Title: Using *Agrobacterium*-Mediated Transformation to Introduce Sunflower Transcription Factor HaWRKY76 to *Oryza sativa* cv. N22 to Confer Drought Tolerance
Application: Drought tolerance, agricultural sustainability, yield improvement
Challenge: Reduce water usage for convenal agriculture; Introduce transgenic drought tolerance
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Introduction

The goal of our project is to confer the seedling drought tolerance observed in the sunflower family (*Asteraceae*) to a rice cultivar, Nagina 22, via transgenic expression of the transcription factor HaWRKY76. We propose that complete success in this endeavor will result in producing a stable line of transgenic rice that requires significantly less water during the seedling stage. The inspiration to use sunflower transcription factor draws from a similar successful transformation carried out in Arabidopsis by Raineri et al in 2015.

This project aim to insert a T-DNA sequence containing the HaWRKY76 gene into the cv.N22 genome, using *Agrobacterium*-mediated transformation. We then aim to regenerate modified N22 line that will stably expresses the HaWRKY76 transcription factor. The final result is expecting that the modified cultivars produce greater yield under drought stress than unmodified controls.

We are selecting the N22 cultivar for the experiment because it is already partially drought tolerant in the flowering stage but not in the seedling stage (Jagadish et al. 2011). Even though the introduced water retention and modified water uptake properties may have unforeseen tradeoffs in terms of development and yield, improving the response to drought stress in the early developmental stages of N22 rice can potentially enhance its tolerance to a larger diversity of environments while maintaining a profitable yield. We are using the commercial *Agrobacterium* transformation plasmid, pRI101-ON from TAKARA BIO Inc. and aiming to insert the HaWRKY76 gene linked to a nuclear localization signal. The modified pRI101-ON-WRKY plasmid will first be amplified by the *E. coli* strain DH5α and then introduced into *Agrobacterium* LBA4404. The pRI101-ON-WRKY plasmid will be inserted into N22 embryogenic calli cells via *Agrobacterium* transformation. We are utilizing a binary vector transformation system in which Agrobacterial cells carrying the pAL4404 helper plasmid are transformed with the pRI101-ON-HaWRKY76-NLS construct; the binary vector system separates the VIR gene and T-DNA onto two different plasmids. This allows us to transfer a longer T-DNA than would be possible using only a single-vector system. Once our T-DNA has entered the plant cells, the NLS will lead the HaWRKY76 gene into host nucleus and be incorporated into the host genome.

The HaWRKY76 transcription factor is involved in the regulation of drought stress responses in sunflower (*Helianthus annuus*), in which the expression increases in response to drought stress (Raineri et al. 2015). HaWRKY76 expression directly results in lower water loss, lower ion leakage, and stomatal closure in the plant, which in turn leads to delayed drought-activated senescence, lower cell membrane damage, and lower water consumption (Raineri et al. 2015). The exact biochemical mechanisms or potential crosstalk with ABA signaling pathways that confer these responses have not yet been established; however, HaWRKY76 has been shown to down-regulate or not affect several marker genes for drought stress and ABA signaling (Raineri et al. 2015). We aim to confer the decreased seedling water

consumption to rice cv.N22 via transgenic expression of HaWRKY76. Rice seedlings are typically planted into flooded conditions since they require a certain amount of root waterlogging for the most productive growth. Although rice is generally tolerant of many environments, a certain level of yield is necessary for many farming operations. A drought year or inconsistent rainfall can bring about severe economic losses, but this circumstance can be mitigated by the performance we are trying to achieve in our modified cultivar. This project revolves around the premise that the introduced presence of HaWRKY76 transcription factors decreases this required level of water, protecting the plants against sudden declines in water availability due to irregular rainfall patterns.

Proposal Rationale

Agriculture is one of the most water intensive industries, and water usage will only increase with the rising world population. As the planet's freshwater supply decreases, there is a pressing need to reduce agricultural water consumption. In addition to developing more efficient farming practices to improve agricultural sustainability, we need to be developing cultivars that can be grown with less water consumption. Drought and irregular rainfall patterns are only worsening with global climate change, ultimately exacerbating the shortage of freshwater for crop irrigation further underlining this push for sustainability.

