planned the experiments. All authors performed the experiments and collected the data. Z.H.L. performed all bioinformatics analyses. All authors analysed the data. Z.H.L., J.V.E. and Z.B.L. designed the research. Z.H.L., S.S., J.V.E. and Z.B.L. wrote the paper with input from all authors.

**Competing interests**
The authors declare no competing interests.

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Data analysis: R v3.4.3, RStudio v1.1.383, Excel v14.4.1, Benchling, clustalw2 v2.1, pal2nal v14, PhyML v20120412, FastQC v0.11.2, BUSCO v3.0.2, Trinity v2.2.0, megahit v1.1.1-2-g02102e1, Trinotate v2.0.2

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Sample sizes were defined based on previous work that found to be sufficient for determining statistically significant results. At least 6 independent replicates were analyzed in each experiment for data presented in Figure 2. n values and statistical tests are presented.

Data exclusions
No data excluded

Replication
Number of biologically independent replicates is presented in the Figure legends and Online Methods, and replicated experiments yielded similar results. Individual replicates (e.g. plants, shoots, flowers and fruits) are indicated and at least 6 independent replicates analyzed for each experiment.

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