CRISPR/CAS9 EDITING OF TANDEMLY DUPLICATED GENES IN *POPULUS TREMULA* × *ALBA*

by

SHAKUNTALA SHARMA

(Under the Direction of Chung-Jui Tsai)

ABSTRACT

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) system has been used to efficiently induce targeted mutagenesis in a variety of organisms. This study assessed the efficiency and patterns of mutations in *Nucleoredoxin1* (*NRX1*) tandem genes by CRISPR/Cas9 in *Populus tremula* × *alba*. Successful mutation in at least one tandem gene has been achieved in nearly all the transgenic lines. The majority of mutations were single-base insertions or deletions, but large-fragment deletions of tandem genes were also detected. Several transgenic lines have lost all of the functional *NRX1* genes, and likely represent total knockouts. Preliminary analysis based on *NRX1.2* suggested that CRISPR/Cas9-editing outcomes have been stable in vegetatively propagated plants. Overall, this study demonstrated that tandemly duplicated genes can be efficiently edited by CRISPR/Cas9 using a single guide-RNA. The novel transgenic lines with various mutations should facilitate functional characterization of *NRX1* in *Populus*.

INDEX WORDS: CRISPR, Cas9, Nucleoredoxin1, genome editing, *Populus*, tandem duplicate

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SHAKUNTALA SHARMA

BS, Institute of Forestry, Tribhuvan University, Nepal, 2009

MS, University of Arkansas Monticello, 2014

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

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SHAKUNTALA SHARMA

Major Professor:	Chung-Jui Tsai
Committee:	Esther van der Knaap
	Caterina Villari

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION

Populus is an economically important tree that has a wide range of uses including pulp, paper and for bioenergy. *Populus* is a prominent tree species that has both huge natural reserves and plantation status in the world. In the United States, *Populus* is the fifth most abundant species in its natural occurrence. Two major plantations of *Populus* has been reported in the United States. The first consists of about 15,000 ha of eastern cottonwood (*Populus deltoids* Bartr. ex March) and was planted in the sixties in the lower Mississippi River Valley (Krinard and Johnson, 1984). In another instance, about 70,000 ha of hybrid poplar were planted in the Pacific Northwest (Stanturf and Zhang 2003). Outside of the United States, *Populus* is extensively planted in other countries. In China alone, 14% of plantation consists of *Populus* (Li et al., 2014; Wang et al., 2016).

Populus has a small genome size and contains abundant genetic diversity in natural populations (Bradshaw et al., 2000). The asexual propagation and interspecific hybridization in *Populus* are easier compared to other tree species (Bradshaw et al., 2000; Taylor, 2002). In addition, methods for genetic transformation and regeneration to create transgenic plants are well established and it has a shorter regeneration interval among tree species (Bradshaw et al., 2000; Taylor, 2002). A draft *Populus trichocarpa* genome was first published in 2006 (Tuskan et al., 2006); the genome has a total size of ~500 Mbp, with greater than 45,000 putative protein coding genes (Tuskan et al., 2006).

Nucleoredoxin1 and Its Role in Oxidative stress

Nucleoredoxins (NRX) belong to the thioredoxin superfamily of redox proteins. Thioredoxin superfamily includes Thioredoxin (TRX), Glutaredoxin (GRX), and Nucleoredoxin (NRX) subfamilies. They are involved in the reduction of the disulfide bridge of target proteins (Meyer et al., 2009). The TRX subfamily consists of a well-conserved WCG/PPC (Trp-Cys-Gly-Pro-Cys) motif that forms either a disulfide bond, or exists as a free thiol, depending on its oxidation state (Holmgren, 1985). TRX is reduced by thioredoxin reductases (Jacquot et al., 1994; Laloi et al., 2001; Rivera-Madrid et al., 1995). Reduced TRX provides reducing power for thiol-containing antioxidant proteins, which in turn protect the cell from oxidative stress by detoxifying ROS. Alternatively, TRXs can directly interact with target proteins by reducing disulfide bonds that may activate or deactivate the target proteins (Gelhaye et al., 2005).

GRXs, on the other hand, have a di-cysteine or mono-cysteine residue that is less conserved with variable surrounding amino acids (Rouhier et al., 2008). In plants, the main function of GRXs is deglutathionylation, which involves a reversible modification of disulfide bonds between glutathione and cysteine (Meyer et al., 2009). GRX is reduced by glutathione reductases.

NRX is a multi-domain protein, with three TRX-like WCG/PPC (Trp-Cys-Gly/Pro-Pro-Cys) domains (Marchal et al., 2014). NRX is reduced by NADPH dependent thioredoxin reductases (Marchal et al., 2014). NRX was first characterized in mice, where it interacts with Disheveled (Dvl), an essential adaptor protein for redox regulation of the Wnt β-catenin signaling pathway, for early growth and stem cell maintenance (Funato and Miki, 2010; Funato et al., 2006). Recent studies on plant *NRX* genes have shown the involvement of NRX in redox

regulation. In *Arabidopsis*, NRX1 maintains catalases in a reduced state (Kneeshaw et al., 2017) and in *Gossypium*, apoplastic NRX1 maintains the redox balance during stress (Li et al., 2016)

TRX is involved in the salicylic acid (SA)-mediated redox signaling in *Arabidopsis* through transcriptional control of many defense genes primarily by activating *Non-expressor of Pathogenesis-Related 1 (NPR1)*. SA also induces expression of at least two *GRXs* in *Arabidopsis* independent of *NPR1*, namely *GRXC9* and *GRXS13* (Blanco et al., 2009; Herrera-Vásquez et al., 2015). In *Arabidopsis*, *NPR1* and *TRX* genes (specifically the *AtTRXh3* and *AtTRXh5* duplicates) are central regulators of SA-mediated oxidative stress (Laloi, 2004; Sweat and Wolpert, 2007; Tada et al., 2008). Upon pathogen invasion, elevated SA activates the expression of cytosolic TRXh5/h3, which in turn modifies the redox state of NPR1 from an oligomeric to a monomeric form. NPR1 in its monomeric form moves into the nucleus where it activates the expression of defense genes (Kinkema et al., 2000; Tada et al., 2008).

Our lab previously engineered *Populus* with endogenously elevated SA to explore the beneficial possibilities of SA manipulation (Xue et al., 2013). Transgenic *Populus* plants overproducing SA exhibit metabolic fingerprints similar to heat-stressed wild type plants (Xue et al., 2013). Transcriptome profiling showed increased expression of orthologs of receptor-like protein kinases and WRKY transcription factors known to be involved in the defense response and SA signaling in *Arabidopsis*. Many oxidoreductases also showed increased expression, which are consistent with redox perturbation by stress and SA signaling. Genes encoding *NRX1*, the only redox genes among the member of the *TRX* superfamily, were found to exhibit an SA-dependent expression in transgenic *Populus. NRX1* expression has not been shown in *Arabidopsis* to increase after SA signaling; instead, SA-mediated defense signaling is dependent on the *TRX-h* subfamily. The genome of *Populus trichocarpa* lacks the orthologs of *Arabidopsis AtTRXh3/5*, rather it has

tandem duplicates of *NRX1* as compared to single-copy *Arabidopsis NRX1*. There are nine tandem duplicates in *Populus trichocarpa* and seven tandem duplicates of *NRX1* in *Populus tremula* × *alba*.

The ortholog of NPR1, which acts as a master regulator of SA-mediated systemic acquired resistance response in *Arabidopsis*, did not respond to variation of SA levels in transgenic *Populus*. Also, predicted *Populus* NPR1 proteins do not have the Cys residues required for redox modification or SA interaction for defense. This suggests that SA-related redox regulations differs between *Populus* and *Arabidopsis*, and that NRX1 could be a potential redox regulator in *Populus* (Xue et al., 2013).

Tandem Gene Duplication

Gene duplication provides raw materials for evolution (Woollard, 2005). It can take place in a gene, a segment of chromosome, whole chromosome, or even whole genome of a species. If a duplicated gene is right next to the original gene, then it is called a tandem duplicate (Leister, 2004). Tandem duplicates are the results of replication slippage, unequal crossing over, and adjacent single-strand break repair (Chen et al., 2005; Jugulam et al., 2014; Vaughn and Bennetzen, 2014).

A potential model suggests that during the replication process, DNA polymerase could dissociate from the template strand, which in turn stops the replication process. When polymerase reattaches to the DNA, it aligns in an incorrect position and copies the same section again (Chen et al., 2005; Viguera et al., 2001). During meiosis, chromosomes align precisely at the recombination site and crossovers do not change the number of genes in the recombinant chromosomes. However, misalignment of chromosomes results in unequal crossing over events that increase the copy of genes on one recombinant chromosome and decrease the copy of the

genes on the other recombinant chromosome, a process known as unequal crossing over (Anderson and Roth, 1981; Jugulam et al., 2014). Recent studies have shown that tandem duplication can also be caused by the long-patch mediated double strand break (DSB) formation followed by NHEJ repair (Schiml et al., 2016; Vaughn and Bennetzen, 2014). In *Arabidopsis* and rice, it was shown that whenever there are single-strand breaks (SSBs) close to one another on complementary strands, long repair patches are formed and repairing of SSBs results in tandem duplication (Schiml et al., 2016; Vaughn and Bennetzen, 2014).