Unlike many other conventional crops like maize, rice requires a large quantity of irrigation in the seedling stage to express its full yield potential. Dependence on rainfall is the largest production constraint for rice production in rainfed based rice economies in the developing world (Wassman et al. 2009). If the expression of the HaWRKY76 transcription factor successfully confers drought tolerance during the critical growth stage of seedlings, rice farmers could potentially save a large amount of freshwater usage. Another obstacle limiting production are irregular rainfall patterns that potentially lengthen a drought period, having detrimental effects on conventional rice yield, especially in agricultural regions lacking back-up irrigation systems. The transgenic N22 cultivar seedling would have a greater survivability through these harsh periods and ultimately produce a better yield compared to the conventional cultivars.

Roughly 75% of the rice produced in the world is from irrigated wetland. Therefore, water usage could be reduced if rice required less water to grow (Bouman et al. 2007). From 2000 to 2004, an average of 591,751,209 tons of rice per year were produced throughout the world; 784,073 cubic megameters (Mm^3) of water were used per year to grow the rice (FAOSTAT. 2009). The creation of drought tolerant rice cultivars could alleviate the steep water requirements for feeding the world, permitting the staple crop to be grown in drier conditions. Ideally, the improved cultivar is expected to produce more yield even while consuming less water. This would increase the profit margins and lower the risk of smaller farming operations that either pay for irrigation or rely on rainfed systems. In the best case scenario, greater yields in rice production could be key in stamping out food shortages and alleviate general economic burdens and living expenses in areas where small rice operations sustain the local economy. Methods to transform rice using *Agrobacterium* have been established and enable the production of transgenic lines. Over the last thirty years, there have been improvements in the procedures to transform monocots, prior to which *Agrobacterium*-mediated transformation was thought to be primarily limited to eudicots (Hiei et al. 1997). As a result, we plan to use disarmed

Agrobacterium strain LBA4404 to introduce the sunflower transgene HaWRKY76 to rice cultivar N22 to confer drought tolerance in seedling stage.

Experimental Design

Hypothesis: We hypothesize that HaWRKY76 gene will confer seedling drought tolerance in the F_2 progeny of transgenic *O. sativa* cv. N22, and function similarly as it is in sunflower, via stable expression of the transcription factor. We also hypothesize that the transgenic N22 cultivar will exhibit greater yield than the control cultivars in a drought conditions.

Specific objectives: We will first clone HaWRKY76 cDNA into pRI101-ON plasmid near a strong constitutive CaMV 35S promoter. After verifying by Sanger sequencing, we will transform *E.coli* strain DH5α to amplify our plasmids. Disarmed LBA4404 *Agrobacterium* will be transformed with the plasmid and then used to introduce the HaWRKY76 gene into rice cultivar N22 genome. Then, we will establish a plant cell culture and select for HaWRKY76-containing cells with kanamycin. If the regeneration process is successful, we will grow these transgenic N22 cells to maturity and self-cross each labeled individual to generate F_1 plants. We will then confirm stable HaWRKY76 expression among F_1 plants using PCR and western blot. If these tests confirm a positive result, then the resulting seeds (F_2) will be grown for experiments that evaluate drought tolerance. They will be grown along with control plants in both sufficient and low water conditions, where the overall growth status and yield will be evaluated and compared.

Experimental Approach

Design proper transgenic vector

Preparation of HaWRKY76 cDNA for cloning - Fresh leaf samples of *H. annuus* will be harvested in tubes at 50-100 mg per lyse and crushed finely. The crushed sample will be treated with TRIzol reagent and followed by a conventional chloroform extraction protocol. Centrifugation is set at low temperature to separate out the RNA-containing upper phase, which will be spun and pelleted at the bottom of the tube. The pellet will be washed a few time and resuspended in EDTA buffer (Thermo Fisher Inc. 2016).

