Identifying Genes Associated with Abiotic Stress Tolerance Suitable for CRISPR/Cas9 Editing in Upland Rice Cultivars Adapted to Acid Soils

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Original article

Keywords: acid soils, Phosphorus use efficiency, CRISPR/Cas9, cultivar improvement

Posted Date: September 14th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-864595/v1

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 genome 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 (non-desirable) 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 and protocols were developed for *SPDT* editing, including vectors targeting the gene, protoplast transfection transient assays, and plant regeneration and transformation protocols by *Agrobacterium* and ribonucleoprotein complexes. 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.

Background

Abiotic stresses associated with rice production in non-irrigated systems include Phosphorus (P) and Nitrogen (P) deficiency, 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 (Mickelbart et al. 2015; Heuer et al. 2017). 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 and 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 (Wang et al. 2014; Chilcoat et al. 2017; Ansari et al. 2020). 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*) (Xu et al. 2006; Bailey-Serres et al. 2010), aluminum toxicity, *Aluminum Resistant Transcription Factor*

(*ART1*) (Yamaji et al. 2009; Famoso et al. 2011; Arbelaez et al. 2017), 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, as well as 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).

In the present study, we provide 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 genome 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; iv) generate transformation and regeneration protocols based on *Agrobacterium* and DNA-free methods (Ribonucleoprotein, RNP) for editing of the *tropical japonica* cultivars under investigation. We further examine quick and reliable methods for evaluating *SPDT*-associated phenotypes and discuss varietal adaptation mechanisms as well as technical and regulatory implications of developing *SPDT*- edited rice varieties.

Materials And Methods

Plant material

The plant material used in the study is described in Table S1. Seed of the two cultivars Llanura11 and Porvenir12 were provided as advanced lines by the International Center for Tropical Agriculture (CIAT) to the Colombian Agricultural Research Corporation (AGROSAVIA), the national institution that evaluated and registered the two cultivars for the acid soil savannas of Colombia. 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 minutes and then washed with sterile water before germination. All seeds were amplified under greenhouse conditions at Cornell University following the procedures manual (Harrington 2016).

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 50µl 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.

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 one day. All seed was treated with fungicide (Captan400, Trilex, and Allegiance), while the *Tos-17 spdt* seed was further treated with gibberellic acid 10 µM for 24 hours at room temperature in the dark to promote germination. Next, 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 90µM P in 180-liter containers, and growth in chambers at 30/26°C - day/night – 12 hours 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 ICP-ES. Seedlings were sampled at 5and 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 and 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 Inductively Coupled Plasma Emission Spectroscopy (ICP-ES) with 3 technical replicates.

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-liter 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 one 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 90µM P control) were normalized against the endogenous actin control 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.

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 (Harrington 2016). 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 20 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 and McCleary 2016). Iron (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.

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.

Selection and verification of guide RNAs (gRNAs)

The best gRNAs flanking *SPDT* for targeted deletion were selected using CRISPR-P v.2.0 (Bioinfo 2016) under the following parameters: Protospacer Adjacent Motif (PAM): NGG *Cas9* of *Streptococcus pyogenes*, nucleotide length: 20, genome: *Oryza sativa* (RAP-DB) against the *SPDT* gene and 5', 3' regions upstream and downstream of the CDS. The selection criteria for the best gRNAs were high on-target scores (> 0.5), low off-target scores (< 0.5), low off-target numbers (< 40), high number of MisMatches (MM) for off-targets (> 2MM), low number of off-targets in genes (< 1). The nucleotide identity of the selected gRNAs was verified using the rice reference genome Nipponbare against the Sanger sequence of cultivars Llanura11 and Porvenir12 at the target region with the primers described in Table S2.

Design and construction of CRISPR/Cas9 vectors

Base vectors from three modules A, B, and C described in Table S4 were used to target the *SPDT* gene. For B module vectors, a previous assembly of the gRNAs alone (case 1) or in pair combinations (case 2) was carried out. The A, B, C vectors were assembled in pTRANS_240, suitable for *Agrobacterium* plant transformation. We followed the protocols used for assembly in module B vectors and in the plant transformation vector described previously (Čermák et al. 2017). Primers used for vector assembly are described in Table S2. Constructs from positive *E. coli Top10* cells (Thermofisher) were confirmed by Sanger sequence.

Protoplast transfection and evaluation

Nipponbare protoplasts were obtained and transformed using polyethylene glycol (PEG) as previously described (Lowder et al. 2015) using the plant vectors assembled with one gRNA and water as control. Total genomic DNA was extracted by the Cetyl Trimethylammonium Bromide (CTAB) method (Fulton et al. 1995), and the presence of cuts indicative of editing events guided by a single gRNA in genomic DNA was detected by the T7 endonuclease assay with the Engen Mutation Detection Kit (NEB) using the primers described in Table S2. Subsequently, the best gRNA pair was selected for further protoplast transfection with the appropriate vector. Genomic DNA isolation, PCR and Sanger sequencing with primers described in Table S2 were performed using the Q5® High-Fidelity DNA Polymerase (NEB) as described above to confirm the *SPDT* deletion.

Agrobacterium tumefaciens transformation protocols

The Plant Transformation Facility at Cornell University perfected the following method for Agrobacterium transformation of Nipponbare, Llanura11, and Porvenir12. The basic steps are the same unless otherwise noted. The recipes for the media are listed in Table S5.

