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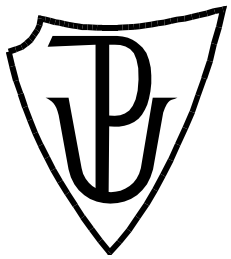

Selection and Screening of Rice Knock-out Lines with Altered Cytokinin Metabolism

BACHELOR THESIS

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I hereby declare that this thesis has been written solely by myself and that all the sources used in this thesis are cited and included in the references part.

In Olomouc 22th August 2013

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Olga Ryparová

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Abstrakt	Pro studium vlivu cytokininů na rostlinu byla vybrána rýže (*Oryza sativa*), která patří mezi organismy s přečteným genomem a jsou dostupné její různé mutantní linie s T-DNA inzercí. V teoretické části jsem se zaměřila na různé způsoby přípravy „loss of function" linií rostlin - inzerce T-DNA, RNAi, zinc-finger nukleáz a TAL nukleáz. Praktická část práce byla zaměřena na screening linií rýže z generace T1 a T2, jejichž mutace byla předpokládána v oblasti genů cytokinin dehydrogenas (CKX3 a CKX11), cytochrom P450 72A1 (CYP450) a isopentenyltransferasy (IPT10). Screening byl proveden pomocí metody PCR a byly vyselektovány heterozygotní a homozygotní linie, ze kterých byla získána semena pro budoucí studium fenotypu.
Klíčová slova	cytokininy, CYP450, IPT, CKX, "loss-of-function", rýže, PCR screening, knock-out linie
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Abstract	Rice (*Oryza sativa*); whose genome was fully sequenced, annotated and its T-DNA insertion lines are available, was chosen to study the influence of knock-out of specific genes involved in cytokinin metabolism. In the theoretical part, I focused on methods for preparation of loss of function mutant lines – T-DNA insertion, RNAi, zinc-finger nucleases and TAL nucleases. In the experimental part of bachelor thesis, I worked on generations T1 and T2 of rice insertion lines. Mutations were expected to occur in the gene regions of cytokinin dehydrogenases (CKX3 and CKX11), cytochrome p450 72A1 (CYP450) and isopentenyl transferase (IPT10). The screening of mutant lines was performed by PCR. Heterozygote and homozygote knock-out lines were selected and their seeds were collected for further studies.
Keywords	cytokinins, CYP450, IPT, CKX, loss-of-function, rice, PCR screening, knock-out lines
Number of pages	60
Number of appendices	–
Language	English

Aim of work

- review on preparation of loss of function lines with focus on T-DNA insertion, RNAi, zinc-finger nucleases and TAL nucleases
- optimization of growing conditions for rice plants
- selection of transformed plants in T1 and T2 generation and verification of T-DNA insertion in expected genes
- screening and selection of knock-out lines with altered cytokinin metabolism

Theoretical part

Techniques to generate mutants for the analysis of gene function in plants

In molecular biology, there are currently available two approaches to the analysis of gene function: loss-of-function and less conventional gain-of-function mutagenesis.

Genomes of various organisms have been completely sequenced and annotated putative genes provide a great quantity of information. The biological function of some genes was identified based on homology to genes of known function and also by functional assays (Wesley et al, 2001). To investigate function of remaining genes, researchers must have applied the approach of reverse genetics – analysis of what phenotypes arise as a result of loss-of-function of specific genes.

Firstly, loss-of-function mutants were generated by chemical treatments, which introduce random mutations into the genome (Ostergaard & Yanofsky, 2004). Later techniques include transposon and T-DNA tagging technology which use tag sequences to identify disrupted genes. The progress in biotechnology enables researches to use more precise techniques and tools for genome editing, such as zinc finger nucleases. In the theoretical part, I cover loss-of-function methods used to generate modified plants with disrupted specific genes including T-DNA insertion, RNAi silencing, zinc finger nucleases and TAL nucleases.