Amplification of HaWRKY76 via reverse transcriptase-PCR -We will amplify the cDNA of our target mRNA using Qiagen OneStep RT-PCR kit and associated protocol. Primers for HaWRKY76 will be ordered or synthesized. The forward primer will contain an NdeI restriction site on the 5' end and be specific to the first 16 nucleotides downstream of the mRNA start codon. The start codon is excluded because the NdeI restriction site already contains an in-frame ATG start codon. The reverse primer will contain a ClaI restriction site on the 5' end that is specific to the last 16 nucleotides upstream the stop codon. The stop codon is also excluded in order to fuse with additional NLS tag. (Qiagen OneStep handbook 2012)

Amplification of SV40 NLS - Genomic samples of SV40 will be used in PCR with the NEB Taq PCR kit to amplify the NLS tag. Similar primers will be used: the forward primer will be specific to the first 10 nucleotides of the NLS coding sequence, while the reverse primer will be specific to the last 10 nucleotides. The resultant PCR product is held at 4ºC and then purified by gel purification. (New England Biolabs)

Clone HaWRKY76 into pRI101-ON vector - The constructed HaWRKY76 and SV40 NLS PCR products will be combined in a reaction vessel containing ClaI to digest, producing the necessary

sticky ends, and then ligated by DNA ligase. A map of his construct is shown in Appendix B. The pRI101-ON vector from ClonTech is selected as it is a binary vector with a MCS preceded by the CaMV 35S constitutive promoter. It contains kanamycin resistance both within (*nptII*) and outside (*nptIII*) of the T-DNA borders for selection in both plant and bacterial hosts (ClonTech). The pRI101-ON vector will be linearized by digestion with NdeI and TspMI, in which neither restriction sites are present within HaWRKY76 so we don't accidentally cleave our gene of interest. Also we choose to use two different restriction enzymes for directional cloning. The construct produced by the ligation of HaWRKY76 and the NLS will also be digested by NdeI and TspMI in order to produce compatible sticky ends, which finally ligated into the linearized vector to create our pRI101-ON-WRKY plasmid. An expected map of the plasmid is shown in Appendix A.

Confirm plasmid fidelity via preliminary transformation and sequencing - We will establish initial plasmid-carrying *E. coli* population by transforming *E. coli* DH5α using the NEB transformation protocol. Once done, tenfold dilutions of the finished suspension will be prepared, and portions of each will be spread onto prewarmed plates containing kanamycin for selection. After incubation at 37ºC, isolated colonies will be suspended in 50 mL portions of Luria broth containing kanamycin and further incubated. Then, plasmid DNA is isolated via QIAprep kit from Qiagen (Thermo Fisher Scientific. 2016) We will then send the plasmid to sequence and confirm whether our pRI101-ON-WRKY plasmid is engineered successfully. Subsets of these samples representing good transformation will be used later to produce a secondary transformed population with a higher copy number of the constructed vector.

Creating transgenic lines of *A. tumefaciens*

Rice embryogenic callus induction - In this study, we chose the rice cultivar N22 and scutellar embryogenic calli will be used in plant transformation and cell culture regeneration. We start by sterilizing about 20 healthy, mature and dry cv. N22 seeds in 70% ethanol (v/v) for no more than 1 minute and pipet out the ethanol solution. The seeds are then washed with freshly premade 50% bleach (v/v) on a shaking platform set at 180 rpm for no more than 30 minutes, immediately followed by washing 8-10 times with sterile distilled water. After the seeds are dried at room temperature for about 5 minutes, they are ready for callus induction. 10-15 healthy seeds are plated on petri dishes containing callus induction MCI media (Caisson laboratories, Catalog no. MSP09). Then, the plates are incubated at 28°C in dark for 14 days and taken out to confirm the formation of embryogenic calli. It is important that we only chose embryogenic calli for subculture, therefore we must recognize and discard the non-embryogenic calli, which appears compact, non-friable and root-like. We will cut each embryogenic calli to 3 pieces with similar sizes and subculture them onto fresh MCI plates at 28°C in dark for 4 days before they are finally ready for transformation.

Transformation of *E.coli* via heat-shock - For a high efficiency transformation, a high plasmid DNA concentration is prefered. We will use the NanoDrop™ to check our pRI101-ON-WRKY plasmid DNA concentration, which ideally would be above 100 ng/µl. Nevertheless, a 20 ng/µl vector concentration will suffice for *E. coli* transformation. DH5α *E. coli* strain will be transformed with our tested pRI101-ON-WRKY vector using the heat-shock method. We will follow the high efficiency transformation protocol manual from New England BioLabs Inc. The transformed bacteria will be cultured in SOC media which claimed to yield a higher transformation efficiencies comparing to conventional LB media (Neb. High Efficiency

