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ORIGINAL RESEARCH

Identifying genes associated with abiotic stress tolerance suitable for CRISPR/Cas9 editing in upland rice cultivars adapted to acid soils

Luz S. Barrero[1,2] | Matthew R. Willmann[3] | Eric J. Craft[4] | Kazi M. Akther[2] | Sandra E. Harrington[2] | Gina A. Garzon-Martinez[1] | Raymond P. Glahn[4] | Miguel A. Piñeros[4] | Susan R. McCouch[2]

[1]Corporacion Colombiana de Investigacion Agropecuaria, AGROSAVIA, Mosquera, Colombia

[2]Plant Breeding & Genetics Section, School of Integrative Plant Science, Cornell University, Ithaca, New York, USA

[3]Plant Transformation Facility, School of Integrative Plant Science, Cornell University, Ithaca, New York, USA

[4]USDA-ARS, Robert W. Holley Center, Ithaca, New York, USA

Correspondence
Susan R. McCouch, Plant Breeding & Genetics Section, School of Integrative Plant Science, Cornell University, Ithaca, NY 14853, USA.
Email: srm4@cornell.edu

Present address
Matthew R. Willmann, USDA-ARS, Robert W. Holley Center, Ithaca, New York, USA.

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Abstract

Five genes of large phenotypic effect known to confer abiotic stress tolerance in rice were selected to characterize allelic variation in commercial Colombian *tropical japonica* upland rice cultivars adapted to drought-prone acid soil environments (cv. Llanura11 and Porvenir12). Allelic variants of the genes *ART1*, *DRO1*, *SUB1A*, *PSTOL1*, and *SPDT* were characterized by PCR and/or Sanger sequencing in the two upland cultivars and compared with the Nipponbare and other reference genomes. Two genes were identified as possible targets for gene editing: *SUB1A* (*Submergence 1A*), to improve tolerance to flooding, and *SPDT* (*SULTR3;4*) (*SULTR-like Phosphorus Distribution Transporter*), to improve phosphorus utilization efficiency and grain quality. Based on technical and regulatory considerations, *SPDT* was targeted for editing. The two upland cultivars were shown to carry the *SPDT* wild-type (nondesirable) allele based on sequencing, RNA expression, and phenotypic evaluations under hydroponic and greenhouse conditions. A gene deletion was designed using the CRISPR/*Cas9* system, and specialized reagents were developed for *SPDT* editing, including vectors targeting the gene and a protoplast transfection transient assay. The desired edits were confirmed in protoplasts and serve as the basis for ongoing plant transformation experiments aiming to improve the P-use efficiency of upland rice grown in acidic soils.

KEYWORDS
acid soils, CRISPR/Cas9, cultivar improvement, phosphorus use efficiency

1 | INTRODUCTION

Abiotic stresses associated with rice production in nonirrigated systems include phosphorus (P) and nitrogen (P) deficiencies, aluminum (Al) toxicity, water deficit or surplus during the growing season, and temperature extremes associated with climate change. These abiotic challenges account for much of the gap between yield potential and actual crop productivity, particularly in developing countries (Mickelbart et al., 2015; Rao et al., 2016). These stresses often occur in combination; for example, heat and drought, or Al toxicity and Pi (P in the inorganic form of orthophosphate) deficiency, or in succession, for example, flood followed by drought (Heuer et al., 2017;

Mickelbart et al., 2015). In highly acidic soils, Al toxicity is associated with numerous other mineral deficiencies and drought, representing a primary factor reducing crop yields (Kochian et al., 2015). Maintaining yield and nutritional quality in variable environments will require intensive research efforts in crop breeding and management (Chaturvedi et al., 2017; Dhankher & Foyer, 2018). Rice (*Oryza sativa* L.) is a staple food for more than 50% of the world's population (Roy et al., 2021), and integrated models of climate change and crop production predict significant yield reductions in the near future, with severe consequences for global food security (Mickelbart et al., 2015; Ray et al., 2019).

The Llanos ecoregion and the foothills of the Colombian Orinoco are part of the great savanna biome comprising half of the African continent, large parts of South America and Australia, and smaller parts of North America and Eurasia (Rincón et al., 2014). The Colombian savanna represents 17 million ha, with approximately 4.6 million ha localized in the flat Altillanura, considered one of the largest land reserves for the expansion of crop production (Amezquita et al., 2013). However, this region is characterized by fragile ecosystems with low fertility, drought-prone, and highly acidic soils, prompting the development of integrated strategies to improve soil productivity and generate adapted cultivars (Amezquita et al., 2013). Among the rice cultivars recently released for this region, two upland *tropical japonica* varieties stand out, CORPOICA Llanura11, also known as Cirad 409 (Guimarães et al., 2020) and CORPOICA Porvenir12 (originated from Line 23), derived from a recurrent selection population PTC11 (Grenier et al., 2015). Llanura11 is prominent because of its importance to farmers and its agro-industrial (Krispies, brewery) and direct consumption market, while Porvenir12 carries resistance to the main disease constraint *Pyricularia* (Colombian Agricultural Institute-ICA-resolutions No. 002581 of 2011 and No. 00024795 of 2018; Saito et al., 2018). Currently, there is great demand for new rice varieties with increased yield and efficient use of water and nutrients, particularly P. A feasible way to tackle this demand is through genetic improvement of target traits in the already adapted cultivars.

Genome editing using the CRISPR/*Cas* system opens opportunities to address the demand for new, improved climate-resilient cultivars (Chen et al., 2019). The system has been widely used to generate precise changes in the genomes of many organisms and is rapidly evolving as an accurate and predictable breeding technique (Chen et al., 2019; Graham et al., 2020). Applications in plant improvement are numerous and include, but are not limited to, enhancing pathogen resistance, drought tolerance, and food product quality (Ansari et al., 2020; Chilcoat et al., 2017; Wang et al., 2014). In rice, genome editing using various nucleases has demonstrated improved disease resistance, crop yield and quality, abiotic stress tolerance, herbicide tolerance, and male sterility (Zafar et al., 2020). Meanwhile, the legislation and regulation of gene-edited crops and agricultural products are evolving rapidly, with several countries classifying transgene-free genome-edited products under the "non-genetically modified organism" (non-GMO) status, except for the EU and New Zealand (Schmidt et al., 2020).

This facilitates the use of genome editing during cultivar development and release of transgene-free edited varieties for use in farmers' fields. In rice, detailed molecular cloning and characterization of genes conferring tolerance to numerous abiotic stresses provides the foundation for genetic improvement of modern cultivars (Mickelbart et al., 2015). Loci harboring genes of known function with large phenotypic effects represent potential targets for gene editing. Among these are genes involved in tolerance to drought, *Deeper Rooting1* (*DRO1*) (Uga et al., 2013), submergence, *Submergence 1A* (*SUB1A*) (Bailey-Serres et al., 2010; Xu et al., 2006), aluminum toxicity, *Aluminum Resistant Transcription Factor* (*ART1*) (Arbelaez et al., 2017; Famoso et al., 2011; Yamaji et al., 2009), P-deficiency, *Phosphorus Starvation Tolerance1* (*PSTOL1*) (Gamuyao et al., 2012; Pariasca-Tanaka et al., 2014), *SULTR-like Phosphorus Distribution Transporter* (*SPDT/SULTR3;4*) (Yamaji et al., 2017), and other traits (Roy et al., 2021). Indeed, rice is a model crop for deploying genes through new breeding technologies due to its amenability for transformation and its extensive genetic and genomic resources (McCouch et al., 2016; Toki et al., 2006; Wang et al., 2018). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated nucleases such as *Cas9* and *Cas12a Cpf1*, base editors, protoplast transfection methods, and transgenic and DNA-free transformation protocols are all part of the rice gene-editing toolbox (Shan et al., 2014; Wang et al., 2017; Yin et al., 2017; Zafar et al., 2020).

The present study provides the foundation for genome editing in the upland cultivars, Llanura11 and Porvenir12, as well in the Nipponbare reference genome, with the goal to enhance abiotic stress tolerance. Specifically, the study aims to (i) characterize allelic variation in the genes *DRO1*, *SUB1A*, *ART1*, *PSTOL1*, and *SPDT* via PCR and sequencing to determine whether the upland varieties carried alleles that might be appropriate targets for gene editing; (ii) evaluate gene expression and phenotypic variation associated with *SPDT* alleles; (iii) generate vectors for targeting *SPDT* using CRISPR/*Cas9* and determine whether the gene can be deleted in protoplasts. Quick and reliable methods for evaluating *SPDT*-associated phenotypes and discussion of varietal adaptation mechanisms as well as technical and regulatory implications of developing *SPDT*- edited rice varieties were further examined.

2 | MATERIALS AND METHODS

2.1 | Plant material

The plant material used in the study is described in Table S1. Seeds of the two cultivars Llanura11 and Porvenir12 were provided by the Colombian Agricultural Research Corporation (AGROSAVIA) through collaboration with the International Center for Tropical Agriculture (CIAT). The lines were developed in the CIAT- French Agricultural Research Centre for International Development (CIRAD) breeding program (Saito et al., 2018). The other materials correspond to rice reference cultivars or related species carrying desirable or undesirable alleles at each of the abiotic stress tolerance genes *ART1*, *DRO1*,

SUB1A, *PSTOL1*, and *SPDT*. Homozygous *Tos17* mutants at *SPDT* were obtained from two lines (ND0047 and NE3502) provided by the Rice Genome Resource Center of the Institute of Crop Science, NARO (RGRC-NICS) in Japan following the provider recommendations (Miyao et al., 2003; Rice Tos17 Insertion Mutant Database, 2013). The seeds were disinfected using sodium hypochlorite (2%) for 30 min and then washed with sterile water before germination. All seeds were amplified under greenhouse conditions at Cornell University (10–14 h of daylight with day/night temperatures ~28/22°C and ~65% humidity).