T-DNA insertion

Transformation by Agrobacterium tumefaciens

Plant cell posses an important feature – totipotency. It enables the production of all differentiated cells in mature plant from a single cell. It brings a great advantage for the genetic engineering because individually modified cells regenerate an entire organism. *Agrobacterium tumefaciens* is a soil bacterium causing a crown gall disease and naturally infects dicotyledonous plants. A. tumefaciens is capable of tumor induction as it carries a large tumor-inducing plasmid (Ti plasmid, Fig. 1.a). Ti plasmid contains two essential parts for the plant transformation - T-DNA with left and right border and *vir* region. During the infection, T-DNA is randomly integrated into the plant genome (Fig. 1.b); moreover, multiple insertions may occur. Afterwards, the cell starts to proliferate and forms tumors. As the energy source, cells use synthesized opine (depending on the strain, it can be nopaline or octopine, Fig. 1.c). In a typical Ti plasmid, there are

enzymes for the hormone synthesis (responsible for tumor proliferation) and enzymes for a successful T-DNA transfer (Brown, 2010).

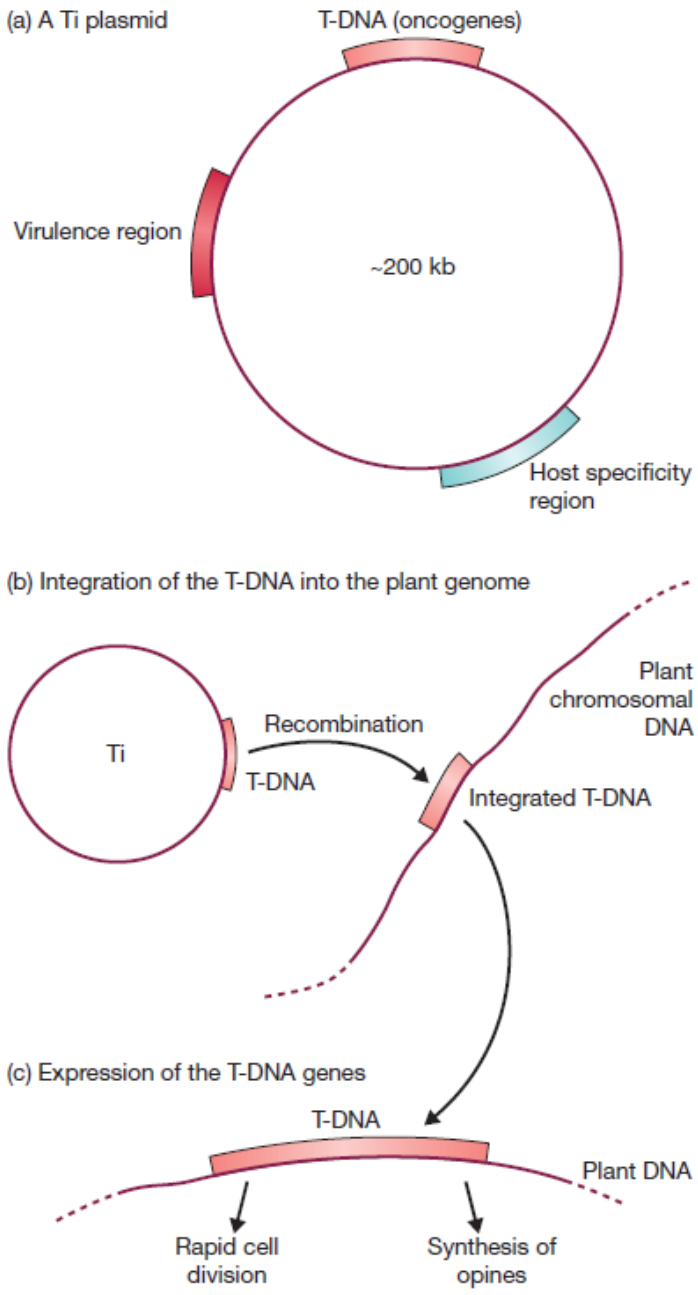


Figure 1: Transformation by Ti plasmid a) Ti plasmid map; b) T-DNA integration; c) Expression of T-DNA genes (taken from Brown, 2010).