Immature seeds were husked and then sterilized as follows. The husked seeds were placed in a 50-ml conical tube and shaken in 70% ethanol for one minute. The ethanol was then replaced with 1–2% sodium hypochlorite, and the tube was placed on a shaker for 30 minutes. In a laminar flow hood, the seeds were then washed with sterile water five times or until the scent of bleach was gone. The seeds were air-dried on sterile filter paper in the laminar flow hood for about 20 minutes. The air-dried seeds were plated on callus induction medium with 25 seeds per standard 100 mm x 15 mm petri plate. The plates were wrapped with PVC tape and incubated in the light (about 40 $\mu E\ m^{-2}\ s^{-1}$) at 28°C. After two weeks, callus was separated from the seed and shoots and put on new callus induction medium, with any callus pieces larger than a pea broken up to increase surface area. After two additional weeks and four days prior to transformation, the callus was chopped with a scalpel to increase surface area and then put on a new callus induction medium.

Agrobacterium tumefaciens strains EHA101 and EHA105 were used for rice transformation. Three days prior to transformation, a single colony of Agrobacterium transfected with the plasmid of interest was streaked on YEP solid medium with the appropriate antibiotics and grown at 28°C for two days. The night before the transformation, this culture was used to start a 5-ml liquid culture of Yeast Extract Peptone (YEP) with the appropriate antibiotics and grown overnight at 28°C in a shaker. Once the culture reached an Optical Density $(OD)_{600}$ of 0.4 or greater, the culture was spun down for 15–20 minutes at 4000 rpm. The medium was poured off while taking care not to lose the pellet. The pellet was resuspended in the infection medium at an OD_{600} of 0.06–0.08.

Four days prior to the transformation, the callus was chopped and put on a new callus-infection medium. On the day of the transformation, the callus was transferred from plates to 50-ml conical tubes such that about 15–20 ml of unpacked callus (based on the markings on the tube) was put in one tube for one transformation to yield transgenic plantlets from 10 events. The callus was pre-washed with 25 ml of infection medium and shaken for one minute. The infection medium was removed using a sterile pipet and replaced with the Agrobacterium solution. The tubes were shaken horizontally at room temperature for 20 minutes. The Agrobacterium solution was removed using a sterile pipet, and the callus was transferred to the top of a stack of sterile filter paper in a glass petri dish and left in the laminar flow hood until dry (about 60 minutes). The callus was then split between two plates of co-culture medium. The plates were wrapped with vent tape and incubated at 25^{o}C in the dark for three days.

After co-culture, the callus was returned to a 50-ml conical tube and washed at least five times (until the final wash is clear) with 25 ml of 200 mg/l timentin. The callus was transferred to the top of a stack of sterile filter paper in a sterile glass petri dish and left in the laminar flow hood until dry and crumbly (30–60 minutes). The callus was then split between two plates of selection I medium wrapped with Polyvinyl chloride (PVC) wrap and incubated at 28^{o}C in the light for 6 days. The callus was then transferred to the selection II medium. The plates were wrapped with PVC wrap and incubated in the light at 28^{o}C for 14 days. At the end of this time, any visible resistant, actively growing callus events were picked and separately placed on selection II medium with 3–7 events placed per plate. The remaining callus was transferred to a new selection II medium, from where additional events were picked after 14 days. Events were grown on selection II medium for 2-week intervals until the amount of resistant callus per event was about 1.5 cm in diameter. At that point, regeneration was initiated.

Regeneration procedures were different for Nipponbare versus the Colombian lines. For all three genotypes, the process began by desiccating the resistant callus. To reduce waste, X-divided petri plates (such as # 25384-316 from VWR) were used for desiccation such that callus from four events could be desiccated separately in one plate. Callus from a single event was placed on a quarter-of-a-circle of sterile filter paper within a well of the petri plate, and the plate was kept wrapped with parafilm for 2–3 days at 28^{o}C in the dark. The length of time for desiccation depended on the amount of callus, with larger amounts of callus requiring more time. After desiccation, the Nipponbare callus was moved to pre-regeneration medium for 6 days before being moved to regeneration medium for another two weeks. Llanura11 and Porvenir12, by contrast, were moved directly from desiccation to regeneration medium for two weeks. Once on regeneration medium, callus of all genotypes was moved to new medium every two weeks until shoots with leaves of at least 1-1.5 cm are visible. Such shoots were then moved to root regeneration medium in Magenta™ boxes.

Plants were ready to move to soil when individual shoots were 8 cm high with extensive roots. To acclimate the plants, the Magenta™ boxes were opened in a growth chamber and 3 cm of sterile water was poured on top of the medium. The boxes were kept open for 5–6 days, adding additional water as necessary to keep the level constant. Prior to moving the plants to soil, the root system was rinsed with water to wash away residual medium.

CRISPR/Cas9 ribonucleoprotein (RNP) gene-editing protocol

For CRISPR/Cas9 gene-editing of rice without creating a transgenic intermediate, the PDS-1000/He™ gene gun system (Bio-Rad) was used to bombard immature embryos with gold microparticles coated with Cas9 in protein form complexed with *in vitro*-transcribed or synthesized gRNAs. Here, 5’ and 3’ gRNAs were individually transcribed *in vitro* using the EnGen® sgRNA Synthesis Kit, *S. pyogenes*, from New England BioLabs. The final synthesis reaction was purified using the RNA Clean and Concentrator with Zymo-Spin IIC Columns from Zymo Research. The RNAs were eluted and quantified using a Nanodrop.

On the day of the bombardment, immature, green seeds were husked and sterilized, as described above. The embryo was carefully released from each seed using sterile forceps and/or scalpels and transferred to the center of a plate of callus induction medium that includes 200 mg/L of timentin. Timentin was used to reduce the risk of contamination in the process of bombardment. Fifty or more embryos were placed side-by-side, scutellum-side up, in the center of the plate such that the middle 1.5 cm circle is filled with embryos.