Although whole genome duplication is the largest contributor of gene duplication, about 15-20% of genes in rice and *Arabidopsis* are tandemly duplicated (Rizzon et al., 2006). Tandem duplicated genes are mainly enriched in functional categories of genes associated with secondary metabolism, disease resistance, abiotic stress, and regulatory function (Kliebenstein et al., 2001; Meyers et al., 2005; Rizzon et al., 2006). Due to the nature of their origin, tandem duplicates often share a similar regulatory region and they are often expressed in a coordinated manner (Schmid et al., 2005). Furthermore, tandem duplicates homogenize each other through unequal crossing over or gene conversion. Consequences of homogenization among tandem duplicates lead to the divergence among non-recombining tandemly arrayed genes (Baumgarten et al., 2003).

CRISPR/Cas9 in Genome Editing

Clustered regularly interspaced short palindromic repeats (CRISPR) is a prokaryotic adaptive immune system. CRISPR locus consists of endogenous protein coding genes and array of short repeated sequences separated by spacers. Organisms that contain a CRISPR system incorporate DNA fragments from invading plasmids and bacteriophages, thereby creating a cellular memory. These incorporated sequences are transcribed into crRNA that recognizes complementary sequences when the same invading plasmid and bacteriophage re-infect. crRNA hybridizes with trans-activating crRNA (tracrRNA) to guide Cas nuclease to cleave the foreign DNA or RNA (Bolotin et al., 2005; Garneau et al., 2010; Rath et al., 2015). Based on differences in components and mechanism of actions, the CRISPR system can be classified into two major classes. The class 1 system includes type I, III, and IV, and has a large complex of several effector proteins. The class 2 system has type II, and V & VI, and it requires one RNA-guided endonuclease to cleave invading foreign DNA (Makarova et al., 2015; Shmakov et al., 2015). The defense mechanism of prokaryotes to invading foreign DNA or RNA involves three stages. First, the DNA fragments are acquired into the host CRISPR locus between crRNA repeats as spacers. In the second stage, Cas proteins are expressed and CRISPR array including spacers are transcribed into pre-crRNA. In the third stage, Cas proteins recognize the target DNA with the guidance of crRNA and they mediate cleavage of invading DNA (Deltcheva et al., 2011; Garneau et al., 2010; Hsu et al., 2014; Rath et al., 2015).

The class 2 type II CRISPR system is widely used for genome editing. A duplex of two RNAs guides Cas9 to cleave the target genome. In the type II system, constitutively expressed tracrRNA base pairs with a CRISPR repeat sequence on the pre-crRNA (Barrangou et al., 2007; Garneau et al., 2010; Jinek et al., 2012). Cas9 together with crRNA-tracrRNA duplex has been

exploited for efficient genome editing (Jinek et al., 2012). To simplify the CRISPR/cas9 system a crRNA and trcrRNA duplex are modified and fused into a chimeric single guide RNA (sgRNA).

This Cas9-sgRNA complex binds to the targeted genome sequence close to a protospacer adjacent motif (PAM) sequence. CRISPR/Cas9 can be designed to precisely target any region on the genome of interest that contains the PAM sequence and target sequence that is complementary to gRNAs (Cho et al., 2013; Cong et al., 2013; Jinek et al., 2012). Cas9 creates double strand breaks (DSBs) at the target site by cleaving three base pairs upstream of the PAM sequence (Hsu et al., 2014). The DSB is repaired by either the non-homologous end joining (NHEJ) pathway or the homology directed repair (HDR) pathway if a homologous template is available at the time of DSB repair. NHEJ repair is error prone, and can result in either insertions or deletions of nucleotides (Jinek et al., 2012; Moynahan and Jasin, 2010). CRISPR/Cas9 specificity for a target site recognition and cleavage is determined by the PAM sequence downstream of the target site and the 7-20 nucleotide sequence that is complementary to gRNA. Many mismatches in the PAM-distal region can be tolerated but not within the 8-12 nucleotides proximal seed sequences (Cong et al., 2013; Hsu et al., 2013; Mali et al., 2013) .

Before repurposing the CRISPR/Cas9 system for genome editing (Jinek et al., 2012), zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were the most commonly used genome editing techniques (Pabo et al., 2001; Wood et al., 2011; Xie and Yang, 2013). ZFNs and TALENs used in genome editing and genome targeting have been illustrated in several species including tobacco and maize (Mahfouz et al., 2011; Shukla et al., 2009; Townsend et al., 2009; Zhang et al., 2013).

ZFN, a fusion protein, consists of an array of zinc finger DNA-binding and DNA-cleavage domains of the bacterial FokI restriction enzyme (Gupta and Musunuru, 2014). Each zinc finger domain can recognize a 3-4 base pair sequence and tandem repeat domains can be designed to target a specific region of the genome (Gupta and Musunuru, 2014; Pabo et al., 2001). However, ZFN are expensive, time consuming to design, and have off-target effects (Bortesi and Fischer, 2015; Ma et al., 2016). Transcription activator-like effectors (TALEs) are proteins secreted by the plant pathogen *Xanthamonas.* TALENs are similar to ZFNs as TALE domains are fused to a FokI nuclease for target recognition and cleavage. TALEs contain tandem repeats, each consisting of 33-35 amino acids and two variable amino that can bind to one of the four nucleotides. TALENs are less expensive and easier to construct than ZFN (Cermak et al., 2011).

CRISPR/Cas9 is a better gene editing tool relative to ZFN and TALEN due to target design simplicity, efficiency, and multiplexed mutations (Bortesi and Fischer, 2015; Ma et al., 2016). CRISPR/Cas9 requires only a gRNA to target a genomic sequence. The DNA target recognition in CRISPR/Cas9 is based on RNA-DNA hybridization, whereas in TALENs and ZFNs it is based on protein-DNA interaction (Hsu et al., 2014).

Application of CRISPR/Cas9 in Plants

CRISPR/Cas9 has been extensively used for gene knockout, genome deletion, disruption of *cis* regulatory elements, and gene knock-in in plants, including *Arabidopsis* (Chen et al., 2017; Fauser et al., 2014; Feng et al., 2014; Gao et al., 2016; Mao et al., 2013; Xing et al., 2014), tobacco (Baltes et al., 2014; Nekrasov et al., 2013; Yin et al., 2015), rice (Liu et al., 2017; Sun et al., 2017; Xu et al., 2014; Zhang et al.; Zheng et al., 2016), maize (Char et al., 2017; Liang et al.,

2014; Xing et al., 2014; Zhu et al., 2016), and tomato (Hashimoto et al., 2018; Mahfouz et al., 2017; Tomlinson et al., 2018).

Similarly, CRISPR was successfully used in woody perennial species like *Populus* to knock-out 4-courmate:CoA ligase (4CL) genes involved in lignin and flavonoid biosynthesis (Zhou et al., 2015) and the phytoene desaturase gene involved in chlorophyll biosynthesis (Fan et al., 2015). Zhou et al. (2015) showed biallelic modification with 100% mutational efficiency that caused perturbation of lignin and flavonoid biosynthesis. Similarly, Fan et al. (2015) showed biallelic homozygous mutations that lead to albino phenotype in *Populus*.

The CRISPR/Cas9 system has also been used to target multiple genes by using a design that co-expresses multiple gRNAs in a single vector (Cong et al., 2013; Li et al., 2013; Zhang et al., 2016). Single gRNA was used to target duplicated genes in *Caenorhabditis elegans* (Xu et al., 2016). Additionally, Lv et al. (2016) used a single gRNA to delete a large-fragment of tandem repeats in human cells.

