For centuries, the United States government granted patents to the first party to invent the claimed subject matter. This first-to-invent patent law system was based upon the US Constitution which gives Congress the authority to promote the useful arts by securing for limited times to inventors certain exclusive rights to their inventions. To support the first-to-invent system, the Patent and Trademark Office developed the interference proceeding by which the PTO determines the person or group first to invent a claimed technology.

In an interference proceeding, the first party to file a patent application on the invention is designated as the senior party; all other parties are designated as junior parties. Interference rules establish a presumption that parties made their inventions in the order of the filing dates of their patent applications. This means that a junior party has an initial burden to prove a date of invention before the senior party. Interference proceedings are complex and can be lengthy. For instance, Monsanto Company won an interference in 2004 focused on technology for *agrobacterium* transformation in dicot plants. The interference ran for 12 years.

On September 16, 2011, President Barack Obama signed into law the America Invents Act. Among other changes, the Act converted the traditional first-to-invent system to a first-to-file system, a change that will eventually eliminate the PTO’s interference proceeding. The switch became effective on Saturday, March 16, 2013. That date became significant in a patent dispute over technology described a year earlier in a *Science* report: CRISPR-based gene editing.

**The Gathering Storm**

During the summer of 2012, Jennifer Doudna (University of California, Berkeley), Emmanuelle Charpentier (Umeå University), and their colleagues published their research about an RNA-guided DNA endonuclease activity that provides a type of adaptive immunity in certain bacteria and archaea. The key components of the system are CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) genes. In the type II CRISPR system, foreign DNA is cleaved into small fragments and incorporated into a CRISPR locus among a series of short nucleotide repeats. After the loci are transcribed, the transcripts are used to produce small RNA molecules that bind with foreign DNA having complementary nucleotide sequences. These RNA guide molecules also bind with activator RNA molecules, which in turn interact with Cas9 endonuclease that cleaves the foreign DNA.

Researchers eagerly adapted CRISPR-Cas9 technology for many applications,
from engineering fungus-resistant crops to generating mosquitoes resistant to the malaria parasite, *Plasmodium falciparum*. The technology could be used to cure single-gene hereditary diseases in humans, some suggested. Science declared CRISPR the 2015 Breakthrough of the Year. By that time, a patent dispute had been inching its way through the PTO. What was at stake? The outcome could determine ownership of multi-billion-dollar patent rights to core CRISPR technology.

In May 2012, the UC Berkeley group filed a patent application on the use of CRISPR-Cas9 gene editing technology in all cells. During October 2013, Feng Zhang of the Broad Institute Inc./Massachusetts Institute of Technology filed a CRISPR technology patent application, which claimed priority to a number of patent applications dating back to December 2012. Thanks to an accelerated examination program, the October application issued in April 2014 as US Patent No. 8,697,359. The ‘359 patent has claims to a method of using a CRISPR-Cas system to alter gene expression in a eukaryotic cell, and to CRISPR-Cas systems designed for that use.

While Broad Institute received additional patents on the technology, the UC Berkeley (UC) patent application crawled through the examination process. The UC application had claims to the use of CRISPR technology to edit genes in both prokaryotic and eukaryotic cells. During April 2015, the UC group asked the PTO to consider an interference proceeding to determine if they or Zhang had been the first to invent the technology.

On January 11, 2016, the PTO declared an interference. The UC group was the senior party, and Broad Institute was the junior party. “This is an absolutely humungous biotech patent dispute,” Jacob Sherkow of New York Law School told *Nature*. “We’re all waiting with bated breath.”

PTO Judges Throttle Interference

During the first phase of an interference, PTO judges and the parties define the scope of the disputed invention. In March 2016, the PTO announced a single “count” – a type of patent claim – that represented the interfering subject matter. The count read: “A method, in a eukaryotic cell, of cleaving or editing a target DNA molecule or modulating transcription of at least one gene encoded thereon, the method comprising . . . .”
The judges also considered motions filed by Broad Institute and UC. One of Broad Institute’s motions was a request for judgment of no-interference-in-fact. Broad Institute reasoned that the interference process should end, because the claims of Broad Institute’s patents and patent application have a eukaryotic limitation. Broad Institute patent claims cover CRISPR gene editing methods in eukaryotic cells, whereas UC’s method claims are “environment-free,” such as claims to cleaving DNA in vitro. As such, Broad Institute’s claimed invention does not interfere with the claims of UC which lack the requirement for eukaryotic cells.