To create the RNP complex, 1X New England Biolabs Buffer 3, 1 µl of an RNase inhibitor, 0.62 µl of EnGen® Spy Cas9 NLS (2 µg of protein; New England Biolabs), and 1 µg of each of the gRNAs were combined in a 10 µl reaction. This protocol was developed by combining pieces from two different published protocols (Svitashev et al. 2016; Liang et al. 2017). The amounts of Cas9 and gRNAs were those used by Liang et al. (2017) and result in an approximate 1:4.89 molar ratio of Cas9:gRNAs. The reaction was incubated at 25^{o}C for 10 minutes. Then, 5 µl of 1.0 µm gold microparticles (100 µg/µl in RNase-free H20) were added and mixed by pipetting. The entire volume was then transferred to the center of a pre-sterilized macrocarrier in a holder. Gold microcarriers, macrocarriers, microcarrier holders, and all other supplies for use with the PDS-1000/He™ gene gun system are available from Bio-Rad. After allowing to air dry in the laminar flow food for two hours, the bombardments were done by manufacturer’s conditions with the following specifications. The petri plate with the rice embryos was placed on a target shelf positioned 6 cm from the disk retaining cap, 1350 psi rupture disks were used, and each sample was bombarded 2–3 times. These conditions were optimized by bombarding rice embryos with a *35S::GUS* plasmid and then staining the embryos after 24–48 hours for transient GUS expression.

After bombardment, the embryos were spread across the plate with the scutellum-side up. Plates were wrapped with PVC tape and placed in the light at 28^{o}C for 2 weeks. Every 2 weeks for 6–8 weeks total, the callus was allowed to multiply before initiating regeneration, as above. All regeneration media and procedures were the same as described above except that no selection agents, timentin, or cefotaxime were included. In the initial RNP experiments with Nipponbare and Llanura11, the callus from all of the bombarded embryos was pooled and 82–166 plantlets were regenerated. In the future, callus will be kept separate from each embryo to help provide a better understanding of the efficiency.

Results

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 are associated with Quantitative trait loci (QTL) variation, while *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*.

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	R
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)*	Y a F et 2 A e 2
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*)	U 2
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;* S*UB1A-1* absent or allele *SUB1A-2*)	Submergence tolerant: Survival for more than 2 weeks under inundation	FR13A, Dhalputtia *(aus)* allele *SUB1A-1* present	X 2
PSTOL1	*Phosphorus Starvation Tolerance1*	Receptor-like cytoplasmic kinase	*PUP1 (PSTOL1)*	Phosphorus (P) acquisition efficiency (PAE); root biomass	Pi deficiency-susceptible	Nipponbare *(temperate japonica*); IR64, IR74 (*Indica*)	Pi deficiency-tolerant	Kasalath *(aus*); CG14 (*Oryza glaberrima)*	G e 2 P e 2
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*)	Y a

At the *ART1* locus, PCR products of identical size (1.5 Kilobases (Kb) were generated using the primers described in Supplementary 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 non-synonymous substitution at position 436 compared to Azucena (Fig. 1). When ART1 sequences from these four *japonica* genomes were compared to those from the *indica* (IR64) and *aus* (Kasalath) reference genomes, they differed by 4 amino acid (aa) substitutions, six small InDels, and an 8-aa InDel at position 387–395 (Fig. 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 Supplementary Table S2. Sequencing of the PCR products showed that 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 (Fig. 2a). The shallow-rooting allele discovered in the *indica* variety IR64 differs from the deep-rooting allele by a one bp deletion in exon 4 at nucleotide position 943 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 the Colombian cultivars using primers K46-CGsp2fw/K46-1 (Suppl Table S2), indicating that they harbor the same desirable P-deficiency tolerant allele found in CG14, an *O. glaberrima* cultivar (Pariasca-Tanaka et al. 2014). Amplification of this allele was weak in Nipponbare, IR64, and Kasalath. Using primers K46-K1F/K46-Ksp3rv, a weak 342 bp amplicon (as well as a ~ 2000 bp amplicon) were observed in Llanura11, and Porvenir12. The 342 bp amplicon was identical in size to that found in the P- deficiency tolerant *aus* reference cultivar, Kasalath (Pariasca-Tanaka et al., 2014; Fig. 2b), while amplification was completely lacking in the P-deficiency intolerant varieties, IR64 and Nipponbare (Gamuyao et al. 2012). Further sequencing of the 258 bp amplicons confirmed that the Colombian cultivars carry the same tolerant allele as CG14, indicating that they share identical polymorphisms as those previously reported between CG14 and Kasalath (Pariasca-Tanaka et al. 2014). Sequencing of the 342 bp

amplicon was not performed, but the overlapping region (from positions 451 to 526) 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) shows three SNPs at each of these primers confirming that the Colombian cultivars are identical to the CG14 allele (Fig. 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) (Suppl. Table S2) while no amplification was observed in the Nipponbare reference nor in either of the Colombian cultivars (Fig. 2c). This indicated that the *SUB1A-1* allele conferring submergence tolerance was missing from the *japonica* varieties, consistent with expectations (Xu et al. 2006; Singh et al. 2010; Bailey-Serres et al. 2010).

At the *SPDT (SULTR3;4)* locus, sequence analysis confirmed that the 670 amino acid protein observed in the two Colombian cultivars was identical to that observed in the reference variety, Nipponbare (Fig. 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; Fig. 3b). Considering technical and country-level regulatory feasibility for the development of gene editing products, we selected *SPDT* for further analyses.