Research Motivation and Specific Objectives

Genetic perturbation of *NRX1* is necessary in order to test the hypothesis that *NRX1* acts downstream of SA to modulate redox-sensitive stress responses in *Populus*. By knocking out *NRX1* in SA-overproducing poplars, for example, it would be possible to assess SA-mediated oxidative stress responses in the presence or absence of *NRX1* to infer *NRX1* function. Therefore, as a first step toward functional characterization of *NRX1*, this study exploited the CRISPR/Cas9 system to target the *NRX1* tandem repeats in *Populus.* Although CRISPR/Cas9 has been used to knockout unlinked gene duplicate in plants, its application to tandemly arrayed genes has not been reported. The specific objectives were:

- To evaluate the knockout efficiency of *NRX1* tandem duplicates in *Populus* by CRIPSR/Cas9
- To examine the pattern and frequency of mutations in *NRX1* tandem genes caused by CRISPR/Cas9
- To assess the stability CRISPR/Cas9 editing outcomes overtime in clonally propagated plants using *NRX1.2* as a test case
- To determine the effects of *NRX1* expression in transgenic lines

CHAPTER 2

EXPERIMENTAL Methods

gRNA Construction for Cloning

The pUC-gRNA plasmid (Addgene #47024) (Jacobs et al., 2015) was used to insert the gRNA sequence. Reverse and forward primers that share 20 bp of the gRNA sequence (gRNA-F and gRNA-R) were designed. The pUC-gRNA plasmid was used as a template for PCR amplification of *Medicago truncatula* U6 promoter and scaffold DNA using high fidelity Q5 2X master mix. The U6 promoter region was amplified using MR and gRNA-R-tailed primers and the scaffold region was amplified using gRNA-F-tailed and pUCR primers at 98^0C for 30 sec; 25 cycles (98^0C for 10 sec, 60^0C for 15 sec, 72^0C for 30-45 sec); and 72^0C for 2 min. Amplified U6 promoter and scaffold were annealed and extended for 10 cycles after which outside primers MR and pUCR were added to the reaction, annealed and extended for 10-15 more cycles at 98^0C for 30 sec; 10 cycles (98^0C for 10 sec, 60^0C for 15 sec, 72^0C for 30-45 sec) and 72^0C for 2 min. The PCR products were digested with restriction enzymes EcoRV and EcoRI in 1X NEB buffer 3.1. The digested products were then gel-purified using 1% agarose and 1X TAE + cytidine gel with Zymoclean Gel DNA Recovery Kit (Zymo Research).

Plasmid Construction

The human codon-optimized P201 Cas9 plasmid (Addgene #59175) (Jacobs et al., 2015). Plasmid p201N was linearized with restriction enzyme *Spel* in 1X NEB buffer at 37^0C. The linearized product was ethanol precipitated with 0.1 volumes of 3M NaOAc and 2.5 volumes of 100% ethanol. The resulting product was then digested with restriction enzyme *SwaI* in 1X

buffer 3.1 at 25^0C. The digestion products were gel-purified using 1% agarose and 1X TAE + cytidine gel with Zymoclean^{TM} gel DNA recovery kit (Zymo Research).

Ligation and Transformation

The gRNA cassette was ligated into linearized p201N Cas9 plasmid in a reaction containing 1 µl of 10X ligation buffer, 0.5 µl of 10 mM ATP, 5 µl of p201N (~ 10 ng), 1 µl of insert, and 0.5 µl of ligase. The ligation mixture was incubated at 14^0C overnight. Transformation was carried out using 2 µl of ligation mixture mixed with 15 µl of competent *E.coli* on ice for 30 minutes. The mixture was then incubated at 42^0C for 30 seconds. Luria-Bertani (LB) broth (500 µl) was added and mixture was incubated at 37^0C for 5 hours with shaking (225 rpm). One hundred µl aliquot of the mixture was then plated onto LB agar media containing 100 mg/l of kanamycin and incubated overnight.

Colony PCR for Screening

After overnight incubation, the resulting colonies were screened using colony PCR. Selected *E.coli* colonies were suspended in 50 µl of colony lysis buffer that contained 100 µl of 1% of Triton X-100, 400 µl of 20 mM Tris, pH 8.5, 200 µl of 2 mM EDTA and incubated at 95^0C for 10 min. The supernatant was transferred to a new tube after centrifuging for 2 min at maximum speed. one µl of lysate was used for colony PCR with the gRNA-specific and p201N-Cas9 specific primers (Table 1) at 94^0C for 5 min; 30 cycles (94^0C for 30 sec, 55^0C for 45 sec, 72^0C for 1 sec); and 72^0C for 5 min. PCR products were run on a 1% agarose, 1X TBE gel and UV visualized. Positive colonies were cultured further for plasmid extraction and Sanger sequencing.

Transformation into Agrobacterium

The confirmed CRISPR/Cas9 expression construct was transformed into Agrobacterium. Five hundred ng of plasmid DNA were added to a tube of competent cells and incubated in ice for 15-30 min followed by snap freezing in liquid nitrogen. After 5 minutes in liquid nitrogen, cells were heat shocked at 37^0C for 5 min and incubated on ice for 5 minutes. One ml of LB was added to the resulting samples and incubated at 28^0C using rotating shaker for 3-4 hours. Fifty-two hundred µl of culture were plated on LB agar media containing 100 mg/l of kanamycin and plates were incubated for 2 days at 28^0 C. Colony PCR was used to confirm the transformation.

Transformation and Generation of Transgenic lines

Leaves from one month-old tissue culture propagated *Populus tremula* × *Populus alba* clone INRA 717-1B4 were used for *Agrobacterium*-mediated transformation (Meilan and Ma, 2006). *Agrobacterium* strains (carrying Cas9 and gRNA genes) were grown overnight in 5ml of LB medium with kanamycin at 28^0C shaker at 225 RPM. The following day, leaves were excised, cut into approximately 30 leaf discs, and placed in the callus induction media (CIM). One ml of Agrobacterium culture was added. Excess liquid was removed after 10 minutes and leaf discs were transferred to a dark closet. After 2 days, the discs were washed in water containing 200 mg/l of timentin, 100 mg/l of kanamycin, and 10 mg/l of hygromycin, transferred to CIM containing 200 mg/ml of T, 300 mg/ml of claforan (C), 100 mg/l of kanamycin, and 10 mg/l of hygromycin, and cultivated in dark until the calli appeared (within a month). An individual callus from each leaf discs was transferred to the shoot induction media (SIM) with 200 mg/ml of timentin, 300 mg/ml of claforan, 100 mg/l of kanamycin, and 10 mg/l of hygromycin.

After one month, individual shoots from each callus were transferred to the shoot elongation media (SEM) containing 200 mg/l timentin, 300 mg/ml of claforan, 100 mg/l of kanamycin, and hygromycin 10 mg/l and grown for a month. Elongated shoots were then transferred in the root induction media with 200 mg/ml of timentin, 50 mg/l of kanamycin, and 5 mg/l of hygromycin. All calli and plants in SIM were subcultured every two weeks whereas plants in SEM and RIM were subcultured every four weeks. Selected transgenic lines were transplanted into the soil and maintained in a greenhouse.

Screening of Transgenic Plants

Editing patterns in each of the seven tandem *NRX1* gene copies in the transgenic lines were analyzed using amplicon sequencing, qPCR, and PCR with gene specific primers. Genomic DNA was extracted from leaves of one-month-old transgenic lines as well as plants have been maintained in tissue culture for two years. Some represented lines were transferred to the greenhouse and propagated through cuttings. Leaves samples were also collected from these plants for DNA extraction.

Genomic DNA extraction

DNA was extracted following Dellaporta et al., (1983). Leaf samples were collected in 1.5 ml micro-centrifuge tubes and snap frozen in liquid nitrogen. The samples were ground under liquid nitrogen using a plastic mini-pestle. Seven hundred fifty µl DNA extraction buffer (50 mM Tris-HCl (pH8), 10 mM EDTA (pH 8), 100 mM NaCl, 1% SDS, and 10 mM β-mercaptoethanol) were added to the frozen powder followed by vortexing. The extract was incubated at 65^0C in a water bath for 20 minutes. A total of 200 µl of ice-cold 5M KOAc was then added to the extract, mixed well and incubated again on ice for 20 minutes. The samples were centrifuged for 10 minutes at 15,000 rpm to pellet DNA. Supernatant was discarded and the

pellet was washed twice with 500 µl of 70% ethanol, centrifuged at 15,000 rpm for 5 minutes, and dried in a speed vac. Finally, the dry pellet was dissolved in 50 µl of H_2O with 10 µg/ml of RNaseA to remove co-purifying RNA. DNA concentration was estimated by gel electrophoresis and comparison with band intensities of a DNA ladder.

Amplicon Sequencing

For amplicon sequencing, consensus primers that amplify 135 bp covering the gRNA target sites of all seven tandem genes were used. In the first round, the target region of interest was amplified using genomic DNA at 95^0C for 30 sec; 25 cycles (98^0C for 10 sec, 60^0C for 15 sec, 72^0C for 30-45 sec); and 72^0C for 5 min. In order to verify amplification, PCR products were run on a 1% agarose, 1X TBE gel and visualized on a UV transilluminator. The PCR products were diluted 1:100, and used as templates for a second PCR at 95^0C for 3 min; 10 cycles (98^0C for 15 sec, 60^0C for 15 sec, 72^0C for 30-45 sec); and 72^0C for 10 min. The second PCR was used to add the final Illumina adapters and barcodes in each of the 59 samples. The resulting PCR products from all 59 samples were pooled and concentrated with DNA clean and concentrator columns (Zymo Research). The pooled samples were run on 1.5% agarose, 1X TAE + cytidine gel and the desired fragments were gel extracted with the Zymoclean Gel DNA Recovery Kit and sent for sequencing. The sequencing data were analyzed using AGESeq following Xue and Tsai (2015).

PCR with Gene Specific Primers

Gene specific primers were designed for each of the seven tandem genes (Table 1). PCR was performed using the gene specific primers listed in the table and PCR products were run on a 1% agarose, 1X TBE gel and visualized on a UV transilluminator.