Transformation Protocol-c2987). After the culturing stage, we will prepare 5 ten-fold serial dilutions in SOC and spread 100 µl of each dilutions onto prewarmed kanamycin selection plates and let these 5 plates incubate overnight at 37°C. On the next day, we will pick one solitary colony where there is no overgrowth and introduce it into a baffled flask containing 50 ml of LB media supplemented with 50 µg/ml kanamycin. After another day of incubation, the culture solution should appear cloudy with an O.D. 600 index around 0.6-0.8. Then, we will use the same QIAprep kit to extract the pRI101-ON-WRKY plasmids in larger quantity and use them to transform *A. tumefaciens.*

Transformation of *A. tumefaciens* via electroporation - We choose to work with LBA4404 *A. tumefaciens* not only because it is electrocompetent but also its disarmed vector, now serves as the helper vector that confers Vir activities and streptomycin resistance. The purified pRI101-ON-WRKY plasmids will be introduced into LBA4404 using electroporation. We will follow the protocol from Bio-Rad electroporation instruction manual. It is crucial that we resuspend the plasmid DNA in ddH_2O or perform a microdialysis to make sure the plasmid DNA is salt-free prior to electroporation. We will follow the preset electroporator condition at 2.5 kV, 200 Ohms, and 25 µF (Bio-Rad. Transformation of Bacteria by Electroporation Protocol). Then we are going to incubate the electroporated cells on a shaker platform at 200 rpm for one hour at 28°C and then prepare 3 ten-fold serial dilutions with YEP broth. We will spread each dilution on prewarmed agarose-YEP plates supplemented with 25 µg/ml streptomycin and 50 µg/ml kanamycin (Sahoo et al. 2011). The plates are incubated upside down at 28°C for 16-24 hours and will be ready to make dipping culture.

Preparation of *Agrobacterium* dipping culture- We start primary *Agrobacterium* culture by picking one solitary colony with a pipet tip and introduce it into a 15 ml test tube containing 5 ml of YEP media with 25 µg/ml streptomycin, 10 µg/ml rifampicin and 50 µg/ml kanamycin. Then we will incubate the test tube for 16-20 hours on a shaker platform at 200 rpm at dark at 28°C. Then, the secondary culture starts by adding 100 ml YEP-streptomycin-kanamycin medium into a 500 ml baffled flask, followed by adding 0.4% of the primary culture and incubate under the same condition. On the following day, the culture solution should appear cloudy with a expected O.D. 600 index approaching 1.0. Then, the cells are pelleted at 4°C by centrifugation at 8000 xg for 15 minutes and resuspended in 100 ml MS medium with 150 µM acetosyringone to lower the O.D. 600 down to 0.3 (Sahoo et al. 2011).

Transformation and Regeneration of *O. sativa* cv. N22

Agrobacterium LBA4404 dipping - After the embryogenic calli are subcultured for 4 days, we take them out of the dark room and carefully immerse them in the 100 ml *Agrobacteria* culture that is just made, for about 20-25 minutes on a slow shaker at 50 rpm. We will dry the calli on a sterile Whatman No.3 filter paper for 5 minutes and transfer them to co-cultivation media MCCM-MCI, and incubate for about 48 hours in dark at 28°C (Sahoo et al. 2011). As soon as *Agrobacterium* growth appears, we wash the calli with sterile distilled water with 250 mg/l cefotaxime for 8-10 times. After the calli are dried, we transfer them to the first selection plates which consist of MCI media supplemented with 250 µg/ml cefotaxime and 50 µg/ml kanamycin in dark at 28°C for 12 days. After the first selection, calli tissue is expected to develop chimeric phenotype so that untransformed plant tissues start to turn black or brown, as a strong negative selection from kanamycin while transformed cells should appear creamish. We will carefully remove all dull-colored tissue and transfer the healthy calli onto secondary selection plates with

the same MCI-kanamycin media in dark at 28°C for 10 days. After the second selection, we will observe the samples under light microscope and expect microcalli formation which is transferred to another sets of MCI-kanamycin media for the third selection in dark at 28°C for 5 days.