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 presence of sufficient Pi (90 µM), P concentrations significantly increased in older leaves (L2) and decreased in the SBR of the mutants as compared to the other three varieties (Fig. 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 (Fig. 4a-b). P concentrations were similar in Nipponbare, Llanura11 (Fig. 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, a 4.4, 5.5, and 2.3-fold increase in *SPDT* expression was detected in Llanura11, Porvenir12, and Nipponbare, respectively, when 0 µM P treatments were compared to the P-sufficient (90 µM Pi) control condition (Fig. 4c).

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 to Nipponbare (reduced by about 15–19%) (Fig. 5a), consistent with the study (Yamaji et al. 2017). Llanura11 and Porvenir12 showed P concentrations similar to Nipponbare for both straw and seed (Fig. 5a). No significant differences were observed in P and phytic acid concentrations in the PS for any lines (Fig. 5b-c).

Fe concentrations in the BS of the mutant lines were higher than in Nipponbare or the Colombian cultivars (Fig. 5d), while there was no clear correspondence between Fe concentration (Fig. 5d) and Fe-bioavailability in the PS (Fig. 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 (Fig. 5e). As with Fe, Zn concentrations were higher in the mutants than in Nipponbare, in both the BS and the PS (Fig. 5f). Fe and Zn concentration differences trend between mutants and wild type in BS is in agreement with the previous study (Yamaji et al. 2017). Zn concentrations were the highest in Llanura11 and Porvenir12 (Fig. 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 from 60:1 in the mutant to 71:1 in Nipponbare and the phytic acid to Zn ranging from 5:1 in Porvenir12 to 8:1 in Nipponbare (Figure S5).

Generation of specialized reagents and protocols for SPDT CRISPR/Cas9 editing

The *SPDT* genotype, phenotype, and RNA expression analyses all indicate that the Colombian cultivars carry the wild-type (less desireable) allele. Based on this conclusion, specialized reagents and protocols were designed to target the deletion of the *SULTR3;4* gene via CRISPR/*Cas9*. This included construction of editing vectors, confirmation of vector gRNA editing by protoplast transfection in Nipponbare (as the reference genome), and development of regeneration and transformation protocols for the Colombian *tropical japonica* cultivars under investigation.

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, Fig. 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 non-genic 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). When combined in pairs, the gRNAs (1U or 3 at the 5'end with 4 or 1D at the 3'-end) are expected to produce a deletion of the gene, and the 1F gRNA is designed to produce a frameshift mutation for use as a back-up editing strategy.

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	TTAAATCGACCTTTTCCTGGTGG	906 nt upstream of start codon	0.77	12	0	n.a.	n.a.	n.a.
1D	TGTTTCTCACGAGTCGCACAGGG	749 nt downstream of stop codon	0.61	5	1	UTR	0.44	4
3	GGATTAAGAGGCGAGTTGGGGGG	190 nt upstream of start codon	0.65	40	0	n.a.	n.a.	n.a.
4	GCACATCTCTGCTCTTGTCAGGG	At stop codon	0.59	19	1	Intron	0.22	4
1F	TCAAGAACCAGTCGTCCGCGCGG	In exon 1	0.90	9	1	CDS	0.26	3

n.a. = non-applicable

Five vectors, each containing a single gRNA were designed and constructed to establish a transient expression assay in Nipponbare protoplasts. The T7 endonuclease that recognizes and cuts mismatches larger than one bp is useful for detecting gRNA targeting and was used with genomic DNA from transfected and control protoplasts. Despite the presence of ghost bands observed in the T7 controls that may reflect non-specific amplicons (gRNAs 1U, 1D, 3, 4), the T7 produced PCR products of the expected size for each gRNA. This indicated that each gRNA resulted in the desired cuts (Fig. 6b). 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 (Fig. 6a). Deletion of the gene is advantageous because it also minimizes country-level regulatory concerns (Chilcoat et al. 2017; Schmidt et al. 2020). Therefore, we next transfected protoplasts 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 (Fig. 6c).

Discussion

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, we characterized alleles found in these varieties at five major-effect genes known to confer Al toxicity tolerance (*ART1*), drought avoidance (*DRO1*), submergence tolerance (*SUB1A*), P-acquisition (*PSTOL1*), and P-use efficiency (*SPDT*) via PCR and sequencing. Desirable alleles were found at *ART1*, *DRO1*, and *PSTOL1*, suggesting that adaptative mechanisms associated with physiological response to soil pH and ionic composition, including root growth angle and overall root system architecture are conferred by these loci 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), and 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 2–3 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 (Xu et al. 2006; Singh et al. 2020). It is also associated with enhanced tolerance to other abiotic stresses, including drought (Fukao et al. 2011; Bin Rahman and Zhang 2016). 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, we found that the gene was absent from the *tropical japonica* Llanura11 and Porvenir12 cultivars (Fig. 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 and 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 could be used, posing fewer technical and regulatory constraints (Chilcoat et al. 2017; Georges and Ray 2017).

The SPDT phenotype was next 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 to the control condition of 90 μM P (Fig. 4c). This represents a smaller induction of gene expression as compared to 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 (Fig. 4b) as opposed to the reported in theprevious study (Yamaji et al. 2017), where lower P concentrations were detected in the *sdpt* mutants as compared to wild type Nipponbare. The differences in gene expression and P concentrations in the 8th leaf in the two studies are likely due to differences in the nutrient solutions used (Magnavaca in the present study vs. Kimura in Yamaji et al. (2017) (Table S3)) 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 (Figs. 4–5). Based on these results, we concluded 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.

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 we could phenotypically differentiate the *SPDT* wild-type from the *spdt* knock-out lines at earlier stages of development, i.e., at the 5-leaf stage in plants growin in Magnavaca solution (Famoso et al. 2011), which has 7.5 times greater ionic strength than the Kimura B½ solution (Table S3). We also grew plants 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.