RNA Extraction

Leaf tissues from seven transgenic lines including wild types were collected from three month-old greenhouse grown plants. Three replicates of each transgenic line were used. Leaf tissues were grounded in liquid nitrogen using a mortar and pestle. Total RNA was extracted with a Direct-Zol extraction kit (Zymo Research) using Plant RNA Reagent (Life Technologies) and Nanodrop™ quantified following manufacturer's instructions. Samples were homogenized by adding 500 µl of plant RNA reagent and vortexed. Samples were incubated at 50^0C for 15 minutes and cooled on ice. Hundred µl of chloroform was added to the samples and centrifuged for 5 minutes. Five hundred fifty µl of supernatant (aqueous phase) was transferred into the new tube with 100 µl of NaCl and 500 µl of 100 % ethanol. The samples were vortexed, transferred to a column and briefly centrifuged. RNA samples were then washed with 400 µl of RNA wash buffer. Eighty µl DNase I cocktail (5 µl of DNase I, 8 µl of DNase buffer and 64 µl of wash buffer) were added to samples and incubated at 37^0C for 15 minutes. RNA pellets were washed twice with 400 µl pre-wash buffer, once with 700 µl of RNA wash buffer and centrifuged at 13000 rpm speed for 1 minute to dry the pellet. The pellet was suspended in 30 µl RNase free ddH_2O and RNA was allowed to rehydrate for 5-10 minutes. RNA quality and concentration were measured with gel electrophoresis and nanodrop.

Quantitative Real Time PCR (qRT-PCR)

cDNA synthesis was carried out using high capacity cDNA reverse transcription kit according to the manufacturer's instructions (Thermo Fisher Scientific, USA). Reactions were set up by mixing 5 µl of master mix (1µl of 10 x RT buffer, 0.4 µl of 25x dNTP, 1µl of 10X RT random primers, 0.5 µl of RNase inhibitor, and 1µl of multiscribeTM reverse transcriptase) with 5µl of 500 ng RNA and incubated at 25^0C/10 min, 37^0C/60 min, 85^0C/5 min.

Real-time PCR was carried out using three biological replicates and 2 technical replications with the Mx3005P QPCR system (Agilent). Reactions were performed in a final volume of 10 µl containing 5 µl of cDNA (1ng), 500 nM 0.25 µl of each forward and reverse primers (Table 1) and 5 µl of Absolute^{TM} QPCR SYBR© Green and ROX mix (Thermo Fisher Scientific, USA) at 95^0C/15 min, 40 cycles (95^0C/30 sec, 60^0C/15 min, 72^0C/1 min, 95^0C/1min, 35^0C/30 sec, 95^0C/30 sec). MxPro QPCR software (Agilent) was used to analyze fluorescence data. Expression levels were calculated relative to the housekeeping gene (Table 1) using the comparative threshold cycle method, $\Delta Ct = \Delta Ct_{\text{gene of interest}} - \Delta Ct_{\text{housekeeping gene}}$, where Ct represents the threshold cycle for target amplification. The $2^{\Delta CT}$ method was used to analyze the relative changes in gene expression from RT-qPCR (Livak and Schmittgen, 2001). Analysis of variance was used to analyze the gene expression data in R.

CHAPTER 3

RESULTS

NRX1 Tandem Duplicates

Populus trichocarpa has nine tandem duplicates of *NRX1*. These duplicates are highly similar, with identity between duplicates ranging from 89 to 99 %. *Populus tremula* × *alba* has eight tandem duplicates of *NRX1*; seven full length (*NRX1.1 to NRX1.7*) and one pseudogene *NRX1.8* with no start and stop codons. It also has pseudogene *NRX1.9* with no start and stop codons in another chromosome. They are also very similar to each other, and identity between *Populus tremula* × *alba NRX1* duplicates ranges from 73 to 97 %.

gRNA design

Two synthetic gRNAs were designed for *NRX1* mutagenesis. The first one (g1) was selected to target all full-length tandem genes but with mismatches to the pseudogene *NRX1.8* (Figure 1). The second one (g2) was chosen to target seven tandem genes, including the pseudogene (Figure 2). gRNAs target sites are located in the first exon of *NRX1* genes.

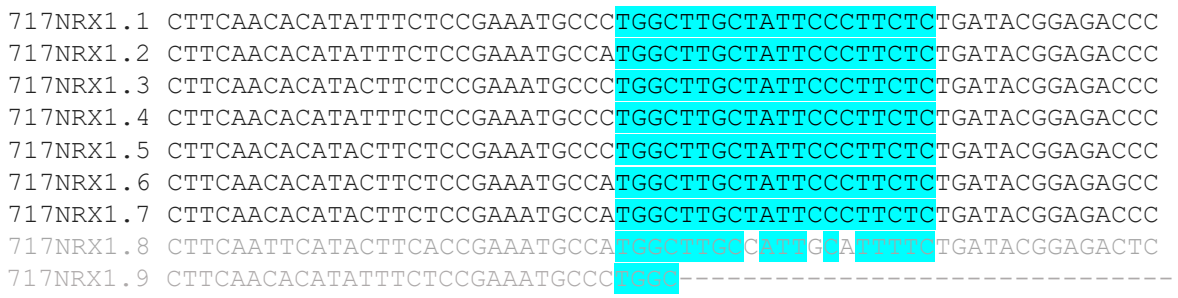


Figure 1: Alignment of *NRX1* tandem genes showing the g1 target sites

Figure 2: Alignment of *NRX1* tandem genes showing the g2 target sites

Generation of Transgenic Plants

The two *NRX1*-targeting CRISPR/Cas9 constructs were transformed into *Populus tremula* × *alba* wild-type and transgenic SA over-producing plants. Three transgenic SA over-producing lines (F10, F55, and F52) were made available from a previous study (Xue et al., 2013).

After *Agrobacterium*-mediated transformation of leaf discs, calli were produced from the g1 construct and whole plant regeneration was obtained in all four backgrounds. However, no calli were produced from the g2 construct in two independent transformation trials. Hereafter, all results were based on the g1 construct that is predicted to target all seven full length *NRX1* tandem genes. A total of 59 transgenic lines were generated from different genetic backgrounds;

15 in wild type (group A), 13 in F10 (group B), 16 in F52 (group C), and 15 in F55 (group D) (Table 2). No phenotypic abnormalities were observed in the transgenic plants.

Confirmation of Transgenes Using PCR

The CRISPR-g1 construct to target *NRX1* genes was based on a vector backbone having an *nptII* as a selectable marker. SA-overproducing plants have *hptII* as a selectable marker (Xue et al., 2013). Therefore, representative transgenic lines from group A were tested with *nptII* gene- specific primers and representative transgenic lines from groups B, C, and D were PCR screened with both *nptII*- and *hptII*-specific primers to confirm the presence of transgenes. The PCR confirmation of transgenes in the genomic DNA of representative lines is shown in Figure 4 and Figure 12. Amplification of *nptII* gene in all transgenic lines confirmed the presence of the *NRX1*- targeting CRISPR/Cas9-g1 T-DNA. Amplification of *hptII* gene in double transgenic lines confirmed the presence of the SA synthase containing T-DNA.

CRISPR/Cas9-Induced Mutation Patterns and Frequencies

The amplicon sequencing data were analyzed to determine the mutation patterns and frequencies induced by the CRISPR/Cas9 system (Figure 5). A variety of mutations were observed, including insertions of one or two nucleotides and deletions of varying lengths. A high proportion (64%) of mutations were deletions, and the majority of them were single-base deletions (38%), followed by single-base insertions (34%) (Figure 5). Of the 59 independent transgenic lines, 17, 17, 4, 16, 3, 12, and 7 lines had 90 to 100% indel mutations in the *NRX1.1, NRX1.2, NRX1.3, NRX1.4, NRX1.5, NRX1.6, and NRX1.7* genes, respectively (Table 3)

CRISPR/Cas9 Efficiency on Tandem Gene Mutations

PCR was carried out to assess if any of the tandem genes were deleted in transgenic lines. Because g1 was predicted to target all *NRX1* tandem genes, simultaneous cleavage at two or more sites would lead to large-fragment deletions. PCR was carried out using consensus primers that amplify all *NRX1* genes (Table 1). As shown in Figure 6, three bands were detected in the wild type; the middle band is *NRX1.2,* the top band represents all other *NRX1* genes, and the bottom band is a truncated *NRX1.9* in another chromosome, lacking start and stop codons and g1 target sequence. Many CRISPR lines such as A1, A2, A4, C5-2, C6-1, C10, and C2-2 failed to amplify the middle band, suggesting potential *NRX1.2* deletions (Figure 6). Some other lines such as A6, C7, A9-2, D1-1, and D5-1 showed weak or no amplification of the upper band or altered sizing of the top two bands compared to the wild type (Figure 6). In order to strengthen these results, PCR on selected transgenic lines with gene-specific primers was carried out. Of the 32 lines analyzed, *NRX1.1, NRX1.2, NRX1.3, NRX1.4, NRX1.5, NRX1.6, and NRX1.7* were absent in 11, 18, 21, 14, 21, 20, and 16 lines, respectively.