After hearing oral arguments in December 2016, the PTO published a decision on February 15 that Broad Institute is correct. The judges terminated the interference.

To determine if an interference proceeds, patent judges apply a two-way test. They must decide if the subject matter of one party’s claim would, if prior art, have anticipated or rendered obvious the subject matter of an opposing party’s claim and vice versa. “In this proceeding, to prevail on its argument that there is no interference,” the judges explained, “Broad must show that the parties’ claims do not meet at least one of the following two conditions:

1) that, if considered to be prior art to UC’s claims, Broad’s involved claims would not anticipate or render obvious UC’s involved claims, or

2) that, if considered to be prior art to Broad’s claims, UC’s involved claims would not anticipate or render obvious Broad’s claims.”

Broad Institute argued that the second of these conditions is not met: UC’s claims would not have rendered Broad Institute’s patent claims obvious. “To make its argument,” the judges wrote, “Broad acknowledges that the UC inventors published results in Jinek 2012 using the prokaryotic CRISPR-Cas9 system in vitro, that is, in a non-cellular experimental environment, before Broad filed its claims directed to the eukaryotic cell environment.” Yet Broad Institute urged that a skilled artisan would not have had a reasonable expectation that the CRISPR-Cas9 system would work successfully in an eukaryotic cell. In part, Broad Institute supported its argument with contemporaneous statements made by researchers at the time that CRISPR-Cas9 was shown to function outside of a prokaryotic cell. For example, Broad Institute cited statements reportedly made by UC inventor Doudna that the Jinek et al. 2012 Science report “was a big success, but there was a problem. We weren’t sure if CRISPR/Cas9 would work in eukaryotes—plant and animal cells.”

The patent judges agreed with Broad Institute that the statements by and attributed to the UC inventors do not demonstrate a reasonable expectation of success. “Although the statements express an eagerness to learn the results of experiments in eukaryotic cells and the importance of such results,” they wrote, “none of them express an expectation that such results would be successful. The contemporaneous commentary by the UC inventors cited by Broad do not indicate that at the time of Jinek 2012 the ordinarily skilled artisan would have reasonably expected the CRISPR-Cas9 system to work in eukaryotic cells.”

UC argued that a reasonable expectation of success is evidenced by the ability of many research groups to use the CRISPR-Cas9 system in eukaryotic cells after publication of Jinek 2012. But the patent judges did not buy this argument. “Regardless of how many groups achieved success in eukaryotic cells,” they said, “we are not persuaded that such success indicates there was an expectation of success before the results from these experiments were known.”

The patent judges summarized their conclusions as follows.

The preponderance of the evidence, including the contemporaneous statements of the inventors and others in the field, as well as the knowledge of ordinarily skilled artisans,
demonstrates that one of ordinary skill would not have had a reasonable expectation of success that CRISPR-Cas9 could be used in a eukaryotic cell. In light of this finding, we determine that if they were prior art, UC’s claims would not have rendered Broad’s claims obvious. Because UC’s claims would not anticipate Broad’s claims either, we conclude that the parties’ claims are not drawn to the same patentable subject matter and that there is no interference-in-fact between them.

Still Waiting to Exhale
The UC patent application should proceed to issuance. During a teleconference, Doudna told reporters that her patent claims would cover the use of CRISPR in all cells, whereas the Broad Institute patent claims would cover the use of CRISPR in plant and animal cells. “They will have a patent on green tennis balls,” she explained. “We will get a patent on all tennis balls.”

In a press release, UC Berkeley suggested that it may appeal the interference decision. Kevin E. Noonan, a biotech patent lawyer with McDonnell Boehnen Hulbert & Berghoff LLP, commented on the possible results of an appeal in his Patent Docs website article. Noonan said that “prevailing in such an appeal may be quite difficult” considering the Federal Circuit’s deference to the PTO’s factual findings, such as whether there is a reasonable expectation of success in an obviousness determination.