Our results indicate 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 (Fig. 4). The original method required 32–42 days to evaluate the Nipponbare phenotype at the 8- or 9-leaf stage (Yamaji et al. 2017), while the modified method required 22 days for Nipponbare, and 25 days for the Colombian varieties to reach the 5-leaf stage. Nipponbare takes 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 liters 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 reduce 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.

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, while Zn is found broadly distributed from the aleurone layer to the inner endosperm, often bound loosely to phytic acid but also found in another storage form (Iwai et al. 2012). In this study, we investigated the hypothesis that reducing this chelant might increase nutrient bioavailability and nutritional benefits by comparing P, Fe, and Zn concentrations of BS and PS in *spdt* mutants and wild-type rice.

We confirmed that 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 (Fig. 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 (Fig. 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 mutans and Colombian genotypes of this study (Fig. 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 to milled rice (Muthayya et al. 2014). The present study confirms higher Fe and Zn concentrations in BS compared to 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 non-ruminant 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).

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 (Rose et al. 2013; Heuer et al. 2017). 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 (Rose et al. 2013; Heuer et al. 2017). 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), with 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, that it can be easily returned to the field after harvest (as a form of mulch) to help fertilize the next season’s crop (Yamaji et al. 2017).

Here we aim 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 to historical mutagenesis techniques (Graham et al. 2020). We generated specialized reagents and protocols to generate an *SPDT* deletion using CRISPR/Cas9 in the *temperate japonica* variety, Nipponbare, and *tropical japonica* upland cultivars, Llanura11 and Porvenir12, as a foundation for the strategy. The reagents included plant transformation vectors containing the *Cas9* and gRNAs to target identical *SPDT* regions in the three cultivars. We confirmed the intended deletion of 7.3 Kb at the *SPDT* locus by transfecting Nipponbare protoplasts with a vector targeting the 5’ and 3’ up- and downstream gene ends (Fig. 6). The protocols included regeneration and transformation methods mediated by Ribonucleoprotein complexes (RNP) and *Agrobacterium tumefaciens* in the three cultivars. Reagents and protocols 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.

Our work establishes the basis for targeted deletion of *SPDT* in upland rice cultivars of interest. 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 non-regulated 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, non-regulated, 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.

In addition, the fact that we developed protocols for RNP mediated transformation as a backup to *Agrobacterium* transformation aims to further ease regulatory and societal concerns. RNP consists of *in vitro* transcribed gRNAs in complex with the *Cas9* protein and is a DNA-free editing method where no foreign DNA is used, and therefore the edits obtained are completely transgene-free (Zhang et al. 2016; Liang et al. 2017). Moreover, the CRISPR/*Cas9* RNP complex can be quickly degraded *in vivo*. Thus, the genome is less prone to *off-target* mutations as compared to editing through *Agrobacterium* transformation where the CRISPR/*Cas9* DNA construct is incorporated in the genome (Liang et al. 2017). This work lays the foundation for generating a DNA-free edited cultivar, either by using the RNP approach directly or by eliminating the transgene via segregation in edited plants transformed using *Agrobacterium*. Further investigation is needed to determine which approach will be the most appropriate in terms of technical feasibility, efficiency, and

regulatory and societal concerns as we continue our efforts to generate *SPDT*-edited lines in different genetic backgrounds to improve P-acquisition and P-use efficiency in the upland rice ecosystem in the Colombian savannas.

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 to a variety of reference genomes using PCR and sequence analysis. The Colombian cultivars carried desirable alleles at three of the loci and non-desirable 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. Editing vectors containing single gRNAs (5 vectors) and pair-wise combinations of the most suitable gRNAs (1 vector) were developed for targeted *SPDT* deletion and successfully used in experiments involving protoplasts to obtain the desired deletion and evaluate gRNA targeting efficacy. Likewise, protocols were developed for plant regeneration and transformation by *Agrobacterium* and RNP as a strategy for DNA free editing in *tropical japonica* germplasm. The study provides an essential foundation for applying CRISPR/*Cas9* editing to improve PUE and grain nutritional quality in upland cultivars.

Abbreviations

AGROSAVIA: Colombian Agricultural Research Corporation

ART1: Aluminum Resistant Transcription Factor

BS: Brown Seed

CIAT: International Center for Tropical Agriculture

DRO1: Deeper Rooting1

CIRAD: French Agricultural Research Centre for International Development

FL: Flag Leaf Blade

GL: Green Leaf Blade

GMO: Genetically Modified Organism

gRNAs: guide RNAs

ICA: Colombian Agriculural Institute

ICP-ES: Inductively Coupled Plasma Emission Spectroscopy

L: Leaf

MM: MisMatches

PAE: P Acquisition Efficiency

PAM: Protospacer Adjacent Motif

PE: P Efficiency

PS: Polished Seed

PSTOL1: Phosphorus Starvation Tolerance1

PUE: P Use Efficiency

R: Root

RGRC-NICS: Rice Genome Resource Center of the Institute of Crop Science, NARO

RNP: Ribonucleoprotein

SBR: Shoot Basal Region

SPDT (SULTR3;4): SULTR-like Phosphorus Distribution Transporter

SUB1A: Submergence 1A

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests

Funding

This work was performed as part of an agreement between AGROSAVIA and Cornell University and was funded by the U.S. National Science Foundation Plant Genome Research Program (**Award #1444511 Quantitative Trait Locus Editing for Crop Improvement through S. McCouch), early vegetative and seed phenotyping was supported by USDA-ARS, and salary support for** LSB was provided by AGROSAVIA through the Colombian Ministry of Agriculture, Colombia.