The *NRX1.2* gene-specific PCR results matched the results from the PCR carried out using consensus primers, including two lines where the top band was absent and the middle band appeared smaller in size (A9-1 and A6). Several transgenic lines exhibited patterns indicative of consecutive tandem gene deletions. Two, five, 10, two, and seven transgenic lines failed to amplify six, five, four, three, and two consecutive tandem gene fragments, respectively. For instance, transgenic line A6 was PCR-positive for only *NRX1.1*, suggesting a large-fragment deletion of consecutive tandem genes from *NRX1.2 to NRX1.7*. On the other hand, transgenic line C5-2 might have lost *NRX1.1* and *NRX1.2* (Table 4).

Comparison of both PCR and amplicon sequencing results showed that many genes were successfully mutated by small indels or large-fragment deletions in some transgenic lines (Table 4). For instance, PCR results showed that *NRX1.2, NRX1.5,* and *NRX1.6* were absent in transgenic line A8, and amplicon sequencing results showed that the remaining four tandem genes had 100% mutations. Similarly, amplicon results showed 93% mutations in *NRX1.4* and *NRX1.7* in line A9, with the remaining five *NRX1* tandem genes absent based on PCR. In another instance, transgenic line A10 lost *NRX1.3* based on PCR results, and exhibited 98% mutations of *NRX1.1*, *NRX1.2*, *NRX1.4*, *NRX1.5*, and *NRX1.6* based on amplicon sequencing. Taken together, transgenic lines A8, A9, A9-2, C2-2, and D1-1 might have lost all functional copies of *NRX1* based on the above results (Table 4).

Monitoring of *NRX1.2* Modification in Vegetatively Propagated Plants

To assess the stability of the *NRX1* tandem gene editing in vegetatively propagated transgenic plants, NRX1.2-specific PCR was performed on samples that have been vegetatively propagated for two years. Deletions detected by gene-specific PCR in the original transformants have been stable after two years. Of the 29 lines analyzed, NRX1.2 was absent in 17 of them from both sampling times (Figure 7).

Expression of *NRX1* in Transgenic Lines

The *NRX1* expression levels were determined using qRT-PCR in seven transgenic lines grown in a greenhouse. These seven lines were selected to represent different rates of mutations based on amplicon sequencing data. Significantly reduced levels of *NRX1* expression were detected in transgenic lines A9-1 and A10 compared to WT (Figure 8).

CHAPTER 4

DISCUSSION

Based on gene-specific PCR, CRISPR-induced mutations occurred in all except one of the transgenic lines analyzed. Although the pattern and extent of mutations varies among the lines, CRISPR/Cas9 editing was highly efficient in causing targeted mutations. The efficiency of CRISPR/Cas9 in *Populus* 4-coumarate: CoA ligase (4CL) gene family has been previously reported (Tsai and Xue, 2015). *Populus* plants are diploid, and one or both alleles of the target gene may be cleaved by CRISPR/Cas9, generating three possible outcomes in transgenic lines, including homozygote, heterozygote, and chimera (Feng et al., 2014). Several studies have shown 100 % biallelic mutations in *Populus* (Zhou et al., 2015), tomato (Brooks et al., 2014) and rice (Xie et al., 2015; Zhou et al., 2014). Based on amplicon sequencing data and gene-specific PCR, nearly all transgenic lines harbored mutations of at least one *NRX1* tandem genes, suggesting a mutational efficiency approaching 100%.

Most of the CRISPR/Cas9-induced mutations are small insertion or deletions (Feng et al., 2014; Pan et al., 2017; Zheng et al., 2016). Most common mutations identified in this study were single-base deletions, followed by single-base insertions, consistent with the results previously reported (Pan et al., 2016). However, 1-bp insertions were predominant in *Arabidopsis* and rice (Feng et al., 2014; Lv et al., 2016). The *NRX1* target sites are located in the first exon, so most of the deletions and insertions are predicted to frame-shift and loss-of-function of the genes. Several transgenic lines lost as many as six consecutive tandem genes (*NRX1.2, NRX1.3, NRX1.4, NRX1.5, NRX1.6,* and *NRX1.7).* It is possible that the whole fragment from *NRX1.2* to *NRX1.7* was deleted in those lines. When a gRNA targets multiple genes close to each other on the same

chromosome, a large-fragmental deletion has been reported in several studies (Li et al., 2013; Lv et al., 2016; Mao et al., 2013; Xie and Yang, 2013; Zhou et al., 2014). For large-fragment deletions to occur, simultaneous cleavages have to occur at the two farthest target sites or multiple consecutive individual genes need to be cleaved at the same time.

Many transgenic lines had lower rates of indel mutations compared to other lines based on amplicon sequencing data. However, PCR using either consensus primers for all *NRX1* genes or gene-specific primers showed that those transgenic lines had lost several genes. This suggests that the fragments of *NRX1* tandem genes were deleted in those lines.

This study performed a preliminary assessment of the stability of CRISPR modifications in vegetatively propagated transgenic plants because *Populus* requires multiple years before sexual reproduction is feasible, and because clonal propagation is the most common method of regeneration in *Populus*. Therefore, it is important to determine stability of mutations in vegetatively propagated *Populus.* Initial results based on *NRX1.2* suggested that the CRISPR/Cas9-mediated changes, based on presence or absence of the gene-specific PCR amplicon, have been stable in vegetatively propagated *Populus*.

New mutations were reported in the subsequent generation of CRISPR-mutated *Arabidopsis*, rice, and soybeans via sexual reproduction (Feng et al., 2014, 2014; Ma et al., 2015; Pan et al., 2017). Additional analysis by amplicon sequencing and with additional gene-specific primers of greenhouse poplar samples will provide information on whether additional indels had occurred by the continuous modification of intact genes by the gRNA-Cas9 complex during vegetative propagation.

NRX1 expression was significantly reduced in some transgenic lines compared to the wild type. However, it was not significantly different among the transgenic lines analyzed, although those lines had different rates of mutations and different numbers of missing tandem genes. This analysis was based on two or three replicates of transgenic lines. In the future, qRT-PCR with more replications of transgenic lines will be needed to verify the *NRX1* expression data.

This study has generated transgenic lines with a series of mutations in *NRX1* tandem genes, affecting one to all seven tandem genes. Transgenic lines A8, A9, A9-2, C2-2, and D1-1 had the most severe mutations potentially lacking functional *NRX1* genes. These materials will be valuable for the functional characterization of *NRX1* in *Populus* in the future.

CHAPTER 5
CONCLUSION

This study showed that it is possible to mutate multiple tandemly duplicated and highly homologous genes by CRISPR/Cas9 using a single guide-RNA. Successful mutation in at least one *NRX1* duplicates has been achieved in nearly all of the transgenic lines, either in the form of gene deletion or small indels. Furthermore, knockout of all *NRX1* tandem genes was achieved in some transgenic lines. Small indels in individual genes as well as large-fragment deletions of tandem genes were also detected. Preliminary analysis suggested that the PCR amplification patterns of *NRX1.2* in a subset of transgenic lines analyzed have been stable in vegetatively propagated plants. These results demonstrate that CRISPR/Cas9 is an efficient tool for creating targeted genome modifications at multiple sites simultaneously, making it ideally suited for mutagenesis of highly repetitive regions. This study also generated novel mutants that will facilitate future research with *Populus NRX1*.

Table 1: Primers used in this study for gRNA construction, cloning, colony PCR, amplicon sequencing and to amplify specific tandem genes