2.2 | PCR and sequence of gene variants

Total genomic DNA was extracted from fresh foliar tissue using the DNeasy Plant Mini Kit (QIAGEN) as the template for PCR of gene regions. For *SPDT*, cDNA obtained from the shoot basal region (SBR, 0.5 cm above the union of root and shoot) as described below (qPCR) was also used as a template. The PCR was carried out in a 50-µl reaction with 40 ng/µl of total DNA or cDNA. The Q5® High-Fidelity DNA Polymerase (NEB) was used following manufacturer instructions. PCR products were visualized on 1% agarose TAE gels. PCR products of the expected size were cleaned using the ExoSAP-IT enzyme (*Thermo Fisher Scientific*) and sequenced by Sanger. For *ART1* and *SPDT*, the complete coding sequence (CDS) was further sequenced. The *ART1* CDS was obtained using primers *ART1*-3F/4b, and the PCR product was cloned using the Zero Blunt® PCR Cloning Kit (Invitrogen). The *SPDT* CDS, as well as intronic, upstream and downstream regions, were obtained from cDNA and genomic templates using different primers (Figure S1). All primers used for PCR and sequencing are described in Table S2. Trimming of noisy sequences was followed by nucleotide or predicted amino acid sequence alignment using the Clustal W 1.83 against the GeneBank reference genes using the GeneStudio version 2.2 software.

2.3 | *SPDT* phenotyping at early vegetative development

The *SPDT* phenotype was evaluated following the method described for Nipponbare (Yamaji et al., 2017) with modifications in the hydroponic nutrient solution and developmental stages targeted for evaluation. One-hundred seeds per cultivar (Llanura11, Porvenir12, and Nipponbare) and 40–50 seeds per *Tos-17 spdt* line (Table S1) were dried at 30°C for 3 days followed by 42–45°C for 1 day. All seeds were treated with fungicide (Captan400, Trilex, and Allegiance), while the *Tos-17 spdt* seeds were further treated with gibberellic acid 10 µM for 24 h at room temperature in the dark to promote germination. Then, seeds were placed on germination paper under Milli-Q water between 30/26°C day/night for 5 days. Up to eight homogeneous seedlings per cultivar/line were transferred to Magnavaca nutrient solution (Famoso et al., 2010; Table S3) at pH 5.6 and 0-µM phosphorous (P) in 30-liter containers. Ten days after germination, the seedlings were transplanted to the same fresh solution, this time with 90-µM P in 180-liter containers, and growth in chambers at 30/26°C—day/night—12 h each, light intensity of 450-mmol photons m^{-2} s^{-1} and continuous aeration. The accuracy of the final concentration of elements in the nutrient solution was confirmed by an inductively coupled plasma emission spectrophotometer (ICP-ES, Thermo Scientific iCAP 7000 series). Seedlings were sampled at 5- and 8-leaf stages and were first washed with a 5-mM $CaCl_2$ solution and then MilliQ water. Samples were taken from roots (R), shoot basal region (SBR), the old leaf (L2), and the youngest leaf (L5 or L8) at the corresponding leaf stage (Figure S2). Follow-up of phenological stages and leaf tracking was carried out as previously reported (Counce et al., 2000; Xing & Zhang, 2010) for each cultivar (Figure S3). All samples were oven-dried at 65–70°C for a week, followed by determination of P by ICP-ES with three technical replicates.

2.4 | Real-time quantitative PCR (RT-qPCR) for *SPDT* at early vegetative development

cDNA was obtained from the SBR of Llanura11, Porvenir12, and Nipponbare seedlings grown under hydroponic conditions as described above. Briefly, 120 seeds per cultivar were treated and germinated in water for 6 days, and 40 homogenous seedlings per cultivar were transferred to Magnavaca nutrient solution, pH 5.6 and 0-µM P in 30-L containers. Twelve days after germination, half of the seedlings were transferred to a fresh solution without phosphorus (0-µM P, treatment) and the other half to a solution containing P (90-µM P, control experiment) for 1 week according to Yamaji et al. (2017). On day 19 after germination, SBR samples were collected, frozen in liquid N2, and stored at −80°C. Total RNA extraction, on-column DNA digestion, and synthesis of single-stranded cDNA were performed using the RNeasy Plant Mini Kit (QIAGEN), the RNase-Free DNase Set (QIAGEN), and the High-Capacity RNA to cDNA kit (Applied Biosystems), respectively, following the manufacturer instructions. RT-qPCR was carried out using the Power SybrGreen Mastermix (Applied Biosystems) and the primers listed in Table S2 according to the manufacturer's instructions. Relative expression levels (at the 90-µM P control) were normalized against the endogenous actin control (Zhao et al., 2016) and calculated using the ΔΔCt method with the RQ-Manager software (Life Technologies). Four independent biological replicates with three technical replicates each were analyzed. Each biological sample was represented by 20 SBRs that were pooled to obtain the amount of RNA required for cDNA synthesis.

2.5 | *SPDT* phenotyping at seed maturity

Seeds were dried (as described above) and sown in germination trays. Seedlings were transplanted to pots and randomly distributed in paddy tanks under greenhouse conditions. Plants were grown to maturity, and samples were taken from the flag leaf blade (FL) (of the main culm/stem), three green leaf blades (GL) (of culms harboring

panicles from three different positions), and from the panicles. Fifteen to twenty grams of dried seeds per sample were de-husked using the TR200 Rice Husker (Kett) and polished using the Pearlest Grain Polisher (Kett) to obtain brown seed (BS) and polished seed (PS). All samples were homogenized and subjected to ICP-ES analyses to determine P, Fe, and Zn. The PS was further analyzed for P and phytic acid concentrations using the Megazyme Phytic Acid Assay Kit (McKie & McCleary, 2016). Fe bioavailability was determined by Fe uptake in Caco-2 cells by ferritin formation in response to exposure to a digest of the PS (Glahn et al., 2002). The molar ratios of phytic acid to Fe and Zn were calculated as the micromoles of phytic acid per gram of PS divided by the micromoles of the element per gram of PS. Six biological replicates per genotype with three technical replicates each were analyzed.

2.6 | Statistical analyses

For hydroponic and greenhouse experiments, the response variables (concentration of P and other elements) were analyzed using a linear model. Residual analysis was carried out to ensure that model assumptions related to normal distribution and homogeneity of variances were met. Multiple comparison of means was performed using the Tukey method. Means were declared significantly different if p values were <0.05 or 0.01. The RStudio package Version 3.6.2 was used for the analyses.

2.7 | Selection and verification of guide RNAs (gRNAs)

The best gRNAs for targeted deletion of *SPDT* were selected using CRISPR-P v.2.0 (Bioinfo, 2016) using the following parameters: Protospacer adjacent motif (PAM): NGG *Cas9* of *Streptococcus pyogenes*, nucleotide length: 20, genome: *O. sativa* (RAP-DB) against the *SPDT* gene and 5′, 3′ regions upstream and downstream of the CDS. The selection criteria included high on-target scores (>0.5), low off-target scores (<0.5), low off-target numbers (<40), high number of Mis-Matches (MM) for off-targets (>2MM), and low number of off-targets in genes (<1). Using the primers described in Table S2, nucleotide sequences of the gRNA target regions in the cultivars Llanura11 and Porvenir12 were generated and compared with corresponding regions of the Nipponbare reference genome.

2.8 | Design and construction of CRISPR/Cas9 vectors

Base vectors from three modules A (pMOD_A1511), B (pMOD_B2112), and C (pMOD_C000) were used to target the *SPDT* gene. For B module vectors, a previous assembly of two gRNAs in pair-wise combinations driven by one PvUbi promoter was carried out. The A, B, C vectors were assembled in pTRANS_240, suitable for *Agrobacterium* plant transformation. The protocols used for assembly in module B vectors and in the plant transformation vector described previously were followed (Čermák et al., 2017). Primers used for vector assembly were described in Table S2. Constructs from positive *Escherichia coli Top10* cells (Thermo Fisher) were confirmed by Sanger sequence.

2.9 | Protoplast transfection and evaluation

Nipponbare protoplasts were obtained and transformed using polyethylene glycol (PEG) as previously described (Lowder et al., 2015) using the plant vector assembled with the best gRNA pair. Total genomic DNA was extracted by the cetyl trimethylammonium bromide (CTAB) method (Fulton et al., 1995). PCR and Sanger sequencing was performed with the primers described in Table S2 using Q5® High-Fidelity DNA Polymerase (NEB) following the manufacturer's instructions to confirm the *SPDT* deletion.

3 | RESULTS

3.1 | Allelic variation at abiotic stress tolerance genes

Five abiotic stress tolerance genes, *ART1*, *DRO1*, *SUB1*, *PSTOL1*, and *SPDT*, previously demonstrated to have large phenotypic effects in rice under field conditions, were examined for allelic variation in two *tropical japonica* upland cultivars, Llanura11 and Porvenir12. Four of the genes were associated with natural variation at Quantitative Trait Loci (QTL), whereas *SPDT* was identified using a *Tos17* mutant screen. Table 1 describes each gene and provides information about desirable and undesirable alleles harbored by reference cultivars. The PCR and sequence analysis of allelic variants in Llanura11 and Porvenir12 identified favorable alleles at *ART1*, *DRO1*, and *PSTOL1*, and null or undesirable alleles at *SUB1A* and *SPDT*.

At the *ART1* locus, PCR products of identical size (1.5 kilobases [kb]) were generated using the primers described in Table S2 in both *tropical japonica* Colombian cultivars as well as in the two *japonica* reference genomes, Nipponbare and Azucena. Sanger sequencing of the PCR products confirmed that the predicted amino acid sequences of the ART1 protein were identical in the two Colombian cultivars and 100% identical to Nipponbare, but they differed by a single nonsynonymous substitution at position 436 bp compared with Azucena (Figure 1). When ART1 sequences from these four *japonica* genomes were compared with those from the *indica* (IR64) and *aus* (Kasalath) reference genomes, they differed by four amino acid (aa) substitutions, six small InDels, and an 8-aa InDel at position 387-395 (Figure 1), consistent with a previous report (Arbelaez et al., 2017).