Authors' contributions

LSB conceived, designed, and performed the experiments, analyzed the data, wrote the paper. MRW developed the the CRISPR Cas9 RNP bombardment, transformation and regeneration protocols. EC contributed to the phenotyping experiments under hydroponic conditions. KMA contributed to DNA extractions, PCRs, sequencing, and preparation of seed samples for phenotyping. SH maintained, amplified, and tracked edited and nonedited 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. SM conceived and supervised the overall research at Cornell University and jointly wrote the paper with LSB. All authors read and approved the final manuscript.

Acknowledgments

We gratefully acknowledge Jose Euripides Baquero, Jaime Humberto Bernal and Samuel Caicedo (r.i.p) (AGROSAVIA) for advice on key traits to further improve rice cultivars of importance to the acid soil Colombian savannas, Lyza Maron for advice on potential genes for characterization, Nathaniel Graham and Daniel Voytas (University of Minnesota) for training LSB in final vector assemblies and protoplast transfection, Jon E. *Shaff* for performing strength comparisons of Magnavaca vs. Kimura solutions, Mary Bodis for technical assistance on P and phytate (Megazyme) measurements and bioavailability assays, and Li Wang (Adam Bogdanove Lab, Cornell University) and Xiuling Cai (Hangzhou University) for providing an unpublished Nipponbare transformation protocol that laid the foundation for the method we publish here.

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Figures

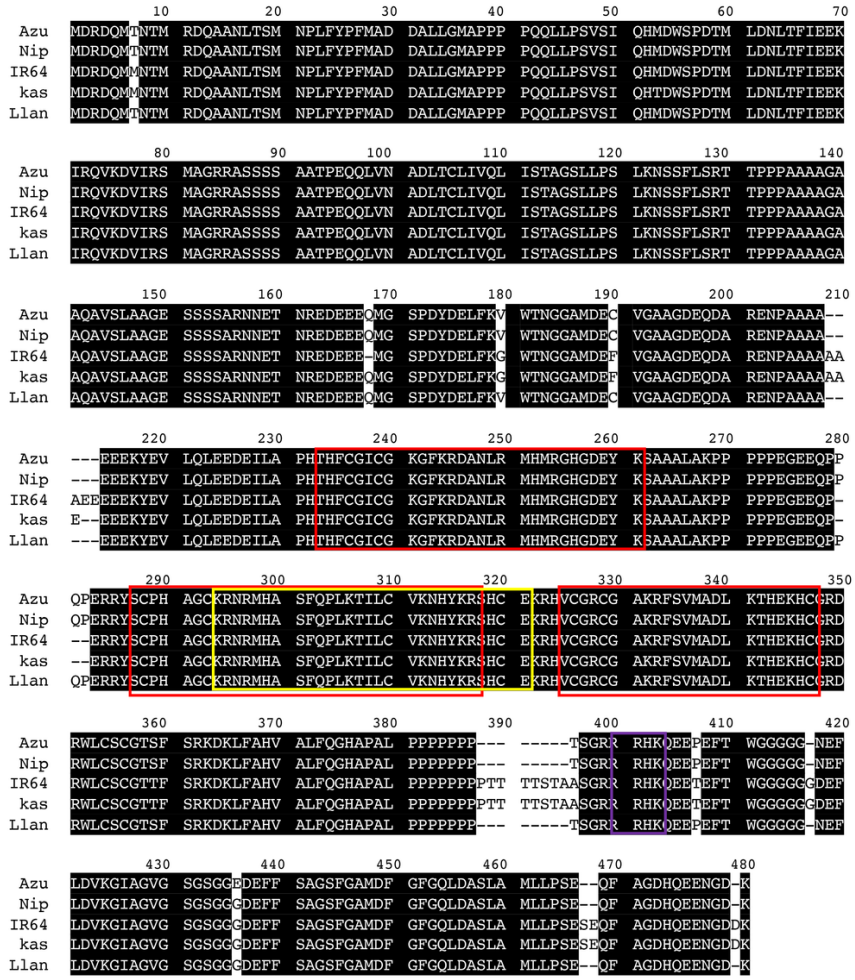

Figure 1

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).

Figure 2

Allelic variation in PCR products and 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 InDel 943 from start of the 5´UTR in the transcribed region according to 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 O. glaberrima (Og) CG14 allele and the 342 pb indicative of the Kasalath (Kas) allele. The nucleotide alignment shows six SNPs (Grey 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.