Forward primer (5' to 3')	Reverse primer (5' to 3')	Purpose
MR: GTTTTCCCAGTCACGACGTTGTA	NRX19gR282R: TGGCTTGCTATTCCCTTCTCAAGCC TACTGGTTCGCTTGA	gRNA construction (one)
NRX19gR282F: GAGAAGGGAATAGCAAGCCAGTTTTAGAG CTAGAAATAGCAAGTT	pUC-R:CGCACAGATGCGTAAGGAGAAA	gRNA construction (one)
MR: GTTTTCCCAGTCACGACGTTGTA	NRX19gR444R: ACTTCAACCTTGATAGACTGACAA GCCTACTGGTTCGCTTGA	gRNA construction (two)
NRX19gR282F: GAGAAGGGAATAGCAAGCCAGTTTTAGAG CTAGAAATAGCAAGTT	pUC-R:CGCACAGATGCGTAAGGAGAAA	gRNA construction (two)
StubiP-R:ACATGCACCTAATTTCACTAGATG	NRX19gR282R: TGGCTTGCTATTCCCTTCTCAAGCC TACTGGTTCGCTTGA	Colony PCR positive (one)
NRX19gR282F: GAGAAGGGAATAGCAAGCCAGTTTTAGAG CTAGAAATAGCAAGTT	StubiP-R:ACATGCACCTAATTTCACTAGAT G	Colony PCR negative (one)
StubiP-R:ACATGCACCTAATTTCACTAGATG	NRX19gR444R: ACTTCAACCTTGATAGACTGACAA GCCTACTGGTTCGCTTGA	Colony PCR positive (two)
NRX19gR444F: GTCAGTCTATCAAGGTTGAAGTTTTAGAGC TAGAAATAGCAAGTT	StubiP-R*:ACATGCACCTAATTTCACTAGA TG	Colony PCR negative (two) *used for sequencing
NRX(217F)tailF: CCTACACGACGCTCTTCCGATCTGAGGTGG TSTTCRTTTCTTCTGA	NRX(350R)tailR: GTTCAGACGTGTGCTCTTCCGATCC CTCTTACTTTGAAYAMTTYCT	Amplicon sequencing
PtNrx(1241): FTGCCTTGGTTAGCCCTTCCATTTG	PtNrx(1380)R: TGTCARGTGCWTCCGAGCTTCCTT	qPCR
APR(476)F:ACTGTGAGGAGATGCAGAAACG CA	ARP(679)R: GCTGTGTCACGGGCATTCAATGYT	qPCR (housekeeping gene)
NRX1.1-4(699)F: TGATTCGTTAAGGGCTGATGTT	NRX1.15(1839)R (test new)	NRX1.1
NRX1.1-4(699)F: TGATTCGTTAAGGGCTGATGTT	NRX1.2(1095)R	NRX1.2
NRX(217)F: GAGGTGGTSTTCRTTTCTTCTGA	NRX1.2(1095)R	NRX1.2
NRX1.1-4(699)F: TGATTCGTTAAGGGCTGATGTT	NRX1.37(1435)R: TCACAGCACATCGTATGGATT	NRX1.3
NRX(217)F:	NRX1.37(1435)R: TCACAGCACATCGTATGGATT	NRX1.3

Forward primer (5' to 3')	Reverse primer (5' to 3')	Purpose
NRX1.1-4(699)F: TGATTCGTTAAGGGCTGATGTT	NRX1.43(1875)R	NRX1.4
NRX1.1-4(699)F: TGATTCGTTAAGGGCTGATGTT	NRX145(1387)R	NRX1.4
NRX1.14(1295)F	717NRX1.R3: GATGYTCCTCGGTAAATGGAA	NRX1.4
NRX1.5(776)F	NRX1.5(1399)R : ATCTGCCATTACAGATAGCAAG	NRX1.5/ NRX1.3
NRX(1-5)F	NRX1.5(1399)R: ATCTGCCATTACAGATAGCAAG	NRX1.5
NRX(217)F: GAGGTGGTSTTCRTTTCTTCTGA	NRX1.5(1399)R: ATCTGCCATTACAGATAGCAAG	NRX1.5
NRX1.67(709)F: TTTGTTTACCCTCTTRATTTAWGGG	NRX1.6(1409)R: CACATCCKATGASCATRSGCTG	NRX1.6
NRX(217)F: GAGGTGGTSTTCRTTTCTTCTGA	NRX1.6(1404)R: CCKATGASCATRSGCTGAAA	NRX1.6
NRX1.67(709)F: TTTGTTTACCCTCTTRATTTAWGGG	NRX1.6(1404)R: CCKATGASCATRSGCTGAAA	NRX1.6
NRX1.67(709)F: TTTGTTTACCCTCTTRATTTAWGGG	NRX1.37(1435)R: TCACAGCACATCGTATGGATT	NRX1.7
NRX(217)F: GAGGTGGTSTTCRTTTCTTCTGA	NRX1.37(1435)R: TCACAGCACATCGTATGGATT	NRX1.7
717NRX1.F6: GCAATTGTTCTAGCMTTTTGAGT	717NRX1.R3: GATGYTCCTCGGTAAATGGAA	all NRX1 genes
NRX1.1-4(699)F: TGATTCGTTAAGGGCTGATGTT	NRX1.14(1359)R: CATTACAGATAGCCAAAAAGGCA	NRX1.1/1.4
NRX1.1-4(699)F: TGATTCGTTAAGGGCTGATGTT	NRX1.2(1095)R	NRX1.2
NRX1.1-4(699)F: TGATTCGTTAAGGGCTGATGTT	NRX1.37(1435)R: TCACAGCACATCGTATGGATT	NRX1.3
NRX1.5(776)F	NRX1.5(1399)R / 1.3 too: ATCTGCCATTACAGATAGCAAG	NRX1.5
NRX1.67(709)F: TTTGTTTACCCTCTTRATTTAWGGG	NRX1.6(1404)R: CCKATGASCATRSGCTGAAA	NRX1.6
NRX1.67(709)F: TTTGTTTACCCTCTTRATTTAWGGG	NRX1.37(1435)R: TCACAGCACATCGTATGGATT	NRX1.7
717NRX1.F6: GCAATTGTTCTAGCMTTTTGAGT	717NRX1.R7: AAGTTCGTGYTTCACTTTCTCTG	all NRX1 genes
NRX(217)F2:GARKTGGTBTTCATTTCTTCTGA	NRX1.1(1837)R :CATGCGACGTTAGCAGAG	NRX1.1
NRX1.1-4(699)F: TGATTCGTTAAGGGCTGATGTT	NRX1.2(1105)R: TGGCCAATTATGACAAGATTAGGG	NRX1.2
NRX1.1-4(699)F: TGATTCGTTAAGGGCTGATGTT	NRX1.37(1435)R: TCACAGCACATCGTATGGATT	NRX1.3
NRX1.1-4(699)F: TGATTCGTTAAGGGCTGATGTT	NRX1.14(1359)R: CATTACAGATAGCCAAAAAGGCA	NRX1.4
NRX1.5(698)F: TTTATGATTAAGAAAAAGCTCCCT	NRX1.5(1399)R : ATCTGCCATTACAGATAGCAAG	NRX1.5
NRX1.67(709)F: TTTGTTTACCCTCTTRATTTAWGGG	NRX1.6(1409)R: CACATCCKATGASCATRSGCTG	NRX1.6

Table 2: Transgenic lines generated in each of the wild type and SA overproducing plants. F10, F52, F55 are SA-overproducing plants (Xue et al., 2013)

Background	**Lines**
Wild type (A)	15
F10 (B)	13
F52 (C)	16
F55 (D)	15

Table 3: CRISPR/Cas9 induced mutation rates in transgenic plants (Indels %)

Backgrounds	Lines	NRX1.1	NRX1.2	NRX1.3	NRX1.4	NRX1.5	NRX1.6	NRX1.7
Wild-type	A1	25	25	35	28			43
	A10	97	98		98			
	A11-1	52	73		52			
	A12-2	47	69		60		51	54
	A2			58				59
	A7		10					
	A7-2		29	25				23
	A8	97	100	99	90			100
	A9	100	91	100	93			97
	A9-2	77	62	54	73		49	89
F10	B-3	98	97		98		97	
	B-5	92	94		94		94	
	B-7	93	97	98	92	98	97	96
	B14-1	98	99	45	98			93
	B2	94	97		92		95	
	B2-2	99	100		100		99	
	B3-1	98	98		98		97	
	B4-1	92	92		96		93	
	B4-2	96	98		98		96	
	B5-1	99	99		98		98	
	B6-1	95	98		98		97	
F52	C1	100	100		99		99	
	C2-2	94	96		96	95		100
	C3-2		99			98	100	99
	C4			100				
	C9				36			

Table 4: CRISPR/Cas9 induced mutations in transgenic plants. ID: small indels seen in amplicon sequencing results, D: gene failed to amplify in PCR, Y band present in PCR, F: faint band present in PCR,