Meanwhile, patent ownership for various uses of CRISPR technology is ambiguous. “Everybody gets to keep their patents,” Noonan told Nature. “This is maximum uncertainty for people because you don’t know if you have to get licenses from both sides.”

And here is another layer of complication. Antonio Regalado in his Technology Review article reported that, by February 2017, the PTO had issued 50 patents related to CRISPR technology. Only 14 were owned by Broad Institute, Massachusetts Institute of Technology, and affiliated groups. These 50 CRISPR patents may represent the first wave of a tsunami. As of February 16, the PTO patent application database includes 310 patent applications with the term CRISPR in the claims.

Selected Sources


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A Powerful CRISPR/Cas9 Platform for Obtaining Precise Gene Knockout Mutagenesis in Cotton (Gossypium hirsutum L.)

Chao Li, Turgay Unver, and Baohong Zhang

Introduction

Cotton is one of the most economically important fiber crops in the world. It has been widely cultivated in approximately 150 countries and provides around US $500 billion in economic benefits to approximately 100 million families worldwide\(^1\). Thus, improvement of cotton fiber quality and yield as well tolerance to environmental abiotic and biotic stresses are a priority for cotton research.

In the past few years, significant progress has been made in sequencing the cotton genome. Currently, the entire genome of three major cultivated cotton species and one potential ancestor of the allotetraploid species\(^2\) have been sequenced. Consequently, knowledge of gene function in cotton has surpassed other plant species, including major crops such as rice and maize. Research on cotton has greatly benefitted from gene function studies, the availability of a highly efficient genetic engineering procedure, and a reliable gene overexpression and knockout tool.

Although there are more than 50 species of cotton, only four are cultivated. Among the four cultivated species, the two are allotetraploid species (AADD; 4n=52)—upland cotton (Gossypium hirsutum) and sea-island cotton (G. barbadense)—have been sequenced\(^3\). Of the two diploid species—G. arboreum (AA; 2n=26) and G. herbaceum (AA; 2n=26)—only G. arboreum\(^4\) has been sequenced. Of the four cultivated species, upland cotton accounts for more than 95% of the cultivated area.

The genome complexity of allotetraploid cotton makes it challenging to conduct gene function studies and to effect genetic improvement through transgenesis. In our study, we used CRISPR/Cas9 and Agrobacterium-mediated genetic transformation to efficiently knockout an individual cotton protein-coding and small regulatory RNA gene. Some of the results have been recently published\(^1\) and the rest are forthcoming.

Clustered regularly interspaced short palindromic repeats (CRISPR) are a component of the bacterial immune system. The CRISPR-associated protein-9 nuclease (Cas9) is used by bacteria to target and destroy foreign RNAs. Over last several years, scientists have modified the CRISPR/Cas9 system to edit the genomes of both plants and animals.

The CRISPR/Cas9 system is composed of a Cas9 nuclease sequence and two noncoding RNA genes, a precursor CRISPR RNA (pre-crRNA), and a trans-activating crRNA (tracrRNA). When the two noncoding RNAs are replaced with an engineered single guide RNA (sgRNA), the sgRNA-Cas9 complex can specifically recognize complementary DNA target sequences that are immediately upstream of a 5'-NGG or 5'-NAG PAM (protospacer adjacent motif) sequence. CRISPR/Cas9 then catalyzes site-specific cleavage 3 to 4 base pairs upstream of the PAM site on the targeted DNA sequence. CRISPR/Cas9 frequently generates DNA double-strand breaks (DSBs) in the desired DNA sites, which can then be repaired through nonhomologous end joining (NHEJ) and homology-directed repair (HDR)\(^1,2\). This process can be used for creating gene mutants or inserting new gene sequences. In our paper\(^1\), we report that CRISPR/Cas9 genome editing system can be successfully used in allotetraploid cotton with high efficiency and high specificity.