At the *DRO1* locus, PCR was used to amplify exons 1 and 2 (667 base pairs [bp]) and exons 3, 4, and 5 (516 bp) using primers described in Table S2. Sequencing of the PCR products showed that

TABLE 1 Description of genes conferring abiotic stress tolerance used in the study

Gene abbreviation	Gene name	Codes for	Major QTL	Phenotypic effect	Undesirable allele phenotype	Reference cultivar (subpopulation) with undesirable allele	Desirable allele phenotype	Reference cultivar (subpopulation or species) with desirable allele	Reference
ART1	*Aluminum Resistant Transcription Factor*	C2H2 zinc-finger transcription factor	*Alt12.1 (ART1)*	Aluminum (Al) tolerance	Al susceptibility	IR64 *(indica)*; Kasalath *(aus)*	Al tolerance	Azucena *(tropical japonica)*; Nipponbare *(temperate japonica)*	Arbelaez et al., 2017; Famoso et al., 2011; Yamaji et al., 2009
DRO1	*Deeper Rooting1*	N-myristoylation sites associated with a membrane protein	*DRO1*	Drought avoidance; controls root growth angle	Shallow-rooting	IR64 *(indica)*	Deep-rooting	Kinandang Patong *(tropical japonica)*; Nipponbare *(temperate japonica)*	Uga et al., 2013
SUB1A	*Submergence1*	Ethylene-response-factor-like gene	*SUB1*	Submergence tolerance	Submergence intolerant: Death after 1 week of inundation	Nipponbare *(temperate japonica*: *SUB1A* absent); IR64 *(indica*; *SUB1A-1* absent or allele *SUB1A-2)*	Submergence tolerant: Survival for more than 2 weeks under inundation	FR13A, Dhalputtia *(aus)* *SUB1A-1* present	Xu et al., 2006
PSTOL1	*Phosphorus Starvation Tolerance1*	Receptor-like cytoplasmic kinase	*PUP1 (PSTOL1)*	Phosphorus (P) acquisition efficiency (PAE); root biomass	Pi deficient - susceptible	Nipponbare *(temperate japonica)*;IR64, IR74 (*Indica*)	Pi deficiency-tolerant	Kasalath *(aus)*; CG14 (*Oryza glaberrima)*	Gamuyao et al., 2012; Pariasca-Tanaka et al., 2014
SPDT (SULTR3;4)	*SULTR-like Phosphorus Distribution Transporter*	Transporter for inorganic P (Pi)	No QTL; isolated by *Tos17* tagging	P use efficiency (PUE); grain quality	High grain-P; low straw -P	Nipponbare *(temperate japonica)*	Grain-P reduced and straw-P increased (by 20–30%)	Nipponbare *(temperate japonica: Tos17* loss-of-function allele at *SPDT)*	Yamaji et al., 2017

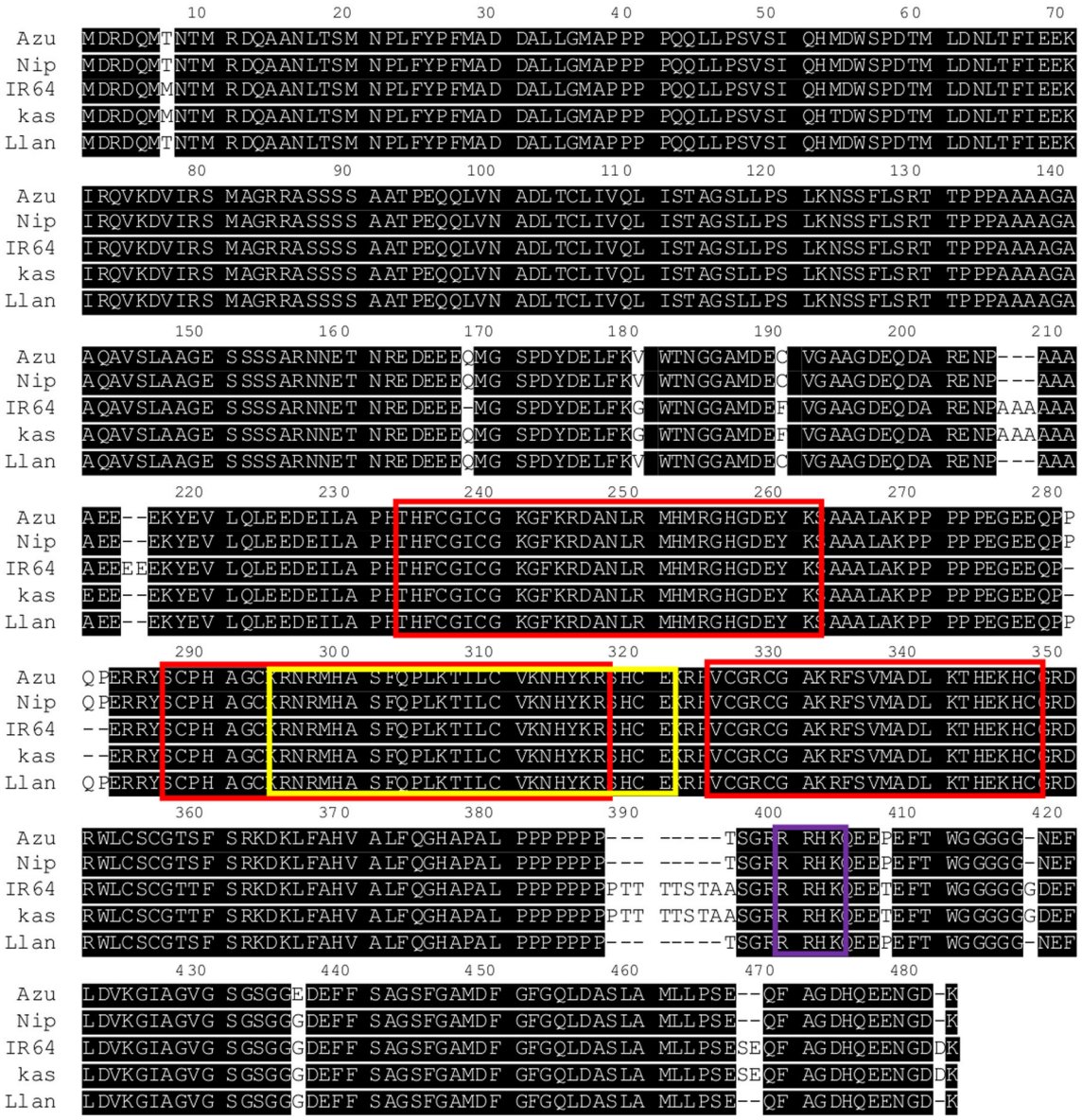

FIGURE 1 Annealing of the predicted aa sequence of the ART1 protein. In Llanura 11 (Llan) and reference *japonica* Azucena (Azu) Genebank accession No. ATU81899.1, Nipponbare (Nip) Genebank accession No. ATU81901.1, *indica* IR64 Genebank accession No. ATU81900.1, and *aus* Kasalath (kas) Genebank accession No. ATU81902.1. Llan is shown as a representative of upland varieties under investigation since Porvenir 12 was identical. Red squares represent the C2H2 zinc finger domains and yellow and purple squares the monopartite and bipartite nuclear localization domains according to Arbelaez et al. (2017). The predicted amino acid sequences of the *japonicas* Llan and Nip differed from Azu by a single nonsynonymous substitution at position 436.

the Colombian cultivars were again identical to each other and carried the desirable deep-rooting allele found in the *tropical japonica* reference variety, *Kinandang* Patong, and the *temperate japonica* reference variety, Nipponbare (Figure 2a). The shallow-rooting allele discovered in the *indica* variety IR64 differed from the deep-rooting allele by a one-bp deletion in exon 4 at position 943 bp from start of the 5′ untranslated region (UTR) in the transcribed region. This single bp change causes a premature stop that produces a truncated protein (Uga et al., 2013).

At the *PSTOL1* locus, a single PCR product of 258 bp was strongly amplified in *Oryza glaberrima* (cv CG14) and in the two Colombian cultivars using primers K46-CGsp2fw/K46-1 (Figure 2b; Table S2), suggesting that they harbor the same P-deficiency tolerant allele (Pariasca-Tanaka et al., 2014). Amplification of the 258-bp allele was weak in Nipponbare, IR64, and Kasalath. Using primers K46-K1F/K46-Ksp3rv, a 342-bp amplicon was strongly amplified in the P-deficiency tolerant *aus* reference cultivar, Kasalath, while amplification of the same amplicon was weak in Llanura11, Porvenir12 and *O. glaberrima* and was completely lacking in the P-deficiency intolerant varieties, IR64 and Nipponbare (Figure 2b) (Gamuyao et al., 2012; Pariasca-Tanaka et al., 2014). Sequencing of the 342-bp amplicon confirmed its identity in Kasalath, but amplification was too weak in