Background	Lines	NRX1.1	NRX1.2	NRX1.3	NRX1.4	NRX1.5	NRX1.6	NRX1.7	No.Genes
Wild-type	A1	Y(25%)	D	D	D	D	Y	43	4
	A2	Y	D	D	D	D	D	59	5
	A3	Y	Y	D	F	Y	Y	Y	1
	A4	D	D	Y	D	D	D	D	6
	A5	D	D	Y	D	D	D	D	6
	A6	Y	D	D	D	D	D	D	6
	A7	Y	D	Y	Y	D	Y	D	3
	A7-2	D	D	F (25%)	Y	D	D	D	5
	A8	D	D	ID	ID	D	D	ID	7
	A9	D	D	D	ID	D	D	ID	7
	A9-2	D	D	D	D	D	D	D	7
	A10	ID	ID	D	ID	F	Y	Y	4
	A11-1	50	ID	Y	D	D	D	D	5
	A12-2	47	ID	D	60	D	D	54	4
	A21-1	F	D	D	Y	D	Y	Y	3
F10	B2	ID	ID		ID	ID	ID	ID	6
	B2-2	ID	ID		ID	ID	ID	ID	6
	B-3B	ID	ID		ID	ID	ID	ID	6
	B3-1	ID	ID		ID	ID	ID	ID	6
	B4-1	ID	ID		ID	ID	ID	ID	6
	B4-2	ID	ID		ID	ID	ID	ID	6
	B-5	ID	ID		ID	ID	ID	ID	6
	B5-1	ID	ID		ID	ID	ID	ID	6
	B6-1	ID	ID		ID	ID	ID	ID	6
	B7	ID	ID	ID	ID	ID	ID	ID	6
	B-9		D						1
	B9-2		D						1
	B14-1	ID	ID	45	ID			ID	4
F52	C1	ID	ID	D	ID	Y	ID	Y	5
	C2-2	ID	D	D	D	ID	D	ID	7
	C3-2		ID			ID	ID	ID	4
	C4			ID					1
	C5-2	F	D	D	Y	Y	Y	Y	2
	C6-1		D						1
	C7	D	Y	D	D	D	D	D	6
	C8-1								
	C9	Y	Y	D	D	D	D	Y	4
	C10		D						1
	C12-1		D						1
	C13-1								
	C15-2								
	C16-2	Y	Y	D	D	Y	D	D	4
	C17-1	D	Y	D	D	D	D	Y	4
	C18	D	D	F	F	D	D	F	4
F55	D1	Y	Y	Y	Y	Y	Y	Y	
	D1-1	F	D	F	D	D	F	D	4
	D2	Y	D		Y	Y	D	F	2
	D3		D						1
	D4		D						1
	D5								
	D5-1	F	Y	D	D	Y	D	D	4
	D6	F	D	D	F	Y	Y	D	3
	D6-1	D	Y	D	Y	D	D	D	5
	D7		D						
	D8	D	D	D	Y	D	Y	D	5
	D10-2		D						1
	D11-1	Y	D	Y	F	F	Y	F	1
	D12-1	D	D	Y	Y	D	Y	Y	3
	D13-2								1
	D23	F	D	D	Y	F	D	D	4

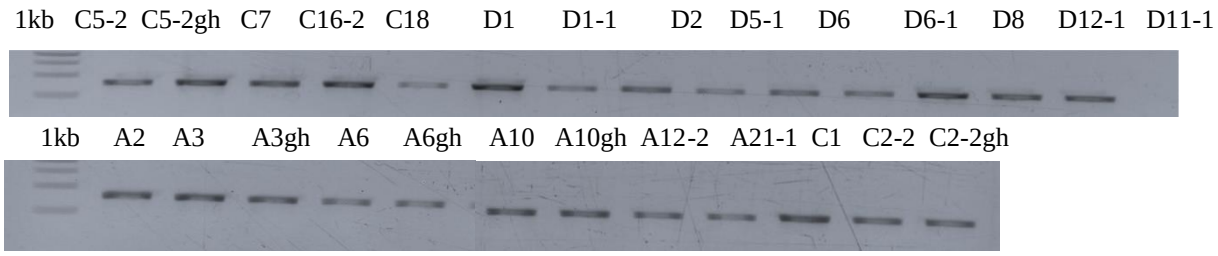


Figure 3: Transgenic lines were confirmed by PCR amplification of genomic DNA using *nptII* gene-specific primers

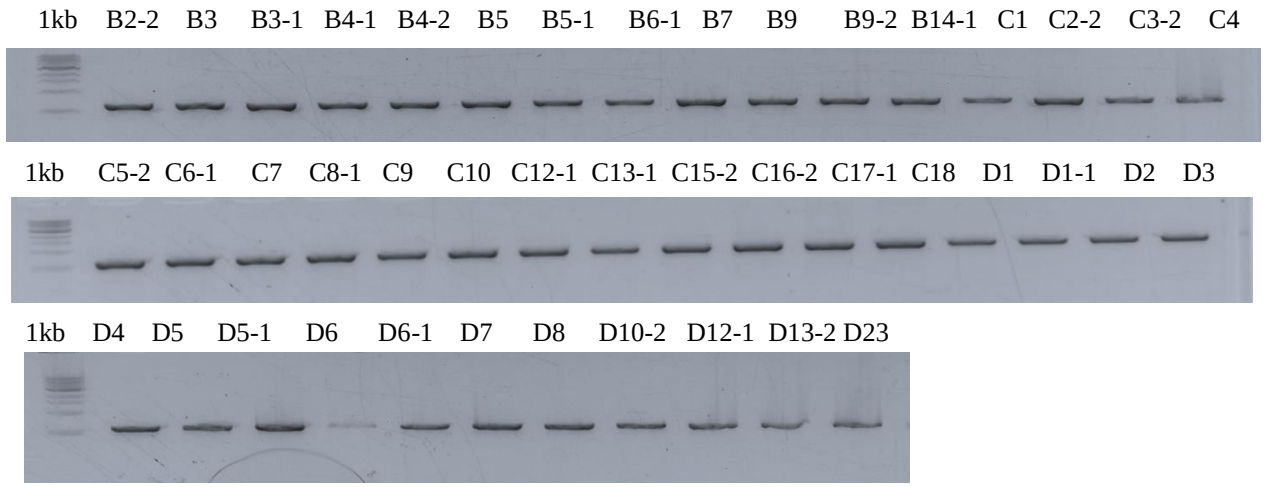


Figure 4: Transgenic lines were confirmed by PCR amplification of genomic DNA using *hptII* gene-specific primers.

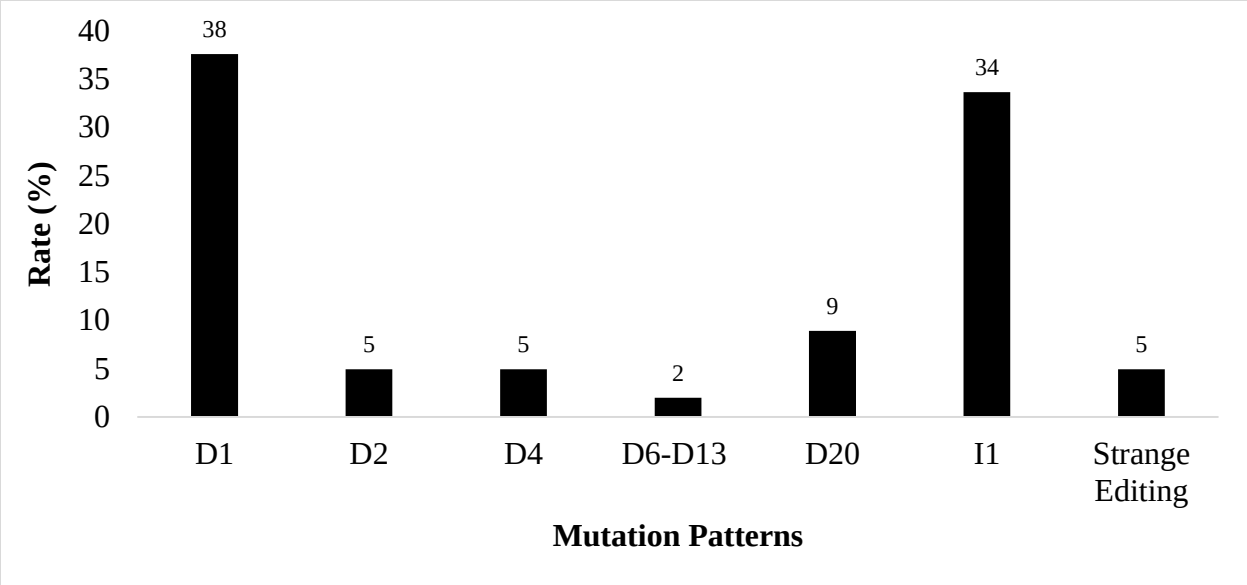


Figure 5: Patterns and frequency of mutations induced by CRISPR/Cas9. Insertion (I), deletion (d) in 19 lines