Molecular biologist can insert genes into the T-DNA region which will be then transferred into the cell. In such modified vectors, genes for tumor growth are deleted and the gene coding an enzyme for deactivation of kanamycin, from the *E. coli* transposon Tn5 with the cauliflower mosaic virus (CaMV) 35S promoter and the Ti

nopaline synthase termination sequence is added as a selectable marker gene. There is a plenty of modified Ti vectors routinely used to transform various species of dicots (Snustad et al., 2012). Scientists also developed strategies for transformation of monocots (Slater et al., 2003). There is another strategy using different type of plasmid. Transformation based on Ri (root-inducing) plasmid of *Agrobacterium rhizogenes* is used for expression of large amount of proteins in plants. Instead of forming crown galls, hairy roots are formed and proteins are secreted into the medium (Brown, 2010).

Strategies for DNA insertion into the plant

Ti plasmid offers a great way of introducing a new DNA sequence into the plant genome. In practice, integration from Ti plasmid showed itself as a tricky part because manipulation with such big plasmids (~ 200 kb in size) is rather difficult. In natural Ti plasmid it is almost impossible to find a unique restriction site. Two strategies have been developed to overcome the difficulty:

The binary vector strategy (Fig. 2)

"Binary" refers to a system with two plasmids because T-DNA sequence does not have to be necessarily attached to the rest of the plasmid. T-DNA is a part of a small plasmid (~ 20 kb) and the second plasmid is in a normal form. This system is also observed in nature in some strains of *Agrobacterium*. The small plasmid is easier to manipulate and contains unique restriction sites. If both vectors are carried by a single *A. tumefaciens*, they complement each other and the integration of foreign DNA is successful (Brown, 2010).

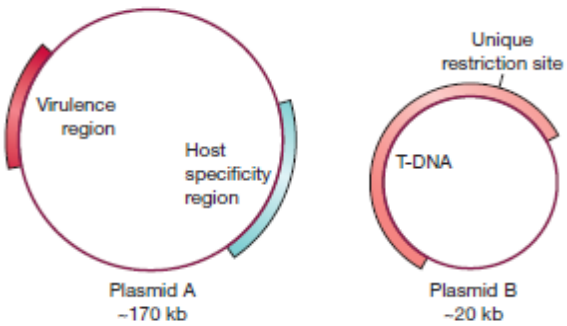


Figure 2: The binary system strategy. The T-DNA of a small plasmid is transferred to the plant chromosomal DNA by enzymes coded by genes on a bigger plasmid. Taken from Brown et al., 2010.

The co-integration strategy (Fig. 3)

This approach uses *Escherichia coli* based plasmid with a part of T-DNA region. If it is carried together with Ti plasmid in one *A. tumefaciens* cell, *E. coli* plasmid will be integrated into the Ti plasmid due to the homology recombination.

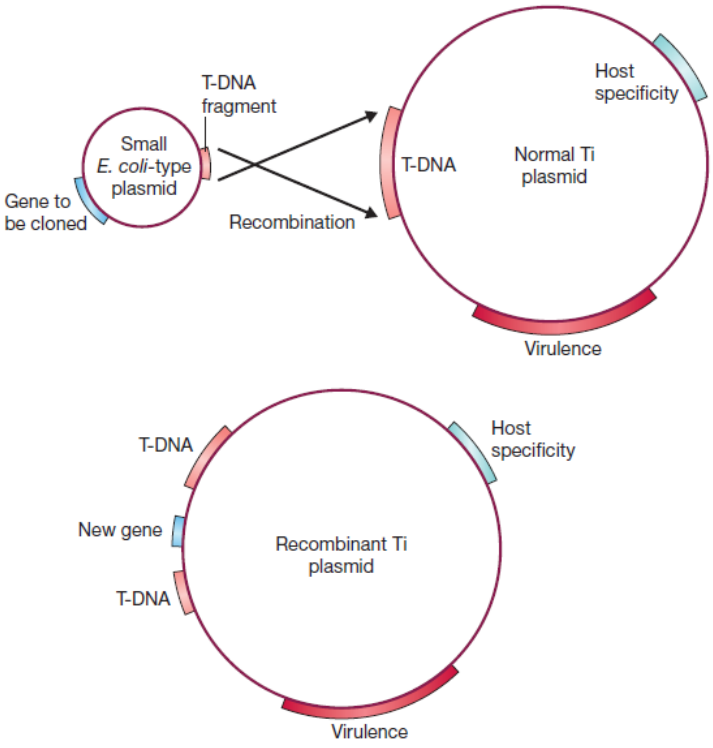


Figure 3: The co-integration strategy. The gene to be cloned is in an E. coli-type plasmid with unique restriction sites and is integrated into the Ti plasmid. During the infection, the new gene is inserted into the chromosomal DNA with the rest of T-DNA. Taken from Brown et al., 2010.