Research strategy and Golden Gate assembly of sgRNAs

The gRNA design is extremely important for a successful genome editing event in which target-site specificity and minimization of any undesired off-target effect is desired. We designed gRNAs based on the upland cotton genome sequence data to meet the following four standards: 1) sgRNAs target sites are unique and only target the tested gene sequences; 2) sgRNAs target sites can be used to test the genome mutation efficacy on both the A and D subgenome of upland cotton; 3) several single-
nucleotide polymorphisms and double-nucleotide polymorphisms near the two sgRNAs target sites can be used to distinguish the identity of the genes of interest, such as \textit{GhMYB25-like} in this report; and 4) there are two highly similar sequences (1 - 3 mismatched nucleotides) with the designed sgRNAs, that can be used to estimate the off-target effect.

We used \textit{GhMYB25-like} as a guide to knockout an individual gene. Two sgRNAs, \textit{GhMYB25-like-sgRNA1} and \textit{GhMYB25-like-sgRNA2}, were designed to target the \textit{GhMYB25-like A} and \textit{GhMYB25-like D} genomic sequences encoded by allotetraploid cotton A subgenome and D subgenome, respectively\textsuperscript{13,14}. The \textit{GhMYB25-like-sgRNA1} and \textit{GhMYB25-like-sgRNA2} expression module was first assembled by direct PCR amplification using a pCBC-DT1T2 template as described in a previous report\textsuperscript{15}. The assembled PCR product was then purified and assembled with the Cas9 gene. The assembled CRISPR/Cas9 vector was chemically transformed into \textit{E. coli} and then \textit{Agrobacterium tumefaciens} (strain EHA105). Finally both sgRNAs and Cas9 were transformed into cotton cultivars YZ1 using the \textit{A. tumefaciens}-mediated genetic transformation as described in the previous reports\textsuperscript{16,17}.

The sequences containing the target cleavage site were amplified using regular PCR. PCR products were cloned using the TA cloning technique and sequenced. The sequences were analyzed and aligned through the NCBI database and DNAman software. At least 50 positive clones were sequenced for each genome knockout event and all potential off-target sites were investigated. Further details and a schedule can be found in Figure 1.

We adopted the CRISPR/Cas 9 platform\textsuperscript{15} developed by Dr. Qi-Jun Chen of China Agricultural University to develop a highly efficient and time-saving CRISPR/Cas9 system. According to the protocol described in their study, two sgRNAs, \textit{GhMYB25-like-sgRNA1} and \textit{GhMYB25-like-sgRNA2}, were quickly assembled into a sgRNA-expressing module with just one round of PCR reaction. The expressions of \textit{GhMYB25-like-sgRNA1} and \textit{GhMYB25-like-sgRNA2} were driven by the Arabidopsis Pol III promoter U6-26p. However, \textit{GhMYB25-like-sgRNA1} and \textit{GhMYB25-like-sgRNA2} have their own terminators, U6-26t. The application of the sgRNA-expressing module vectors facilitates the assembly process, and also guarantees the accuracy of the sgRNA expression cassettes. In this case, one day was sufficient to accomplish PCR amplification and PCR products purification. In the following step, Type II restriction endonuclease (REase) Bsai was used to seamlessly integrate the maize-codon optimized \textit{Cas9} and two \textit{GhMYB25-like} sgRNA-expressing cassettes; this step can be finished in six hours.

**Cotton transformation and evaluation of CRISPR/Cas9-mediated mutagenesis**

\textit{Agrobacterium tumefaciens}-mediated transformation was used to engineer gRNA/Cas9 into cotton plant cells, as in previous reports\textsuperscript{16-18}. After several rounds of subculture and antibiotic selection, somatic embryos were obtained from embryogenic callus generated from cotton hypocotyl segments. These antibiotic-resistant embryos were cultured on hygromycin-containing modified MS medium until the plantlets were generated. Two plantlets from each independent transgenic event were selected and used for subsequent mutation analysis.