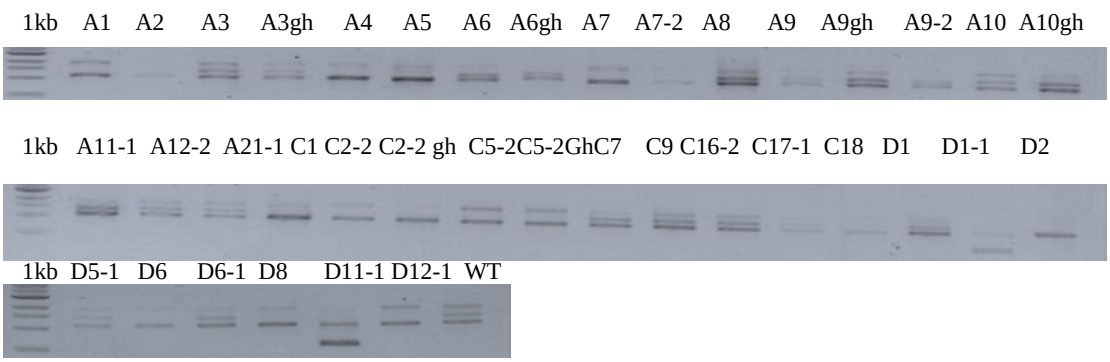
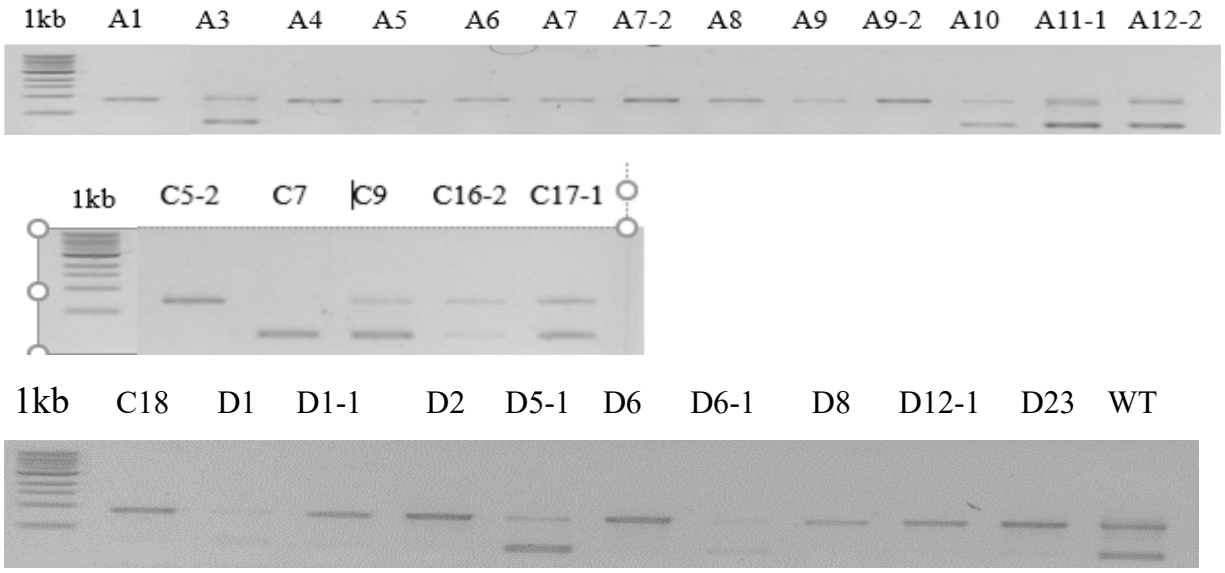


Figure 6: Detection of *NRX1* genes in transgenic lines using primers that amplify all *NRX1* tandem genes.gh: samples collected from greenhouse

(a)



(b)

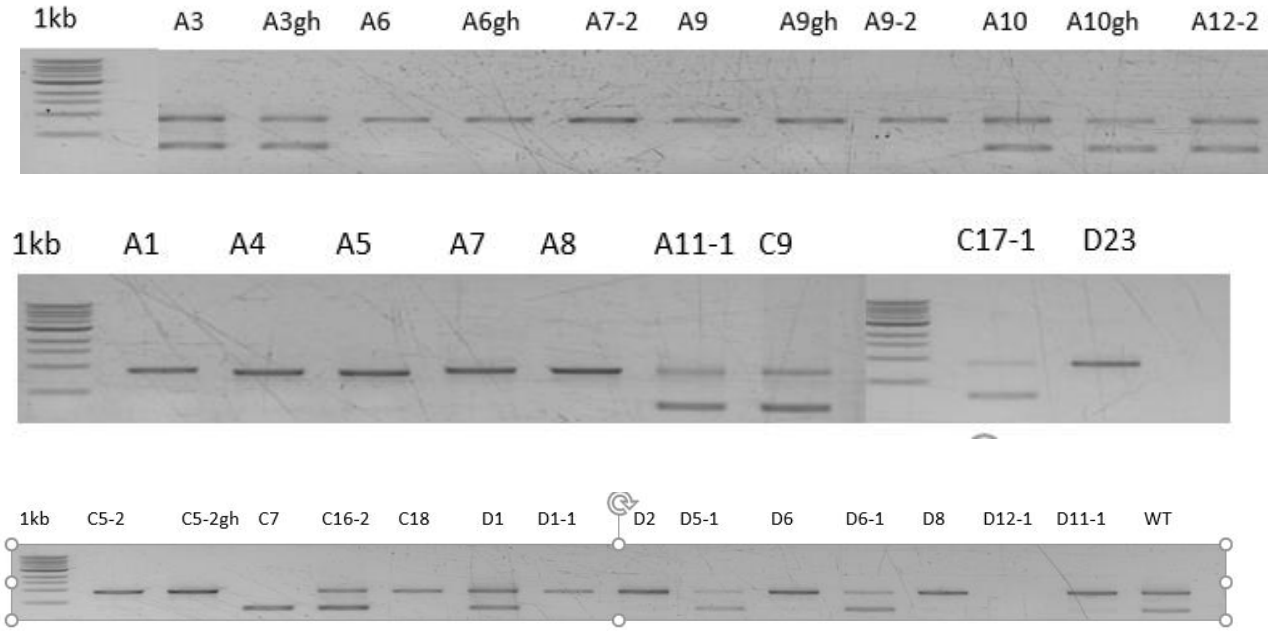


Figure 7: Detection of *NRX1.2* genes in transgenic lines a) in original transformants and b) in vegetatively propagated transgenic lines after two years. gh represents samples collected from greenhouse.

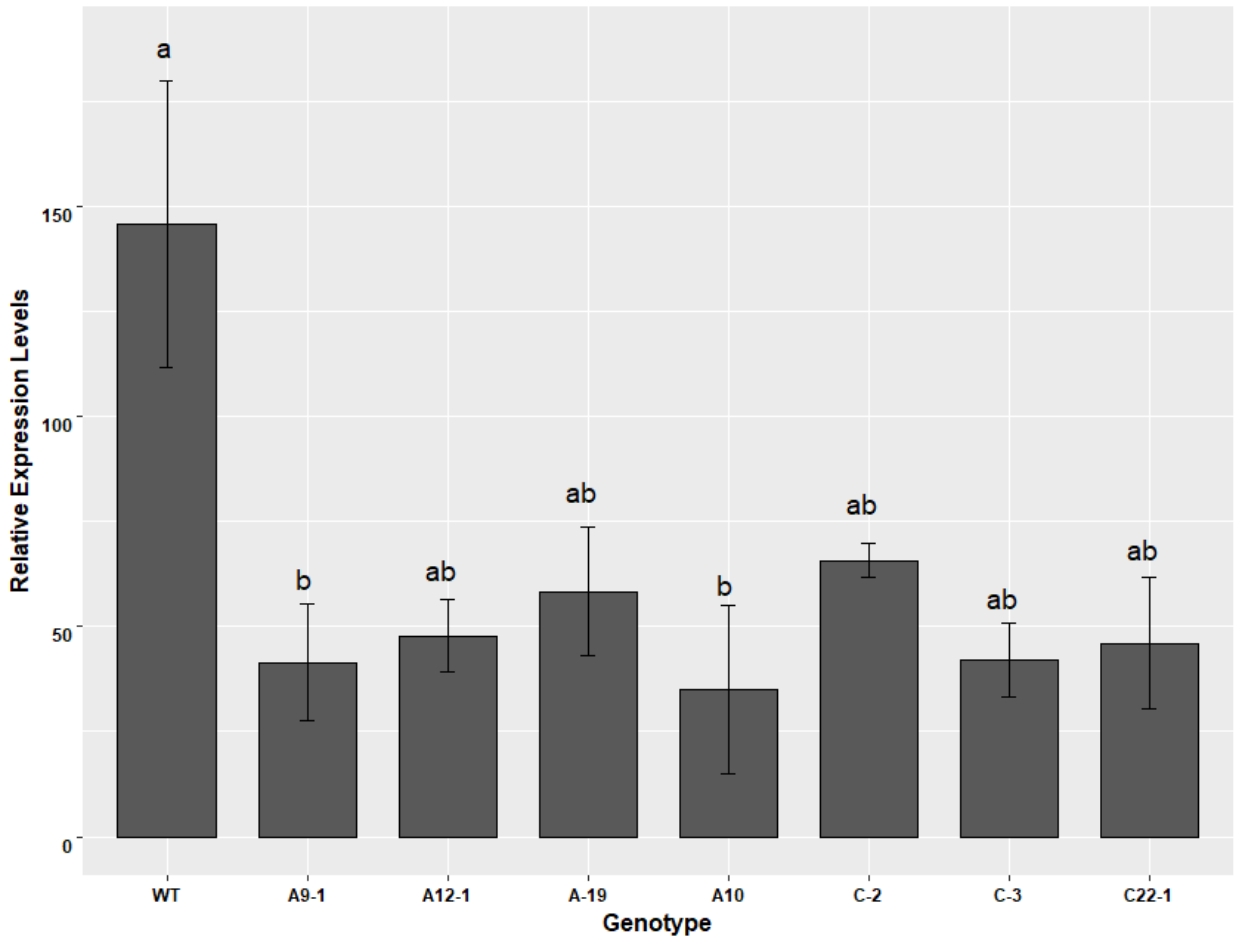


Figure 8: qPCR analysis of *NRX1* gene expression in transgenic lines. Bars represents mean ± SD expression of at least three biological replicates, except A12-1, C3 and C22-1, which included two biological replicates

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