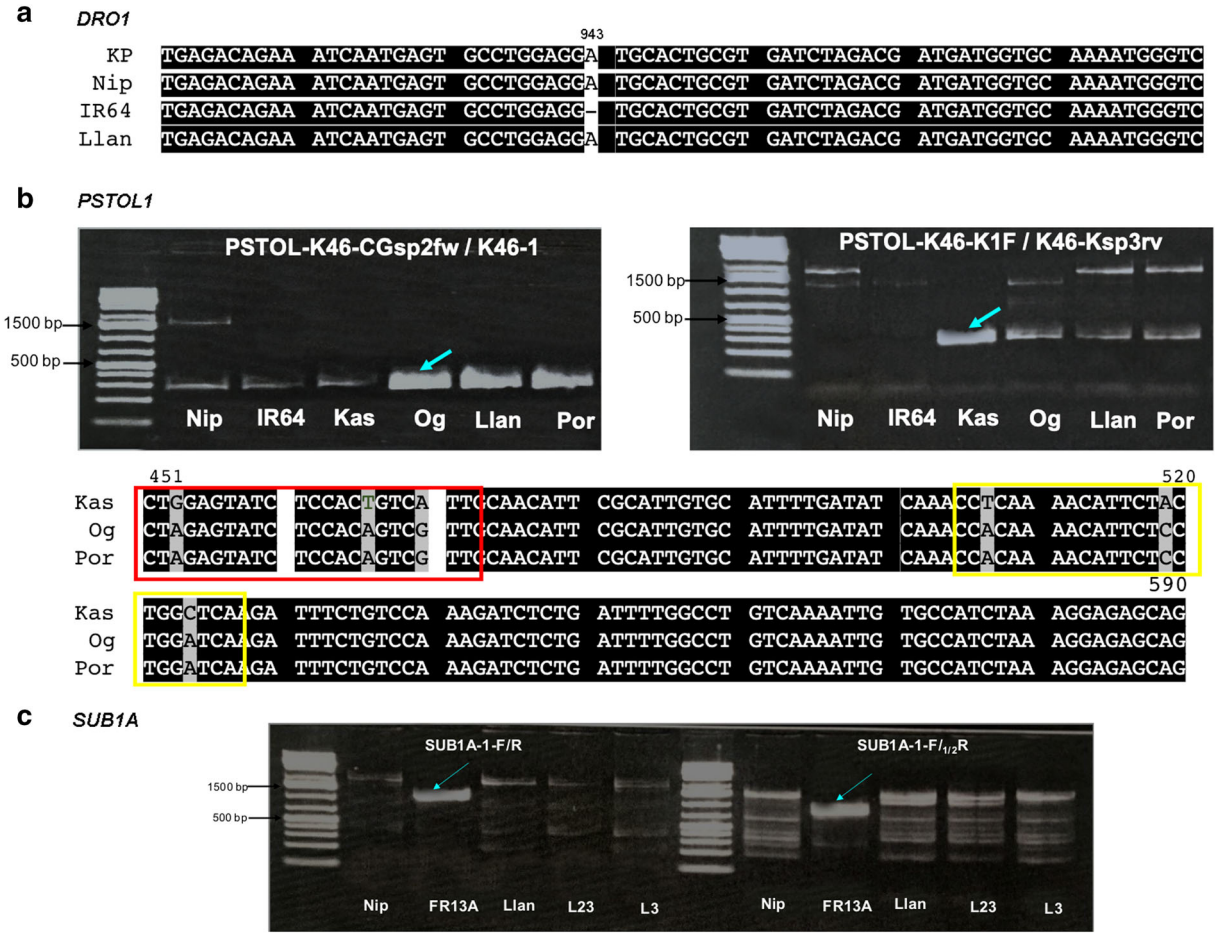

FIGURE 2 Allelic variation in PCR products and/or sequences of three genes. (a) *DRO1* nucleotide alignment at exon 4 in Llanura 11 (Llan), the reference *japonica* Kinandang Patong (KP) Genebank accession (Gb) No. AB689741.1, Nipponbare Gb No. AP005570, and the *indica* IR64 Gb No. AB68742.1, showing the one nucleotide InDel at 943 bp from start of the 5′ UTR in the transcribed region as first reported by Uga et al. (2013). (b) *PSTOL1* PCRs using primers indicated above each gel (Table S2). Blue arrows show the expected PCR product in base pairs (bp): the 258 pb indicative of the *Oryza glaberrima* (Og) CG14 allele and the 342 pb indicative of the Kasalath (Kas) allele. The nucleotide alignment shows six SNPs (gray boxes) at primers K46-CGsp2 (red square) and K46-Ksp3 (yellow square) between the reference Kas Gb No. AB458444.1 and Og according to Pariasca-Tanaka et al. (2014). (c) *SUB1A-1* PCRs using primers indicated above each gel (Table S2). Blue arrows show expected PCR products: the 1015 (left) and 825 pb product (right) as confirmed by sequence corresponded to the FR13A allele Gb No. DQ011598.1 (Xu et al., 2006). The Llan sequence is shown as representative of upland cultivars since both Llan and Porvenir 12 (Por) were identical.

Llanura11, Porvenir12, and CG14 to allow sequencing. However, sequencing of the stronger 258-pb amplicon in these three materials indicated that the overlapping region in the 258 and 342 amplicons (from positions 451 to 526) between primers K46-CGsp2 (the forward primer of the 258 pb amplicon) and K46-Ksp3 (the reverse primer of the 342 pb amplicon) harbored three SNPs at each of these primers, confirming that the Colombian cultivars were identical to the CG14 allele (Figure 2b).

At the *SUB1A* locus, a single strong PCR product was observed in the submergence-tolerant *aus* variety FR13A (Xu et al., 2006) using primers 1F/1R (1015-bp product) or 1F (GN15f)/1/2R (OFR1) (825-bp product) (Table S2), whereas no amplification was observed in the Nipponbare reference nor in either of the Colombian cultivars (Figure 2c). This indicated that the *SUB1A-1* allele conferring submergence tolerance was missing from the *japonica* varieties, consistent with expectations (Bailey-Serres et al., 2010; Singh et al., 2010; Xu et al., 2006).

At the *SPDT* (*SULTR3;4*) locus, sequence analysis confirmed that the 670 amino acid protein predicted in the two Colombian cultivars was identical to that predicted in the reference variety, Nipponbare (Figure 3a; Yamaji et al., 2017). This wild-type allele differed from the allele observed in the P-use efficient *Tos17* mutant lines (ND0047 and NE3502) (Yamaji et al., 2017; Figure 3b). Considering technical and country-level regulatory feasibility for the development of gene editing products, *SPDT* was selected for further analyses.

3.2 | *SPDT* phenotyping and RNA expression at early vegetative development

The *SPDT* phenotype was studied in the two Colombian cultivars (both carried the *SPDT* allele), Nipponbare (*SPDT*), and the two *Tos17* mutant lines (*spdt*) during the early stages of vegetative development grown in hydroponic conditions. At the 5- and 8-leaf stages, in the

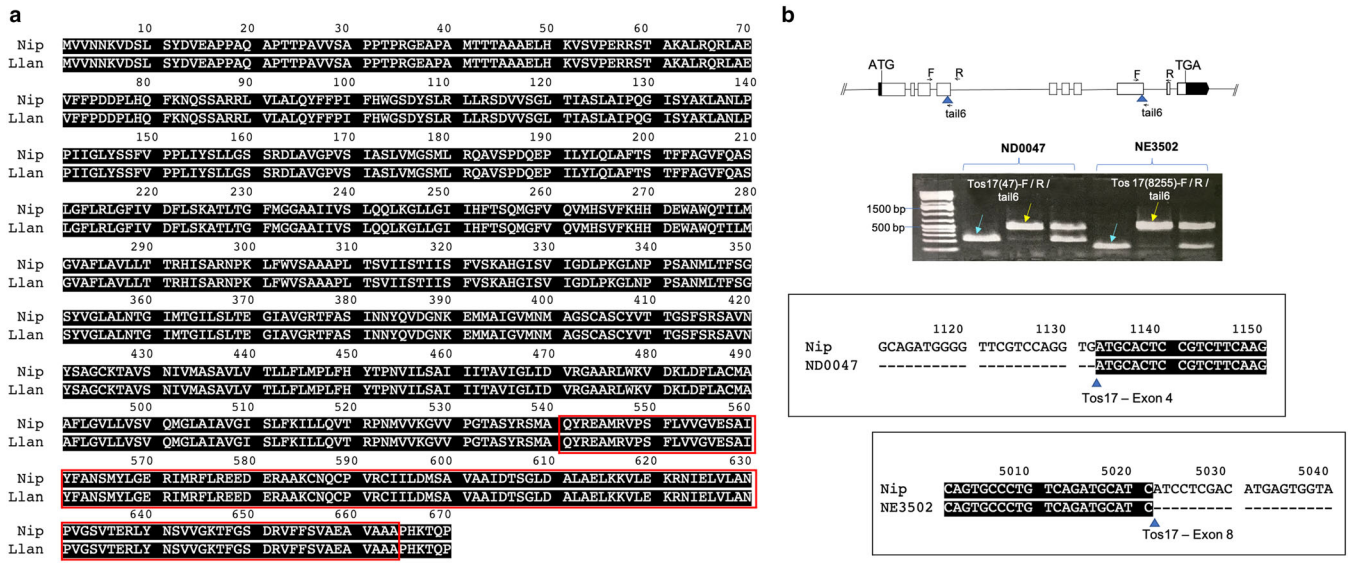

FIGURE 3 Allelic variation in sequences and PCR products of SPDT. (a) Annealing of the predicted aa sequence of SPDT in Nipponbare (Nip) (Os06g0143700) and Llanura 11 (Llan), as a representative of upland cultivars. The sulfate transporter (SULTR) anti-sigma STAS domain is shown as predicted by InterPro-EMBL-EBI (red box). STAS is conserved in the SULTR family though SPDT is the first characterized transporter for Pi in this family (Yamaji et al., 2017). (b) Structure of the *SPDT* gene showing the position of the Tos17 insertions (blue triangles) targeting exons 4 and 8 (white boxes) in lines ND0047 and NE3502, respectively, and the position of the 3-primers (F, R and tail 6) used for amplification of the wild-type and mutant alleles (Miyao et al., 2003; Yamaji et al., 2017; Table S2). The gel shows mutant homozygous amplicons (blue arrows) of ~250 bp (in ND0047) and ~150 pb (in NE3502), and wild type homozygous amplicons (yellow arrows) of 476 bp (ND0047) and 521 bp (NE3502) along with heterozygous plants. The nucleotide annealing of wild type versus mutant alleles shows the Tos17 insertions (Genebank accessions AG25438.1 and AG214407.1).

presence of sufficient Pi (90 μM), P concentrations significantly increased in older leaves (L2) and decreased in the SBR of the mutants as compared with the other three varieties (Figure 4a,b). These differences are consistent with previous observations for Nipponbare at the 8-leaf stage (Yamaji et al., 2017). No differences in P concentration were detected in the youngest leaves at the 5- and 8-leaf stages (Figure 4a,b). P concentrations were comparable in Nipponbare, Llanura11 (Figure 4; Figure S4), and Porvenir12 (Figure S4). Expression of the *SPDT* gene was induced in the SBR of both Colombian cultivars and in Nipponbare under P-deficiency conditions. In the present study, 4.4-, 5.5-, and 2.3-fold increases in *SPDT* expression were detected in Llanura11, Porvenir12, and Nipponbare, respectively, when 0-μM P treatments were compared with the P-sufficient (90-μM Pi) control condition (Figure 4c).