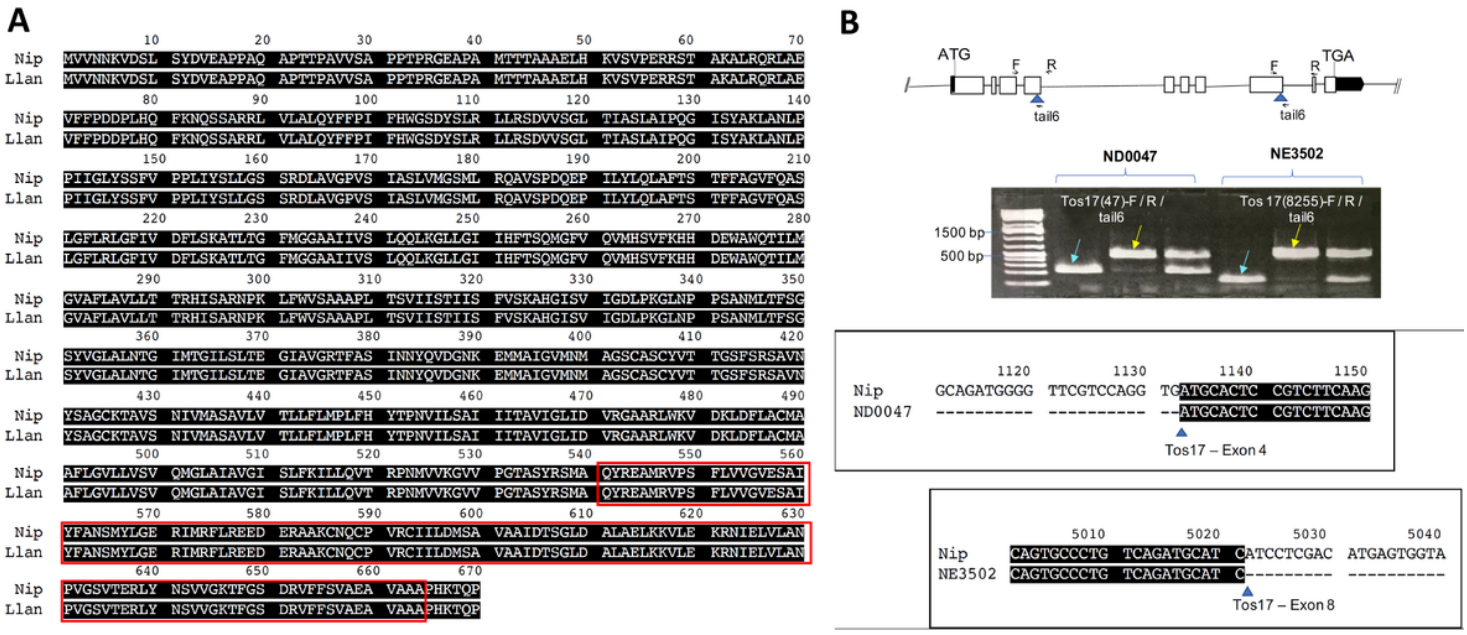

Figure 3

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 vs. mutant alleles shows the Tos17 insertions (Genebank accessions AG25438.1 and AG214407.1).

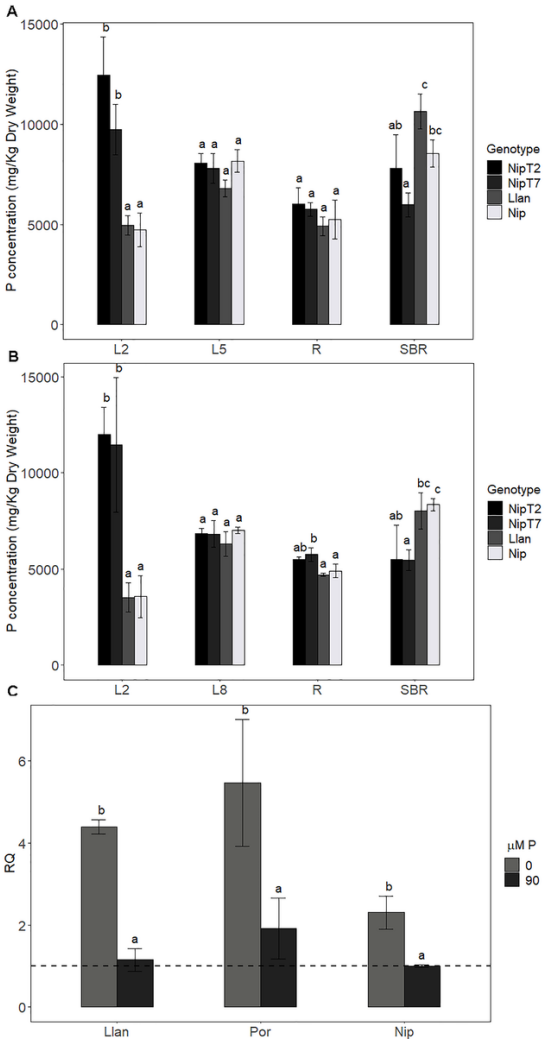

Figure 4

Phenotyping and SPDT expression at early vegetative development under hydroponic conditions. A) and 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 (90µM). C) Relative quantification (RQ= 2-ΔΔ𝑡) in the SBR of seedlings exposed to P-deficiency (0 µM) and P-sufficiency (90µM) relative to the control condition (90µM P, dashed line) using Actin as internal standard. 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.