Generation of loss-of-function mutants

When T-DNA region is inserted into the plant DNA, a particular gene might be disrupted what leads in majority of cases to its improper expression. Similarly, genetic elements called transposons can change a location within the genome and doing so, they may disrupt the gene function as in the case of T-DNA insertion. Exploitation of both principles generates knock-out mutants. Once a single or multiple copy of T-DNA is integrated into DNA, it is possible to identify its location in a particular gene or genes, respectively. Currently, there are available thousands of mutants in various plants, including crops (Snustad et al., 2012). The databases with knock-out lines of rice are enlisted in table 1.

Table 1: Sequence index-tagged mutant resources in rice. The information on different sequence index-tagged mutant resources is integrated in the genome browsers of the Rice Annotation Project Database (RAP-DB) (http://rapdb.dna.affrc.go.jp/) and the Salk Institute Genomic Analysis Laboratory (SIGnAL) (http://signal.salk.edu/). Taken from Kuromori et al., 2009.

Insertion type	Resource name	Institution	Numbers mapped to genome	Website for the resource	Reference
T-DNA	POSTECH	POSTECH (Korea)	84,680	http://www.postech.ac.kr/life/pfg/risd/index.html	Jeon et al., 2000
T-DNA	RDM	Huazhong Agricultural University (China)	15,971	http://rmd.ncpgr.cn	Zhang et al., 2006
T-DNA	TRIM	Academic Sinica (Taiwan)	11,646	http://trim.sinica.edu.tw/	Hsing et al., 2007
T-DNA	SHIP	SIPPE (China)	6,761	http://ship.plantsignal.cn/index.do	Fu et al., 2009
T-DNA	ZJU	Zhejiang University (China)	714	http://www.genomics.zju.edu.cn/ricetdna.html	Chen et al., 2003
T-DNA, Tos17	OTL	CIRAD-Genoplante (France)	28,324	http://urgi.versailles.inra.fr/OryzaTagLine/	Sallaud et al., 2004
TOS17	NIAS	NIAS (Japan)	17,937	http://www.dna.affrc.go.jp/database/	Miyao et al., 2003
Ds, Spm	UCD	University of California Davis (USA)	13,666	http://www-plb.ucdavis.edu/Labs/sundar/Rice Genomics.htm	Kolesnik et al., 2004
Ds	CSIRO	CSIRO (Australia)	589	http://www.pi.csiro.au/fgrttpub/	Eamens et al., 2004
Ds	GSNU	Gyeongsang National University (Korea)	1,046	not available	Kim et al., 2004
Ds	EU-OSTID	A consortium of seven laboratories (EU)	1,301	http://orygenesdb.cirad.fr	van Enckevort et al., 2005

Advantages

Plant transformation mediated by *A. tumefaciens* has become the most used method to introduce novel genes into plants. Once the transformation protocol is established, nearly any plant can be transformed. A large amount of rice loss-of-function mutants with T-DNA insertion is available (Kuromori et al., 2009). Tag sequences within the Ti plasmid enable the identification of the interrupted genes (Kondou et al., 2010).

Disadvantages

Both A. tumefaciens (carrying Ti plasmid) and A. rhizogenes (carrying Ri plasmid) naturally infect only dicots. Monocotyledonous plants include majority of important crops, but techniques for their transformation based on Agrobacterium infection were developed later and are not very efficient. Monocots also showed difficulties in the whole plant regeneration after the transformation. This problem was overcome with the use of biolistics techniques when the plasmid is introduced into the embryo by bombardment with microprojectiles. The foreign gene is integrated into the plant chromosome randomly.