3.3 | *SPDT* phenotyping at seed maturity

The *SPDT* phenotype was also evaluated at seed maturity under greenhouse conditions in the Colombian cultivars using Nipponbare (*SPDT wt*) and *Tos17* mutants as references. Higher P concentrations were observed in leaf tissue, specifically in the FL and GL, while lower P concentrations were observed in the BS of the mutants compared with Nipponbare (reduced by about 15–19%) (Figure 5a), consistent with a previous study (Yamaji et al., 2017). Llanura11 and Porvenir12 showed P concentrations similar to Nipponbare for both straw and seed (Figure 5a). No significant differences were observed in P and phytic acid concentrations in the PS for any lines (Figure 5b,c).

Fe concentrations in the BS of the mutant lines were higher than in Nipponbare or the Colombian cultivars (Figure 5d), while there was no clear correspondence between Fe concentration (Figure 5d) and Fe-bioavailability in the PS (Figure 5e). The latter measured as Fe uptake based on the Caco-2 cell ferritin formation assay showed no significant differences among any of the lines (Figure 5e). As with Fe, Zn concentrations were higher in the mutants than in Nipponbare, in both the BS and the PS (Figure 5f). Fe and Zn concentration differences trend between mutants and wild type in BS is in agreement with a previous study (Yamaji et al., 2017). Zn concentrations were the highest in Llanura11 and Porvenir12 (Figure 5f). The molar ratios of phytic acid: Fe and phytic acid: Zn of PS were not significantly different among cultivars, with the phytic acid: Fe ranging between 60:1 in the mutant and 71:1 in Nipponbare and the phytic acid to Zn ranging between 5:1 in Porvenir12 and 8:1 in Nipponbare (Figure S5).

3.4 | Generation of specialized reagents for *SPDT* CRISPR/Cas9 editing

The *SPDT* genotype, phenotype, and RNA expression analyses all indicated that the Colombian cultivars carried the wild-type (less desirable) allele. Based on this conclusion, specialized reagents were designed to target the deletion of the *SULTR3;4* gene via CRISPR/

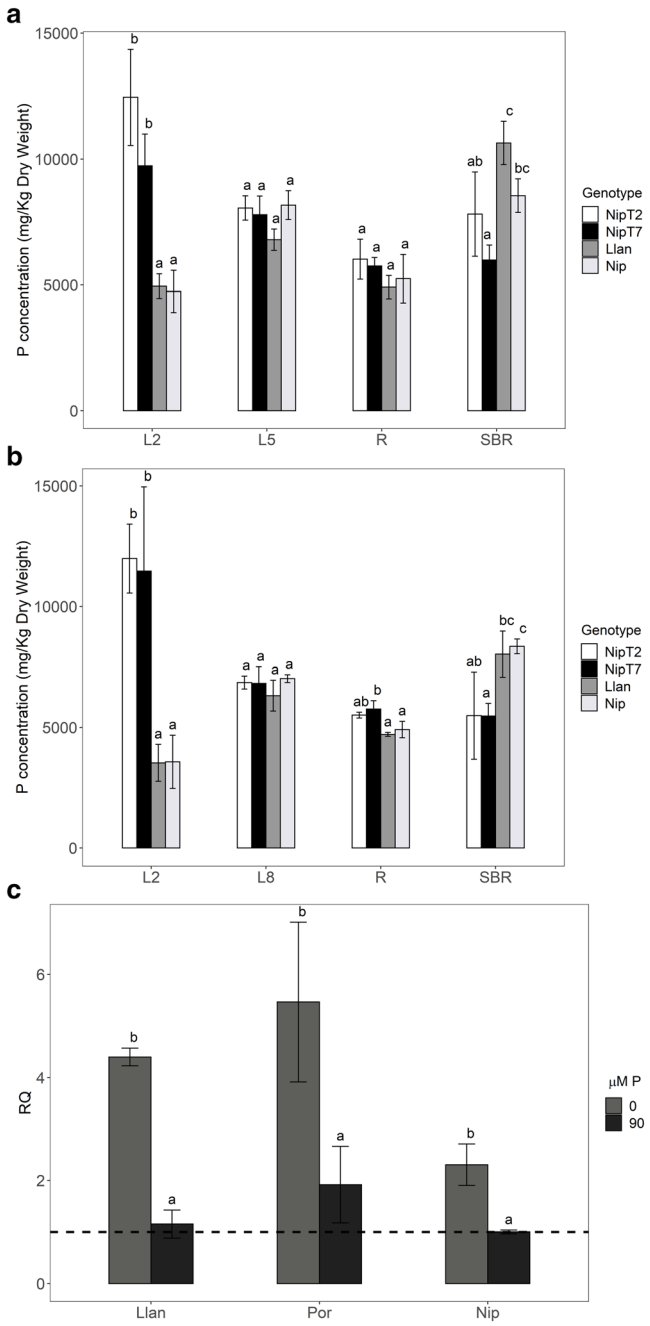

FIGURE 4 Phenotyping and *SPDT* expression at early vegetative development under hydroponic conditions. (a,b) P concentrations shown for the 5- and 8-leaf developmental stages, respectively, in leaf 2 (L2), leaf 5 (L5), leaf 8 (L8), root (R), and shoot basal region (SBR) under P-sufficiency conditions (90 µM). Six to eight biological replicates were used. (c) Relative quantification (RQ = 2-ΔΔCt) in the SBR of seedlings exposed to P-deficiency (0 µM) and P-sufficiency (90 µM) relative to the control condition (90-µM P, dashed line) using Actin as internal standard. Four biological replicates were used. Significant differences are shown by different letters at $p < 0.01$ and standard deviations are shown after Tukey test. NipT2 = *spdt* mutant NE3502; NipT7 = *spdt* mutant ND0047; Llan = Llanura 11; Nip = *spdt* wild-type Nipponbare.

Cas9. This included construction of editing vectors and confirmation of vector gRNA editing by protoplast transfection in Nipponbare (as the reference genome).

Using the genome sequence information from the Nipponbare as the reference genome, five gRNAs targeting the *SPDT* (*SULTR3;4*) gene were designed (1U, 1D, 3, 4, and 1F) (Table 2, Figure 6a). The gRNAs met the following criteria: High on-target scores (0.59 to 0.90), relatively low number of off-targets (5 to 19, except gRNA 3 with 40) preferentially located in nongenic regions, and if located in genes, low off-target scores (0.22 to 0.44) with a high number of mismatches (3–4). These selection criteria were selected to give a low probability of off-target editing by CRISPR/*Cas9* in rice (Tang et al., 2018). Any of the four possible pair-wise combinations of selected gRNAs (1U or 3 at the 5′ end with 4 or 1D at the 3′ end) were expected to produce a deletion of the gene. The 1F gRNA, designed to produce a frame-shift mutation resulting in an early stop codon, was to be used only as a back-up editing strategy in case any of the four possible pair-wise combinations (described above) failed to work.

The 1U-1D gRNA pair was selected for further work based on the low number of predicted off-target sites in the Nipponbare genome (12 and 5, respectively Table 2). Moreover, this pair is expected to delete the entire gene using a template-free approach with the 1U-gRNA targeting a sequence upstream of the transcriptional start site and the 1D-gRNA targeting a sequence downstream of the stop codon (Figure 6a). Most importantly, the gRNA regions in the target *SPDT* gene showed conserved nucleotide identity among the Colombian cultivars and the reference Nipponbare (Figure 6b). Deletion of the gene is advantageous because it minimizes country-level regulatory concerns (Chilcoat et al., 2017; Schmidt et al., 2020). Therefore, protoplasts were transfected with a vector containing the 1U-1D gRNA pair. The PCR using protoplast genomic DNA produced a band of the expected size (0.37 kb), indicating that the desired *SPDT* deletion (about 7.3 kb) had occurred. This was confirmed by Sanger sequencing (Figure 6c).