Co-cultivation and calli selection - After the embryogenic calli have been infected with *Agrobacterium*, the calli will be dried on clean filter paper. Then, the calli will be transferred to a standard Murashige and Skoog (MS) medium. Furthermore, 10 g/L glucose and 150 μM acetosyringone will be added to aid the co-cultivation of the calli and *Agrobacterium* (Sheikholeslam and Weeks 1987). These plates will be kept at pH 5.2 and incubated at 26-28˚C for 2 days in the dark. (Sahoo et al. 2011) Following the incubation, the calli will be rinsed with a solution of 250 μg/mL cefotaxime and distilled water 10 times. Cefotaxime is a cephalosporin antibiotic that is largely non-toxic to plants, but is used to eliminate Gram-negative bacteria (Toku-E 2012). Thus, the cefotaxime solution will kill excess *Agrobacterium* and any other contaminants, leaving healthy, transformed calli free of bacteria. These calli will be transferred to a selection medium with 250 μg/mL cefotaxime and 50 μg/mL kanamycin to select for calli that have gained the kanamycin resistance from the pRI101-ON-WRKY plasmid. The calli will be incubated for 12 days on this selection medium at 26-28˚C in the dark. After the 12-day incubation period, the calli will be inspected, separating the healthy calli from the discolored calli. The healthy calli will be transferred to new plates with fresh selection medium, still containing the cefotaxime and kanamycin. Secondary selection will take place after 10 days of incubation at 26-28˚C in the dark. The calli that form microcalli will be transferred to a 3rd selection medium for the final round of incubation for 5 days. (Sahoo et al. 2011)

Regeneration of selected calli - The microcalli will be transferred to a regeneration medium, which is the MCI medium with kinetin and 1-naphthaleneacetic acid (NAA). The MS salts and maltose provide nutrients to the plant cells, while kinetin and NAA are plant growth regulators to aid in regeneration of full plantlets. Additionally, 250 μg/mL cefotaxime and 30 μg/mL kanamycin will be added to the regeneration medium, to further prevent contamination and ensure that the microcalli are properly transformed. Initially, the microcalli will be incubated on this regeneration medium gellified with 1% agarose at 26-28˚C for 7 days in the dark. This first phase of regeneration will be followed by a second phase, for which the microcalli will be transferred to another regeneration medium, gellified with 0.8% agarose to maximize regeneration frequency. The microcalli will be incubated for 4 more days, with light. By the end of the second phase of regeneration, the calli should have began to regenerate shoots. To develop the roots, the regenerated shoots will be transferred to jars with a standard rooting medium. The jars will be incubated at 26-28˚C for 7 days with light. (Sahoo et al. 2011)

Transplant and self-cross - After both shoots and roots are established, the plantlets will be transplanted into pots with 6 kg of clay loam soil and 2.0 g $(NH_4)_2SO_4$, 1.0 g KCl, and 1.0 g single superphosphate, with one plantlet per pot. 30 days after transplantation, additional fertilizer will be added along with 2.5 g of $(NH_4)_2SO_4$. Each rice plant will be grown under the normal flooded conditions to grow rice. These plants will be grown to maturity at temperatures of 29˚C during the day and 21˚C at night. After about 2 months, the rice will reach reproductive maturity and they will be ready to pollinate (Jagadish et al. 2011). Once flowers have emerged and the plants are ready to be pollinated, each inflorescence will be bagged with pollination bags

in order to prevent cross-pollination. The rice will continue to be grown at 29˚C during the day and 21˚C at night. After about 30 days, the reproductive phase will end and each rice plant should have only been self-fertilized. After pollination has taken place, it will take about 30 days to produce mature seeds. These seeds are the F_1 generation, which will be grown out and self-fertilized again to obtain the F_2 seed (GRiSP 2013). The F_2 seeds will be harvested and tested using PCR test (Forward primer: 5' GTAAAACGACGGCCAGT 3'; Reverse primer: 5' CAGGAAACAGCTATGAC 3' which should yield a ~2kb DNA band) and a Western blot test to confirm that the transgene and expressed products are present.