To detect a genome DNA deletion, a pair of specific primers with a genome similar to \textit{GhMYB25-like A} and \textit{GhMYB25-like D} were used to amplify potentially truncated cleavage products. Small PCR products were recovered from the amplification products, suggesting that the precise cleavage events occurred in the designated genome regions of \textit{GhMYB25-like} genes. To further validate this and investigate the cleavage sequence sites, 20 positive PCR-generated colonies were selected for further sequencing analyses. The genomic DNAs of all 80 selected colonies were deleted partially by CRISPR/Cas 9, either from the \textit{GhMYB25-like A} DNA site or from the \textit{GhMYB25-like D} DNA site. The proportion of double cleavage breaks was quantified by the signal intensity of each band, as measured by ImageJ software (https://imagej.nih.gov/ij/download.html). The majority of the cleavage DNA length was around 268bp. These results suggest that the CRISPR/Cas9 genome editing system has the potential to efficiently create gene knockout in cotton.
One single gRNA also worked perfectly on an individual target site; of the 160 tested samples, 159 exhibited a few nucleotide deletions (from 1 nt to 7 nt). This suggests that both GhMYB25-like-sgRNA1 and GhMYB25-like-sgRNA2 effectively and precisely guided cas9-mediated genome cleavage. Given the high-efficiency effect on both GhMYB25-like A and GhMYB25-like D genome sequences, this CRISPR/Cas9 genome editing system has the potential to generate DNA level knockout mutations on complex allotetraploid cotton genome.

Off-target analyses

The possibility of creating ‘off target effects’ from genome editing is commonly discussed. For many reasons the off target effect of a genome editing technology should be minimized. To test the potential of off target effects of CRISPR/Cas 9 in this study, two putative off-target sequences—the GhMYB4-like genomic sequences, which are similar to GhMYB25-like and have three and one mismatched nucleotides with GhMYB25-like-sgRNA1 and GhMYB25-like-sgRNA2, respectively—were used to search for off-target events. We were unable to find any cleavage products of GhMYB4-like genomic sequences in our study. This suggests that the CRISPR/Cas9 genome editing system has a high specificity in cotton.

Conclusions

We developed a highly efficient CRISPR/Cas 9 genome editing tool for cotton gene function studies. Using the GhMYB25-like gene as a template, both single gRNA and double gRNAs generated cotton genome knockout mutants efficiently. The majority of the genome knockout mutants were genome deletion mutants, with 1 to 7 nt deletions for individual single gRNAs. Double gRNAs also worked perfectly on an individual gene in this study; 168 nt deletion is common for GhMYB25-like transcription factor gene using the GhMYB25-like-sgRNA1 and GhMYB25-like-sgRNA2 gRNAs.

This platform represents a new approach that will significantly enhance cotton genetic research.
More importantly, this CRISPR/Cas9 platform will strengthen cotton molecular breeding and improve cotton yield and quality as well as tolerance to different environmental abiotic and biotic stress by targeting individual protein-coding and small regulatory RNAs (Fig. 2).

Figure 2. Proposed application of CRISPR/Cas9-mediated genome editing in cotton

Acknowledgements

The CRISPR-Cas9 vectors were kindly provided by Dr. Qi-jun Chen of China Agricultural University. This work is partially supported by the Cotton Incorporated. The major results were previously published in Scientific Reports with the following citation: Li, C., Unver, T. & Zhang, B.H. (2017) A high-efficiency CRISPR/Cas9 system for targeted mutagenesis in cotton (Gossypium hirsutum L.). Scientific Reports, 7:43902. doi: 10.1038/srep43902.

References

African Rice for Low-input Agriculture Systems

Jos van Boxtel

African smallholder farms experiencing low inputs like fertilizer and water look forward to having rice varieties that can thrive and be more productive under these conditions. The development of low-input rice was an objective of the African Agricultural Technology Foundation (AATF)\(^1\) when, in the early 2000s, they searched for biotechnological applications that could improve the livelihood of African farmers. Since 2006, in the spirit of the Golden Rice project, AATF and collaborators have been working steadily on the development of such rice. Recent work by Selvaraj et al. (2016) published in *Plant Biotechnology Journal*\(^2\) describes the first results of this intercontinental collaboration. Three cycles of confined field trials in two environments showed that rice overexpressing a nitrogen use efficiency (NUE) gene can outperform controls under limiting nitrogen (N) applications.

These preliminary trials, performed by researchers from the International Center for Tropical Agriculture (CIAT)\(^3\) in Columbia, were done in preparation for a series of trials in three African countries, Uganda, Ghana, and Nigeria. The results of the trials were encouraging; consequently, the project is initiating the deregulation process in Africa to prepare for future commercial production.