4 | DISCUSSION

4.1 | Abiotic stress alleles harbored by Colombian cultivars shed light on adaptation mechanisms and needs for further genetic improvement

The rice cultivars Llanura11 and Porvenir12 are cultivated as *tropical japonica* upland varieties in the savannas of Colombia, which form part of the great Savanna biome (Amezquita et al., 2013; Rincón et al., 2014; Saito et al., 2018). These ecosystems are characterized by highly acidic, low fertility soils associated with Al toxicity, P deficiency, and drought alternating with high precipitation. To investigate the potential for targeted genetic improvement, alleles found in these varieties at five major-effect genes known to confer Al toxicity tolerance (*ART1*), drought avoidance (*DRO1*), submergence tolerance

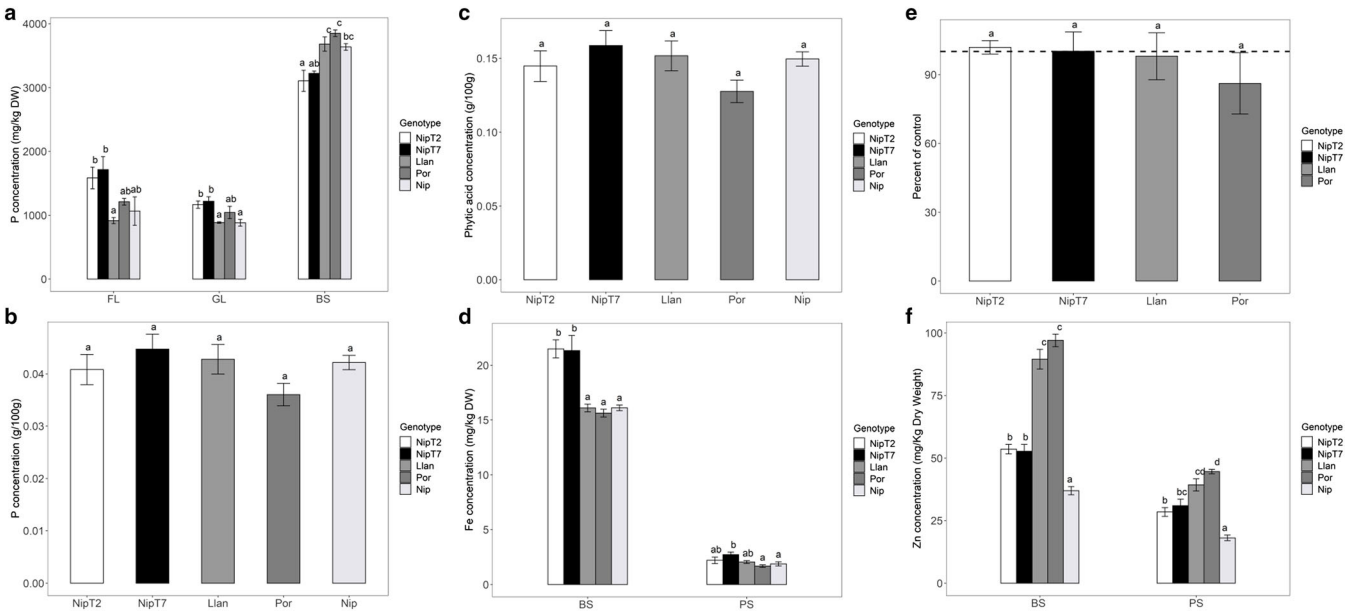

FIGURE 5 Phenotyping at seed maturity stage under greenhouse conditions. (a) P concentration in flag leaf (FL), green leaves (GL), and brown seed (BS) determined by ICP. (b,c) P and phytic acid concentrations in polished seed (PS) determined by Megazyme. (d) Fe and (f) Zn concentrations in BS and PS determined by ICP. (e) Fe-bioavailability in PS relative to the control Nipponbare (4335-ng ferritin/mg protein, 100% represented by the dashed line) was measured by Ferritin formation in Caco-2 cells in vitro assay. Six biological replicates were used. Significant differences are shown by different letters at $p < 0.05$ and standard errors are shown after Tukey test. NipT2 = *spdt* mutant NE3502; NipT7 = *spdt* mutant ND0047; Llan = Llanura 11; Por = Porvenir 12; Nip = *spdt* wild-type Nipponbare.

TABLE 2 Description of gRNAs selected for targeting the *SPDT* gene based on CRISPR-P v.2.0

gRNA	gRNA sequence (PAM site underlined)	Position in *SPDT* gene	*On-target* Score	Number of *off-targets*	Number of *off-targets* in genes	Localization in off-target gene	*Off-target* gen score	Number of mistmatches in *off-target* gene
1U	TTAAATCGACCTTTTCCTGG**TGG**	906 nt upstream of start codon	0.77	12	0	n.a.	n.a.	n.a.
1D	TGTTTCTCACGAGTCGCACA**GGG**	749 nt downstream of stop codon	0.61	5	1	UTR	0.44	4
3	GGATTAAGAGGCGAGTTGGG**GGG**	190 nt upstream of start codon	0.65	40	0	n.a.	n.a.	n.a.
4	GCACATCTCTGCTCTTGTCA**GGG**	At stop codon	0.59	19	1	Intron	0.22	4
1F	TCAAGAACCAGTCGTCCGCG**CGG**	In exon 1	0.90	9	1	CDS	0.26	3

Note: n.a. = nonapplicable.

(*SUB1A*), P-acquisition (*PSTOL1*), and P-use efficiency (*SPDT*) were characterized via PCR and sequencing. Desirable alleles were found at *ART1*, *DRO1*, and *PSTOL1*, suggesting that the Colombian cultivars contained adaptative mechanisms associated with physiological response to soil pH and ionic composition. The mechanisms conferred by these loci altered root growth angle and overall root system architecture in response to drought-prone acidic soil environments. In fact, Llanura11, also known as Cirad 409 (Grenier C., personal communication), is characterized by a strong root system under irrigated conditions and an increased total root volume under drought conditions (Guimarães et al., 2020).

The fact that the Colombian cultivars carry Al tolerant alleles at *ART1*, a deep rooting allele at *DRO1*, and a likely P-deficiency tolerant allele at *PSTOL1* is consistent with their adaptation to acid soils. In rice varieties that are susceptible to Al toxicity (a ubiquitous feature of low pH soils), root growth is severely limited (Famoso et al., 2011; Kochian et al., 2015), making it difficult for roots to explore the soil and reach deeper horizons to find water (Uga et al., 2013) and/or to find an immobile nutrient such as Pi (Heuer et al., 2017). Thus, favorable alleles at these three loci would be mutually complementary in promoting root growth in acid soils.

The present study suggests that the desirable *PSTOL1* allele found in the Colombian cultivars shares ancestry with the *PSTOL1* allele found in CG14, a cultivar of African rice, *O. glaberrima*. This allele is different from the allele originally cloned from the *aus* variety Kalalath (Gamuyao et al., 2012; Pariasca-Tanaka et al., 2014). It showed a 35 base-pair substitution when aligned to the Kasalath allele, which facilitated the development of the Kasalath (342-bp amplicon) and CG14 (258-bp amplicon) allele-specific markers (Pariasca-Tanaka et al., 2014). The 258-bp amplicon in the two Colombian cultivars and