Testing drought tolerance on F_2 transgenic plants - We will take 10 transformed F_2 cv. N22 seeds that is produced from our lab into a 1x5 rectangle pot in rows with 0.5 m long for each seed and 10 untransformed N22 seeds into a 1x5 rectangle pot in rows with 0.5 m long for each seed. Nitrogen, phosphorus, and potassium (NPK) will be applied at the rate of 120:30:30 kg ha–1. The seeds and pots will be placed in the greenhouse with 30/24 °C day/night cycle. 30W high-pressure sodium lights (P. L. Light Systems, Beamsville. ON Canada) is used to maintain the photosynthesis with 16/8 h photoperiod. Peters Excel fertilizer (Scotts-Sierra Horticultural Products Company, Marysville, OH) is used daily to maintain the nutrient. Until 45 days, the seeds will be irrigated by using the sprinkle twice a week from the establishment and vegetative growth. The drought stress will start after this period by not irrigating the plants until soil water tension fell below –50 kPa at 30 cm soil depth. This method is efficient to measure drought tolerance (Lafitte et al., 2004). We will then record the data for plant height and grain yield. Plant height measure the maturity from the soil surface to the apical and yield shows how the transformed gene affect the rice performances compared to untransformed rice plant during the drought stress. In the end, we can evaluate the measurements using null-hypothesis testing such as chi-square test and t-test and decide whether the transformed gene is significantly affecting the plants.

Potential Pitfalls and Potential Solutions: There are a few typical problems that frequently showing up in plant transformation experiments. The vector engineering may fail due to unspecified restriction digestion, which would require going back to the sequence and identifying better cloning sites. The bacterial transformation may be inefficient due to low initial plasmid concentration, so more than one batch of solution may be needed to retry the transformation. In addition, we may have to adjust antibiotic concentration to optimize the transformation. The plant cell cultures could be contaminated if not supplemented with the optimized antibodies, thus sometimes we need to search for our own optimized conditions. Also, microcalli may fail to develop after the third selection as a result of early root development; we can prevent this scenario by constantly monitoring the third selection stage. The self-cross process may not be perfect because many insects inhabit greenhouses and become unwanted pollinators, thus pest control is also important. In addition, the F_2 sample size may not be large enough to generate confident results when analyzed with t-test, potentially making the results inconsistent. For this, we could self-fertilize cv. N22 for one more generation which would yield many more F_3 seeds to work with.

Limitations of our Design: The pursuit of drought tolerance in rice cultivars has been carried out by way of genetic engineering, molecular breeding, and conventional breeding in the past.

Based on the achievements of previous studies, we expect some unintended and unforeseeable complications in the experimental process, but we do not expect to come across an insurmountable roadblock in developing the cultivar. Our transformed rice will preserve water by closing stomata in hot and stressful environments, thus reducing transpiration with an ABA-independent mechanism. This induced stomatal closure could result in reduced carbon dioxide intake, which may negatively affect yield. However, previous HaWRKY76 transformation studies reported increased yields, despite the possibility of any negative effects from reduced CO_2 intake. And with global outcast of declining available fresh water for agriculture, this CO_2 trade-off for an increased water efficiency is an essential one. While this cultivar could be hugely beneficial in areas like India, Philippines, China and Indonesia, where fluctuating weather patterns greatly affect rainfed agricultural practices, social acceptability of this technology is a strong impediment for its adoption. General public across the world, including these countries, remain skeptical of implications of genetically engineered crops and governments largely have not endorsed then bowing to public pressure. For this project to ever see the light of the day in general marketplace, there either needs to be an overhaul of public perception of GM crops or more realistically, this trait needs to be introgressed into commercially successful cultivars via traditional breeding.

Timeline and Schedule

Year 1: Successfully modified pRI101-ON plasmid and cloned HaWRKY76 and NLS
Transform *E. coli* DH5α and *A. tumefaciens* LBA4404 with pRI101-ON-HaWRKY76-NLS
Germinate N22 seedlings and induce calli formation
Grow Parental plants from transformed calli

Year 2: Harvest seeds from parental plants and grow F1 generation
Self-cross F1 generation
Harvest seeds from selfed F1 generation

Year 3: Grow F2 generation and control N22 plants
Sample leaf tissue and conduct PCR and Western to confirm HaWRKY76 expression
Genotype F2 individuals and enroll stably expressed transgenics in drought study
Conduct drought study
Harvest tissues to analyze yield and potentially biomass accumulation

Potential Impact and Expected Outcome

If the experiment is successful, this type of transformation could potentially lead to water conservation on a very large scale. While this might not be directly accepted, we anticipate this cultivar to spur the interest of rice breeders across the world in adopting this trait. As this trait has the potential to increase yields and increase water usage efficiency, it aligns the interests of short term gains as well as long term sustainability of the environment creating a mutual benefit. This would attract breeders from both water-abundant regions and drought-prone regions. On a broader scale, potentially introgressing this trait into commercially successful varieties across the world would not only encourage people to reevaluate the potential values of GM crops in general, but also have a significant environmental impact that positively responds to climate change and addresses global water scarcity to the public.