**Coming together of stakeholders**

In 2005, AATF was scouting for agricultural biotechnologies to enrich the livelihood of smallholder farmers by increasing productivity in traditional low-input agriculture systems. They had several traits in mind—insect resistance, drought tolerance, and salt tolerance (ST). NUE was first considered when the United States Agency for International Development
(USAID), one of AATF’s main supporters, suggested contacting Arcadia Biosciences of Davis, California. For several years, Arcadia had been working on developing NUE in dicot crops, but they were also examining salt tolerance and drought tolerance in main agricultural crops. Using the dissemination of Golden Rice as a guide, in 2006 AATF, USAID, and Arcadia discussed the development of a climate change-resistant rice crop for Africa with no intellectual property strings attached. A preliminary agreement was reached in December 2006 in which Arcadia donated their technology royalty-free for humanitarian purposes and, when finalized in 2008, all parties had agreed to a production cost-based commercialization of the final crop.

Although Arcadia owned several of the genetic elements and enabling technologies that were planned for use in this project, the need for a few additional genetic elements remained. Consequently, UC Davis-based Public Intellectual Property Resources for Agriculture (PIPRA) was engaged to assist. PIPRA negotiated freedom to operate (FTO) licenses for several of the technologies proprietary to UC Davis.

The inaugural project meeting was held in Davis in late 2008. Since it was important to incorporate data from past similar projects, a ‘lessons-learned’ session was held, bringing together scientists at the forefront of the Golden Rice project, including renowned rice breeder Dr. Gurdev Khush. One important outcome of the discussions was the choice of germplasm in which the trait genes would be expressed. Based on consumer-choice in Africa, yield-increase potential, N-response potential, and amenability to tissue culture and Agrobacterium-mediated transformation, the WARDA-developed variety ‘NERICA-4’, emerged as the frontrunner. Ten years later this proved to be a golden pick. Not only is NERICA-4 showing a tremendous adaptability to different growing conditions, it currently remains as one of the most wide-spread and highly appreciated elite rice varieties in Sub-Saharan Africa.

To develop the climate change-resistant rice, a pipeline procedure was used to transfer only the NUE gene into NERICA-4, while Arcadia further optimized their in-house water use efficiency (WUE) technology. The second pipeline combined NUE, WUE, and ST, giving its trademarked name to the entire project, NEWEST. With all players in position and all tasks assigned, the project was launched in the laboratory in 2009.

**Development of field trial-ready rice lines**

While the tissue culture conditions and an Agrobacterium-mediated transformation protocol for NERICA-4 were being optimized at Arcadia, binary vectors were being simultaneously cloned at PIPRA and Arcadia.

Two binary vectors were constructed for Agrobacterium-mediated co-transformation. Vector pARC321 contained the 986 bp rice antiquitin promoter (pOsAnt1), the 1449 bp cDNA of barley alanine aminotransferase (HvAlaAT), and a 252 bp 3’nos fragment (Fig. 1). Binary vector pPIPRA543 (not shown) contained the selectable marker neomycin phosphotransferase (nptII), conferring resistance to the antibiotic kanamycin and controlled by the figwort mosaic virus 34S (pFMV34S) promoter and 3’mas terminator.

**Figure 1: T-DNA of binary vector pARC321 used for co-transformation of NERICA-4.** pOsAnt1, promoter of the rice antiquitin 1 gene; HvAlaAT, barley alanine aminotransferase gene; tnos, 3’UTR of nopaline synthase gene. Source: Selvaraj et al. 2016
While the FMV34S promoter was reported to control constitutive gene expression\(^9\), unpublished work on pOsAnt1 indicated that this promoter can be induced by several stresses, of which saline stress was the most strongly affecting.

pARC321 and pPIPRA543 were independently transformed into *Agrobacterium tumefaciens* strain EHA105\(^10\), and NERICA-4 was co-transformed using callus induced from mature dry seed\(^11\). Healthy rooted T0 plantlets were potted into synthetic soil, acclimated for one week in growth chambers, and grown to maturity in a greenhouse.

The co-transformation approach allowed identification of those events in the T\(_1\) generation which contained the inserted selectable marker T-DNA that was unlinked to pARC321 T-DNA and therefore able to be segregated. From the initial 800 generated T\(_0\) events, 15 were eventually advanced to the T\(_3\) stage, fulfilling all requirements for field testing, i.e., plants were homozygous, with a single copy insertion of T-DNA, and marker-free. Three cycles of trials with a subset of six of these events were reported by Selvaraj et al.