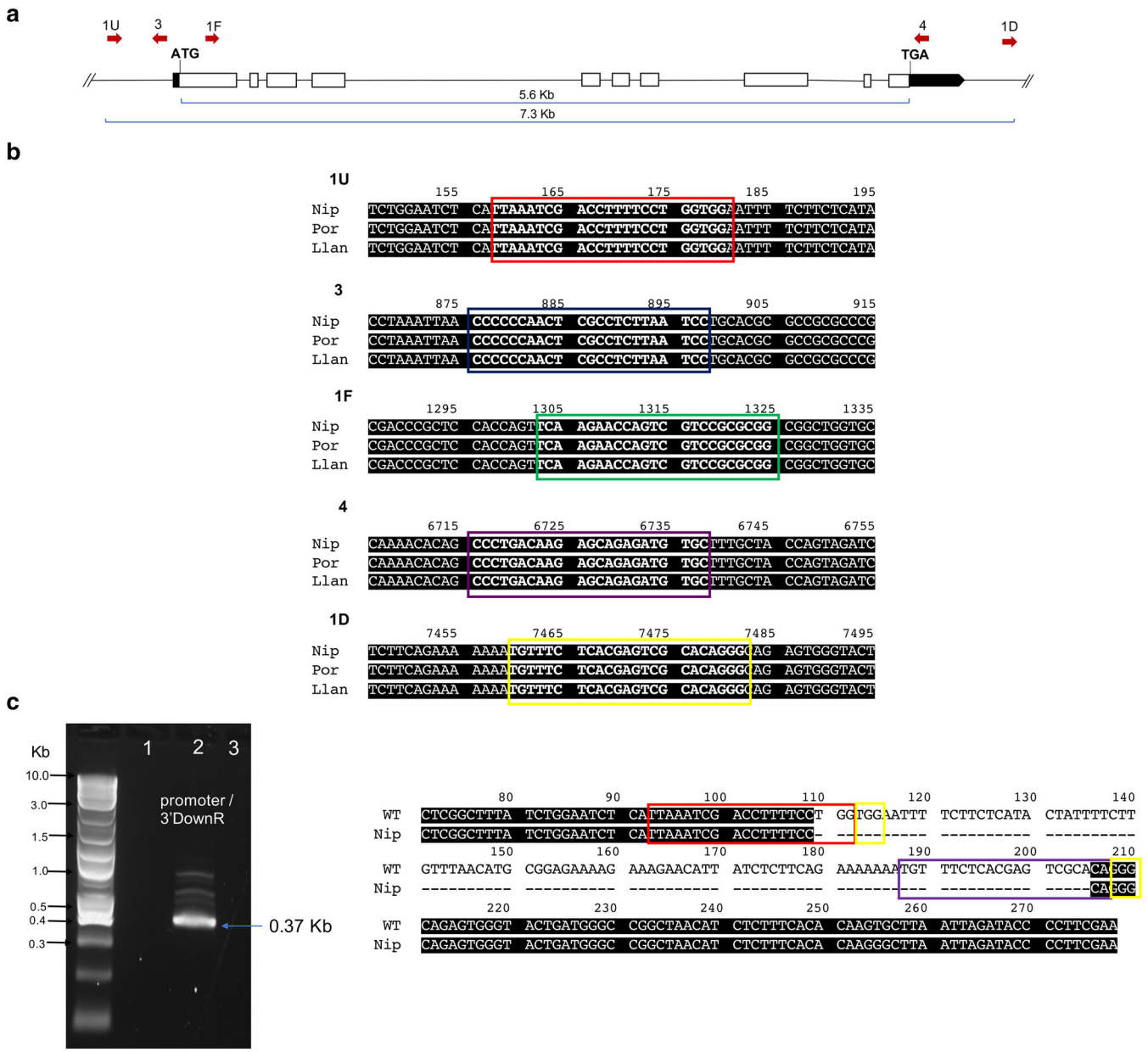

FIGURE 6 Effectiveness of the *SDPT* gRNAs in cv. Nipponbare protoplasts. (a) Position of the selected gRNAs for deletion of the *SPDT* gene (red arrows). The exons (white boxes), UTR region (black boxes), and the start and termination codons (adapted from Yamaji et al., 2017) are indicated. The size of the gene from start to stop codon (5.6 kb) and the size of the expected deletion using 1U and 1D gRNAs (7.3 kb) are underlined. (b) Nucleotide sequences corresponding to the regions of gRNA annealing in the two Colombian cultivars Por = Porvenir12, Llan = Llanura11 and the reference Nip = Nipponbare used to target the *SPDT* gene. gRNAs are indicated in colored boxes. (c) PCR from DNA protoplasts transformed with vector containing the 1U-1D gRNA pair using the primers indicated above the gel (Table S2). The sequence of the expected 370-bp band indicative of the *SPDT* deletion (line 2) was aligned with the wild-type (WT) gene. The 1U (red box), 1D (Purple box) gRNAs, and the PAM sites (Yellow boxes) are indicated. To facilitate visualization, most of the WT 7.3-kb deleted region is omitted and only the 5′ and 3′ ends are shown.

in *O. glaberrima* carried six nucleotide substitutions as previously reported (Figure 2, Pariasca-Tanaka et al., 2014). The fact that a faint 342-bp band in these three cultivars and a faint 258-bp band in Kasalath were also observed may be explained by weak primer annealing due to nucleotide substitutions between the Kasalath and CG14 alleles. In fact, the K46-K1F/ K46-Ksp3rv primer pair specific for the 342-bp Kasalath allele has two polymorphisms in the forward primer and three in the reverse primer, while the K46-CGsp2fw/K46-1 specific for the 258-bp *O. glaberrima* CG14 allele has three polymorphisms in the forward primer (Pariasca-Tanaka et al., 2014).

The Colombian cultivar Llanura 11 was developed by conventional cross breeding with various progenitors (IRAT 146/Oryzica Sabana10//CT10035-43-4-M-3) (ICA resolution No. 002581 of 2011). This line was developed as part of a recurrent selection

population where identification of promising lines adapted to the Colombian savannah eventually gave rise to Porvenir12 (PCT-11\0\0\2,Bo\2\1>133-M-5-1-4-3-M) (Grenier et al., 2015; ICA resolution No. 00024795 of 2018). Tracing the CG14 allele back through the ancestral lines may clarify its origin in the Colombian cultivars. On the other hand, while it appears to be favorable, it remains to be determined whether the CG14 allele confers a similar P-uptake advantage as the Kasalath allele in the cultivars under investigation.

In contrast to *ART1*, *DRO1*, and *PSTOL1*, adaptive alleles at *SUB1A* and *SPDT* were lacking, suggesting that targeted introgression and/or gene editing of favorable alleles at these loci could enhance the performance of Llanura11 and Porvenir12. The *SUB1* locus is known to harbor a gene family consisting of two to three tandemly arrayed members on chromosome 9 (Fukao et al., 2006), with the *SUB1A* family member conferring tolerance to complete submergence in the *aus* variety FR13A for up to 14 days (Singh et al., 2020; Xu et al., 2006). It is also associated with enhanced tolerance to other abiotic stresses, including drought (Bin Rahman & Zhang, 2016; Fukao et al., 2011). The *SUB1A* gene is absent in the Nipponbare reference genome and other *japonica* varieties due to an inversion and deletion (Singh et al., 2010). Consistent with this, the gene was absent from the *tropical japonica* Llanura11 and Porvenir12 cultivars (Figure 2). Although the Colombian savannas are characterized by a strong dry season, there is also an important water surplus due to precipitation and even floods (Rincón et al., 2014), suggesting that marker-assisted introgression of *SUB1A* may be the most efficient way to generate climate-resilient varieties for these environments.

SPDT, a gene involved in internal P use efficiency (PUE), was cloned in cv. Nipponbare by transposon *Tos17* tagging (Yamaji et al., 2017). The loss-of-function desirable allele causes less P to be allocated to the grain (by about 20%) and consequently a potential increase of bioavailability of essential nutrients (Fe, Zn) without affecting seed germination and grain yield (Yamaji et al., 2017). At the same time, more P is assigned to the straw, which remains in the field after harvest and could be used as a fertilizer (Rose et al., 2013; Yamaji et al., 2017). The sequence of this gene locus in the upland cultivars under investigation showed that the wild-type allele was present. Thus, further cultivar improvement by knocking out *SPDT* offers an interesting possibility to generate high PUE and grain quality cultivars.

Given that directed DNA insertion is both technically challenging and presents regulatory hurdles (because it would be subjected to the restrictions imposed on genetically modified organisms [GMOs]) (Georges & Ray, 2017; Wang et al., 2017), it would be advisable to introgress the *SUB1A* gene into the two Colombian varieties using marker-assisted selection (MAS). This approach has successfully introduced submergence tolerance into varieties grown on thousands of hectares in flood-prone regions of Asia and Africa (Mickelbart et al., 2015). On the other hand, editing the *SPDT* gene through a targeted gene deletion offers a practical solution, given that a DNA-free editing strategy or an *Agrobacterium* mediated transformation editing strategy producing transgene-free products could be used, posing fewer technical and regulatory constraints (Chilcoat et al., 2017; Georges & Ray, 2017).

The SPDT phenotype was then examined in the Colombian cultivars and compared with Nipponbare (SPDT *wt*) and the two *Tos17* mutant lines (*spdt*). This was done by evaluating gene expression (using qPCR) and organ P concentration at the early vegetative and seed maturity stages. For qPCR, the SBR was targeted for analysis based on a report showing that this tissue produced the most significant *SPDT* differential expression using 0 and 90-μM P treatments under hydroponic conditions (Yamaji et al., 2017). The induction of *SPDT* expression under 0-μM P in the reference Nipponbare was 2.3-fold higher compared with the control condition of 90-μM P (Figure 4c). This represents a smaller induction of gene expression as compared with that observed in the previous study for Nipponbare (6.5-fold increase) (Yamaji et al., 2017). There was also no difference in P concentration in the youngest leaf 8 at the 8-leaf stage (Figure 4b) as opposed to the study by Yamaji et al. (2017), where lower P concentrations were detected in the *sdpt* mutants as compared with wild type Nipponbare. The differences in gene expression and P concentrations in the eighth leaf in the two studies are likely due to differences in the nutrient solutions used (Table S3; Magnavaca in the present study vs. Kimura in Yamaji et al., 2017) and/or small differences in experimental conditions.

Llanura11 and Porvenir12 were most similar to Nipponbare in terms of induced RNA expression under P-deficiency conditions, increased P concentration in older leaves during early vegetative development (5- and 8-leaf stages) and in flag and green leaves at seed maturity, and reduced P concentrations in BS (Figures 4 and 5). These results strongly suggested that the two Colombian cultivars carry the wild-type *SPDT* allele and are therefore likely to benefit from deletion of the *SULTR3;4* gene, mimicking the results observed in the ND0047 and NE3502 mutant lines.

4.2 | Early phenotyping for fast screening of *spdt* genotypes

The *SPDT* phenotype was previously evaluated at the 8-leaf stage using Kimura B solution at half-strength (Yamaji et al., 2017). One objective of this study was to determine whether the *SPDT* wild-type from the *spdt* knock-out lines at earlier stages of development could be phenotypically differentiated, that is, at the 5-leaf stage in plants growing in Magnavaca solution (Famoso et al., 2011), which has 7.5 times greater ionic strength than the Kimura B½ solution (Table S3). Plants were also grown in a growth chamber rather than in a greenhouse, which helped to minimize environmental variation, providing greater consistency of temperature and luminosity, regardless of the time of year.

The current results indicated that the earlier 5-leaf stage of development and higher ionic strength solution under growth chamber conditions can be used to reliably evaluate the phenotype under hydroponic conditions, saving time and resources (Figure 4). The original method required 32–42 days to evaluate the Nipponbare phenotype at the 8- or 9-leaf stage (Yamaji et al., 2017), whereas the modified method required 22 days for Nipponbare, and 25 days for

the Colombian varieties to reach the 5-leaf stage. Nipponbare took 33 days and the Colombian varieties 42 days to develop to the 8-leaf stage in the Magnavaca solution under growth chamber conditions (Figure S3). The higher ionic strength of the growth solution, a high volume ratio (about 10 L of solution/plant), and shorter growth periods resulted in minimal nutrient depletion from the solutions, as indicated by routinary ICP analysis of the Magnavaca solution throughout the experiment. These modifications reduced water usage, minimal replacement of microelements, and overall less labor. Therefore, this modified protocol represents an improved technique to quickly screen for desirable *spdt* mutants at the seedling stage.

4.3 | Low P brown seed as a potential source of bioavailable nutrients

Varieties that transfer less P to developing grains offer environmental and nutritional benefits (Rose et al., 2013). Phytic acid salt (phytate) is the main form of P in cereal grains where it acts as an antinutrient, decreasing the bioavailability of other essential nutrients, such as Fe and Zn (Perera et al., 2018). Phytate accumulates in the bran or aleurone layer of brown rice seed where it can chelate Fe, K, and Ca, whereas Zn is found broadly distributed from the aleurone layer to the inner endosperm, often bound to phytic acid but also found in another storage form (Iwai et al., 2012). In this study, the hypothesis that reducing this chelant might increase nutrient bioavailability and nutritional benefits was investigated by comparing P, Fe, and Zn concentrations of BS and PS in *spdt* mutants and wild-type rice.