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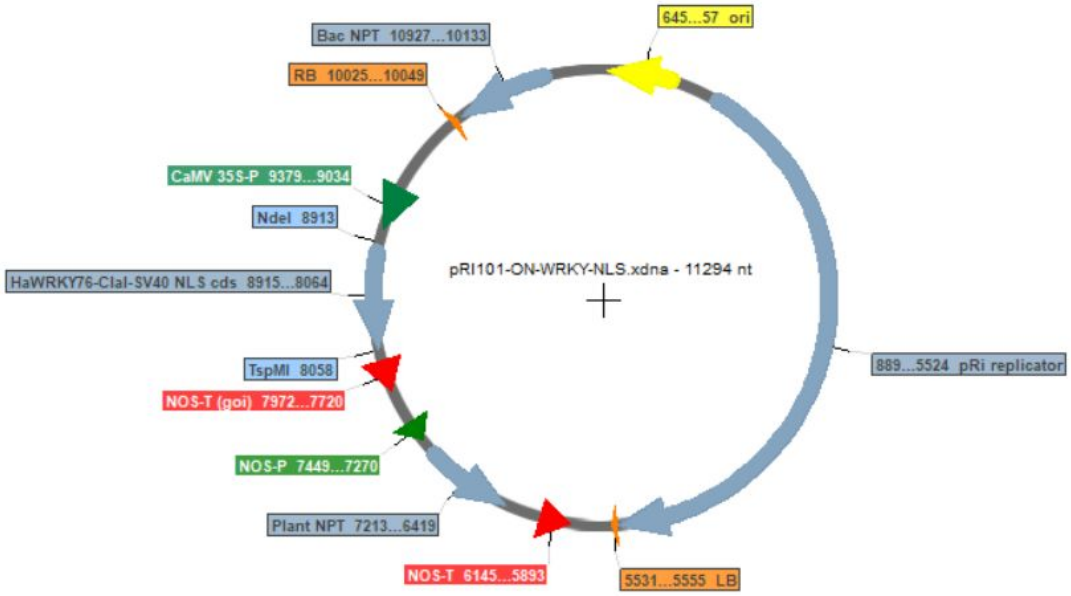
Appendix A: Map of the designed plasmid vector

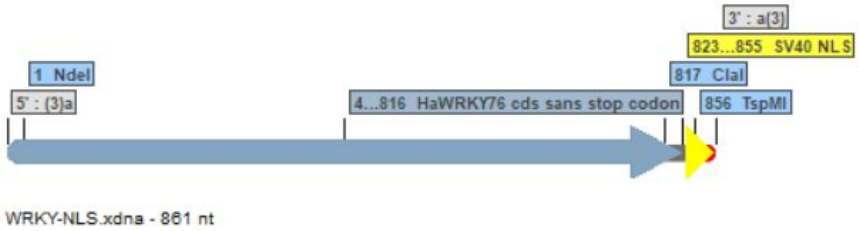
Map constructed using Serial Cloner. Features are labelled with position in base pairs.



Features (clockwise from position 0):

ori - *E. coli* origin of replication
pRi replicator - facilitates *Agrobacterium* replication
LB - T-DNA left border
NOS-T - Nopaline synthase terminator
Plant NPT - Kanamycin resistance gene for plants
NOS-P - Nopaline synthase promoter
NOS-T (goi) - Nopaline synthase terminator affecting transgene
HaWRKY76-ClaI-SV40 NLS cds - Insert containing constructed coding sequence for fusion protein of HaWRKY76 and C-terminus nuclear localization sequence. Ligated directionally using NdeI and TspMI.
CaMV 35S-P - Constitutive promoter from cauliflower mosaic virus 35S gene
RB - T-DNA right border
Bac NPT - Kanamycin resistance gene for bacteria

Appendix B: Map of insert before digestion and ligation into plasmid vector



Features (left to right):

NdeI - Restriction site for ligation, also includes start codon
HaWRKY76 cds - Coding sequence for target gene; stop codon removed to create fusion protein
ClaI - Restriction site for NLS fusing; 6-cutter preserves reading frame
SV40 NLS - Coding sequence for nuclear localization signal from SV40; also contains stop codon.
TspMI - Restriction site for ligation.