**Trials showing rice with increased performance under limiting N conditions**

The first challenge trials with six events were conducted by CIAT in Palmira and Santa Rosa, Colombia. While the African target countries Uganda, Ghana, and Nigeria were in the process of acquiring all necessary permits for trials in their countries, CIAT identified the events to be used for later trials. The CIAT team tested all six events in the lowland flooded fields at the station in Palmira for two years (2012 and 2013), followed by one year (2014) testing under upland rain-fed conditions in the hills of Santa Rosa.

In all three trials, two events consistently outperformed controls for grain yield (Fig. 2). Yields for event NUE-2, which received half the amount of nitrogen (90 kg/ha) of the local farmers’ rate, were 20–30% higher than controls. Also the performance of event NUE-6 was notable, although less impressive than NUE-2.

Hydroponic studies that were simultaneously conducted by CIAT (Fig. 3) indicated that early establishment of bushier and thickened roots were most likely primarily responsible for the increased N scavenging in early plant stages. The resulting early storage of N in pOsAnt1-*HvAlaAT* plants seemed to provide the source for increased tiller and panicle number, leading to increased grain yield under limited N conditions.

Furthermore, the increased yield was correlated…
with altered metabolites (increased levels of glycine, valine, and alanine in both leaves and roots) and accumulation of N in late vegetative stages, but not in seed. The trait was stable in both rice growing ecologies over three years of testing.

**Expanding Opportunities**

In 2013, researchers in Uganda (NARO-NaCRRI)\(^\text{12}\) and Ghana (CSIR-CRI)\(^\text{13}\) began field trials with NERICA-4 NUE events (Fig. 4). The trials were expanded to include the full set of 15 available trial-ready events. The levels of N applied to rice plots were much lower than those applied at CIAT. Sub-Saharan Africa smallholder farmers typically apply 30–60 kg/ha N to rice fields at best, or rely mostly on the application of manure. For comparison, trials were adjusted to ‘the real world’ situation for Africa, with N application rates reduced to 0, 30, 60 and 90 kg/ha. Besides NUE-2, two additional events were identified that had high grain yield at the rates applied. The rice yields were lower in Uganda and Ghana, compared to CIAT, most likely due to the high degree of variation between countries. However, yields were still increased by 10 to 15% compared to controls. In 2016, Nigeria (NCRI)\(^\text{14}\) was included in the trials, and all four countries simultaneously conducted a trial under similar conditions with a subset of the eight best performing events. Currently (2017), two lead events have been selected for regulatory data trials to test their applicability for future commercialization in Africa.

Production of this NUE crop should have significant economic and environmental benefits in both low and high input agricultural systems. By using co-transformation, we were able to generate events in which T-DNA of the gene of interest segregated from the selectable marker T-DNA, giving these transgenic plants an advantage for commercialization in areas for which NERICA-4 was selected.

As a possible additional benefit to farmers who grow NUE plants, Arcadia is investigating the effect that the reduction of nitrogen fertilizer application and more efficient uptake by NUE plants has on the emission of nitrous oxide, a potent greenhouse gas emitted from flooded rice paddy fields toward the end of the growing season. It could be an added incentive for farmers to earn carbon credits for emitting less greenhouse gases while maintaining yields with less fertilizer in high input agricultural systems. To this effect, Arcadia developed a methodology for calculating emissions reductions in agriculture, and in 2012, that methodology was approved by the executive board of the Clean Development Mechanism of the United Nations Framework Convention on Climate Change.

![Figure 4: Confined NERICA-4 NUE trial at NaCRRI, Uganda in 2016. Source Kayode Sanni, AATF, 2016.](image-url)
References
1. AATF: http://aatf-africa.org/
3. CIAT: http://www.cgiar.org/about-us/research-centers/international-center-for-tropical-agriculture-ciat/
12. NACRRI: http://nacrri.go.ug/
13. CRI: https://www.cropsresearch.org/
14. NCRI: http://www.ncribadeggi.org.ng/

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