Higher P concentrations were detected in BS in *SPDT* wild-type genotypes (Nipponbare and Colombian cultivars) compared with *spdt* mutants, while no differences were found in PS for either P or phytate concentrations, nor for Fe bioavailability (Figure 5). This lack of correspondence between Fe concentration and Fe uptake determined by the caco-2 cell bioavailability assay has been previously reported for brown rice (Glahn et al., 2002).

The molar ratios of phytic acid:Fe were very high (Table S5), indicating that not much Fe in the PS is contributing from a nutritional perspective. This is in agreement with the fact that only 4.335 ng of ferritin/mg of protein was formed in the control Nipponbare with no significant differences among genotypes (Figure 5e); therefore, not much Fe is likely to be delivered from the PS. On the other hand, the molar ratios of phytate:Zn were much lower (Figure S5). Typical Zn levels in polished rice are low (8–12 ppm), but there is a wide genetic variability in brown (7.3 to 52.7 ppm) and polished (8 to 38 ppm) rice (Babu et al., 2020). The primary inhibitor of Zn is phytate (Lönnerdal, 2000) and more Zn, as observed in the mutants and Colombian genotypes of this study (Figure 5f), usually means more absorbed Zn. However, to our knowledge, there is not a good method for assessing Zn bioavailability.

Brown or unmilled rice is known to have higher vitamin and mineral content compared with milled rice (Muthayya et al., 2014). The present study confirmed higher Fe and Zn concentrations in BS compared with PS. However, many of the nutritional benefits of brown rice cannot be not realized if nutrient bioavailability is compromised by high levels of phytate in BS. Thus, low P *spdt* mutants that accumulate less phytate in the bran offer a possible solution for breeders interested in developing cereal varieties with high concentrations of bioavailable Fe and Zn as part of the human diet and also as a component of animal feed. Low phytate grain would be particularly useful in nonruminant livestock feeds, including poultry, swine, and fish feeds, where P and inositol in phytic acid are generally not bioavailable, and P-deficiency is a problem, while at the same time it may help reduce P excretion that contributes to environmental problems, such as eutrophication of waterways (Perera et al., 2018).

4.4 | Technical, regulatory, and societal implications of editing the *SPDT* gene

P is an essential micronutrient, and P-deficiency is a major constraint for crop yield; thus, to obtain high yields, regular applications of P-fertilizer are needed, but the supply of phosphate rock is limited (Rose et al., 2013). P efficiency (PE), the capacity of plants to tolerate stress caused by P-deficiency, can be achieved by increasing P acquisition efficiency (PAE) or PUE traits (Heuer et al., 2017; Rose et al., 2013). The latter has prompted more recent attention and has been envisaged that breeding crops by lowering grain P concentration is one approach to increase PUE in cereal systems since less P removed from the field could lower fertilizer requirements, saving production costs to farmers (Rose et al., 2013). For example, the low phytic acid (*lpa*) recessive mutation *lpa1-1* in barley, harbored by the US commercial cultivar "Herald," reduces total grain P by 10–20% and lowers phytate levels without a penalty on subsequent crop yields (Bregitzer et al., 2007; Ye et al., 2011).

In rice, the genetic architecture of PUE was investigated using GWAS with a rice diversity panel grown in a hydroponic system to ensure uniform access to P (Wissuwa et al., 2015). The study identified loci associated with PUE on four chromosomes, with the chromosome 1 haplotype showing high priority based on association with candidate genes of potential utility in plant breeding. Alternatively, novel variation can be generated by mutation breeding (Heuer et al., 2017; Rose et al., 2013). In fact, transporters involved in delivering phosphate to developing seeds and synthesis of phytic acid have been discovered through mutation screens, identifying key genes belonging to the Sulfate Transporter (SULTR) family. One of them, *OsSULTR3;3*, was discovered in two *lpa* mutants developed to improve the nutritional value of rice grains. Disruption of *OsSULTR3;3* in these mutants leads to reduced concentrations of total grain P (19–28%) and grain phytate (35–45%) (Zhao et al., 2016). Moreover, the rice *SULTR3;3* gene is closely related to the barley sulfate transporter *LPA1* (Ye et al., 2011; Zhao et al., 2016). More recently, *OsSULTR3;4* (referred to as *SPDT* in this study), the first characterized transporter for inorganic P in this family, was shown to be involved in P allocation to rice grain, with the desired mutation reducing P (by 20%) as well as reducing phytate concentration. These discoveries point to a potential role in improving PUE in rice cropping systems such that disruption of

the Pi transporter leads to retention of P in the straw. Use of the straw as a form of mulch or green manure would enable growers to easily return P to the field after harvest to help fertilize the next season's crop (Yamaji et al., 2017).

The current study aims to leverage knowledge about *SPDT* to enhance PUE in two upland Colombian cultivars using CRISPR-associated endonuclease as a targeted form of mutagenesis. This system has great precision and minimal risk of introducing *off-target* variation in the genome compared with historical mutagenesis techniques (Graham et al., 2020). Whether this will be the case for the Colombian cultivars awaits confirmation based on whole genome sequencing of edited versus wild type genotypes.

Specialized reagents were generated to produce an *SPDT* deletion using CRISPR/Cas9 and the approach was tested in the *temperate japonica* variety, Nipponbare before applying it to the *tropical japonica* upland cultivars, Llanura11 and Porvenir12. As a first step, experiments were performed to confirm that editing occurred as expected in Nipponbare protoplasts. This is important because Nipponbare is the reference genome from which the gRNAs were designed (as described in the methods) and in which gene function was originally studied (Yamaji et al., 2017). Further sequencing of the target *SPDT* region in Llanura11 and Porvenir12 confirmed that it matched the Nipponbare reference (Figures 3a and 6b). Therefore, it was reasonable to expect that the desired edit in the *SPDT* gene may also occur in the Colombian varieties using the selected gRNAs. Plant transformation in both the Colombian and the Nipponbare varieties is currently underway using both transgenic (via *Agrobacterium*) and nontransgenic (via Ribonucleoprotein complexes) methods.

The reagents included plant transformation vectors containing the *Cas9* and gRNAs to target identical *SPDT* regions in the three cultivars. The intended deletion of 7.3 kb at the *SPDT* locus was confirmed by transfecting Nipponbare protoplasts with a vector targeting the 5′ and 3′ upstream and downstream gene ends (Figure 6). These are essential tools for proof-of-concept to determine whether *SPDT* can be subjected to a targeted deletion in cultivars of interest; whether desired edits, if obtained in plants, produce the improved phenotype to enhance PUE and grain quality under acid soils; and whether individual effects of P reduction in the grain, based on deletions of *SULTR3;3* and *SULTR3;4*, can be leveraged by pyramiding mutations in both genes in a single cultivar.

The current work establishes the basis for targeted deletion of *SPDT* in upland rice cultivars of interest to breeders. Gene deletions are preferred over gene insertions because they tend to present fewer societal and regulatory concerns. Currently, genome edits involving deletions are usually classified into the Site Directed Nuclease-1 (SDN-1) category, as long as there is no addition of foreign DNA (Schmidt et al., 2020). SDN-1 edits follow the standards of conventional mutagenesis with categorization based on the product and are considered nonregulated as GMOs in most countries except the European Union and New Zealand (Schmidt et al., 2020). The first *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) genome-edited resistant rice, where promoter elements of the sucrose transporter genes *SWEET* were targeted through CRISPR/Cas9 (Oliva et al., 2019) was declared transgene-free, nonregulated, and equivalent to what could be accomplished with conventional breeding in Colombia and the USA (Agdaily, 2020). This opens the path for approval of other DNA-free editing products such as those proposed in this study for the Colombian savannas.

5 | CONCLUSIONS

Allelic variation at five major-effect abiotic stress tolerance genes (*ART1*, *DRO1*, *PSTOL1*, *SUB1A*, *SPDT*) was studied in two upland Colombian rice cultivars (Llanura11 and Porvenir12) and compared with a variety of reference genomes using PCR and sequence analysis. The Colombian cultivars carried desirable alleles at three of the loci and nondesirable alleles at *SUB1A* and *SPDT*, providing targets for further genetic improvement. Based on technical and regulatory criteria, the *SPDT* gene, involved in P use efficiency (PUE) and nutritional quality of the grain, was targeted for deletion via a gene knock-out strategy using CRISPR/Cas9. An improved phenotyping pipeline was established to evaluate P concentrations at the early vegetative stage and used in combination with evaluation of P concentrations in the grain at seed maturity to establish the foundation for *SPDT* editing in the Colombian cultivars. An editing vector containing a pair-wise combination of the most suitable gRNAs was developed for targeted *SPDT* deletion and successfully used in experiments involving protoplasts to obtain the desired deletion. This study provides an essential foundation for applying CRISPR/*Cas9* editing to improve PUE and grain nutritional quality in upland *tropical japonica* cultivars.

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AUTHOR CONTRIBUTIONS

LSB conceived, designed, and performed the experiments, analyzed the data, wrote the paper. MRW contributed to conception and design of gene editing. EC contributed to the phenotyping experiments under hydroponic conditions. KMA contributed to genotyping, DNA extractions, PCRs, sequencing, and preparation of seed samples for phenotyping. SH maintained, amplified, and tracked plants and seed stocks. GGM analyzed the data, prepared figures. RG conceived and advised on nutrient evaluation of seed. MAP conceived and advised on early development phenotyping. SRM conceived and supervised the overall research at Cornell University and jointly wrote the paper with LSB. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Luz S. Barrero https://orcid.org/0000-0003-2138-8705
Gina A. Garzon-Martinez https://orcid.org/0000-0001-5620-9055
Miguel A. Piñeros https://orcid.org/0000-0002-7166-1848
Susan R. McCouch https://orcid.org/0000-0001-9246-3106

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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