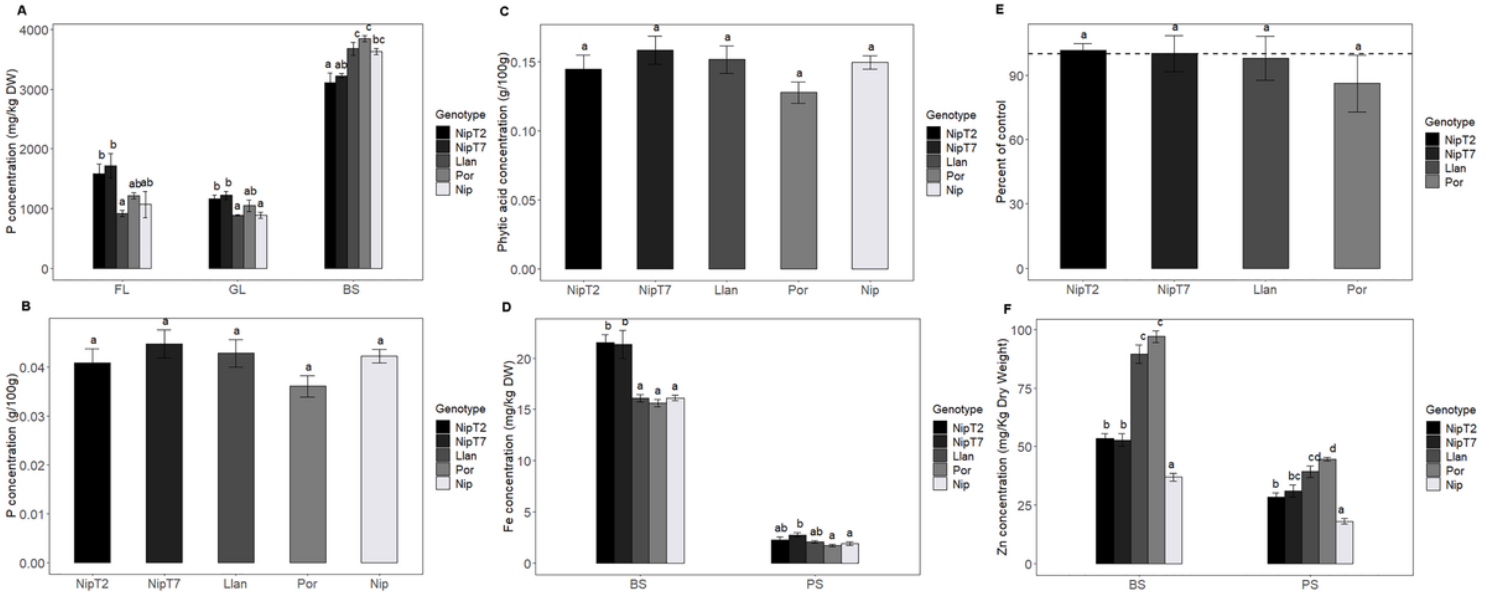

Figure 5

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) and 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 (4,335 ng ferritin/mg protein,100% represented by the dashed line) was measured by Ferritin formation in Caco-2 cells in vitro assay. 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.

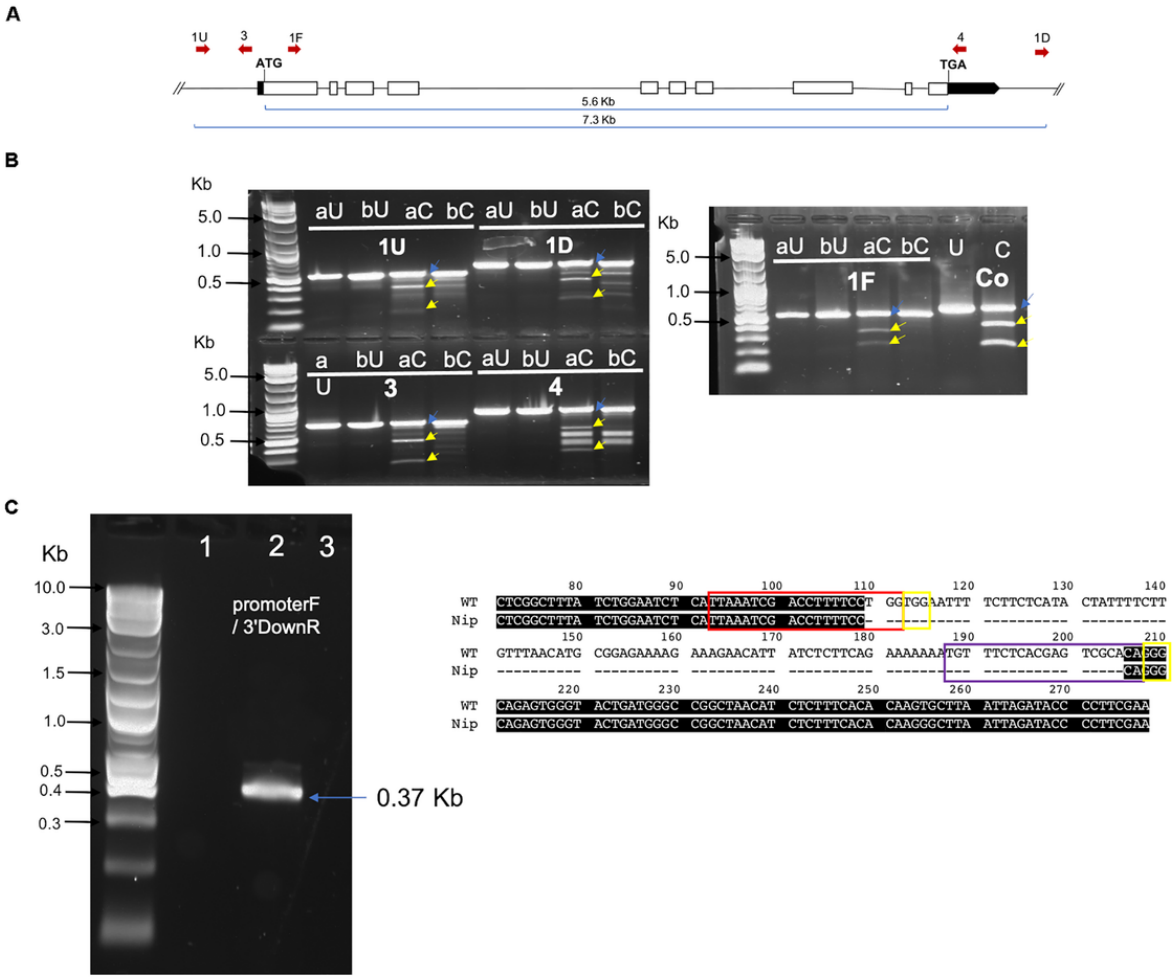

Figure 6

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) T7 endonuclease assay for evaluation of single gRNAs. Four lanes are shown for each gRNA (1U, 1D, 3, 4, 1F) and two lanes for the T7 control Kit (Co). Letters denote: Uncut genomic DNA (U), T7 cut DNA (C), protoplast DNA transformed with vector containing the gRNA (a), and protoplast DNA from water control (b). The blue and yellow arrows indicate the uncut and cut PCR products of expected size for each gRNA. 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.

Supplementary Files

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