Many plant genes belong to the gene families and a single gene disruption does not need to reveal a gene function, because mutants do not show clear phenotypes due to complementation by other paralogous genes (Kondou et al., 2010). Single knock-out

lines for paralogous genes can be crossed to to test for phenotypes but it may involve time-consuming generating of crosses with specific mutant alleles.

RNA-mediated silencing

Early research

RNA interference (RNAi) was observed for the first time in 1990 after introducing a chimeric chalcone synthase (CHS) gene under control of strong constitutive promoter into petunia with pigmented petals. Purpose of the experiment was to darken flowers of transgenic plants by overexpression of pigment producing gene - CHS. Nonetheless, the introduction resulted in the suppresion of the homologous gene and white flowers were produced. The level of petal mRNAs of CHS was 50-fold lower than in WT plants, although there was no alteration in the developmental timing of mRNA expression of CHS (Napoli et al. 1990).The mechanism of this phenomenon was heavily studied in years following after original observations. Similar results were found in other organisms and early research on gene silencing in transgenic plants was reviewed in the paper (Matzke & Matzke, 1995).

Discovery

In 1998, A. Fire and C. Mello injected various forms of RNA encoding mex-3 gene into worm *Caenorhabditis elegans*. Mex-3 mRNA is known to accumulate in worm gonads and embryos. The probes were designed to label and quantify amount of mex-3 mRNA. Those embryos, which were injected with anti-sense mex-3 RNA (ssRNA complementary to mex-3 mRNA) and those, which were not injected at all, were labeled. However, embryos with injected RNA were not labeled as intensively as controls. Whereas embryos injected with ds mex-3 RNA were not labeled at all. Therefore, double-stranded RNA is a strong silencer and single-stranded antisense RNA has weaker effect on the silencing of the gene expression. In this experiment, it was proved for the first time that RNA interference is induced by dsRNA (Fire et al., 1998). Both scientists were awarded Nobel Prize for the discovery in 2006.

RNA-mediated gene silencing has been termed RNA interference. It is an evolutionary conserved mechanism of the regulation of gene expression which can be understood as an ancient part of immune system. In genetic engineering it was developed as a powerful tool for silencing of one or more genes in various eukaryotic organisms, including crops. In modern breeding techniques, it can result in creating genetically

modified plants with enhanced agronomical traits, such as virus resistance, improved storage capacity and health benefits (Mansoor et al., 2006).

Antisense RNA

The first commercial exploitation of antisense RNA was performed before the actual discovery of RNA interference mechanism. Polygalacturonase is an enzyme which hydrolyzes components of the tomato cell walls. It is a part of the ripening process. An antisense strategy was used to reduce the PG activity and thus to slow-down the process of ripening. The PG gene from tomato was isolated and cloned. Recombinant cDNA was later used to make a gene which coded for a complementary RNA to PG mRNA. The gene was attached in a reverse position to a promoter in Ti plasmid. Afterwards, the vector was inserted into tomato protoplasts.

When the antisense RNA was expressed, the production of PG was blocked and tomatoes exhibited longer shelf life than do common tomatoes (Sheehy et al., 1988).

Mechanism of RNA silencing pathways

RNAi is a process where small non-coding RNA molecules, 21 – 28 nucleotides long short interfering RNAs (siRNAs) or microRNAs (miRNAs), are processed from longer double-stranded RNA (dsRNA) by dicers. Dicers are double-stranded RNA-specific endonucleases and can trigger specific RNA degradation. In the cytoplasm, small dsRNA molecules are incorporated into ribonucleoprotein particles and one of the strands is eliminated. Then, siRNAs or miRNAs are base-paired with a target sequence in mRNA. It leads to the prevention of gene expression in a homology dependent fashion. The RNA-protein particle formed is named RNA-induced Silencing Complex (RISC). RISC cut the target mRNA which is then degraded. When cleavage occurs, the RNAs involved are usually referred to as siRNAs. Some of the siRNAs are derived from RNA viruses or from transcription of transposons and transgenes. This is a powerful way to protect against viral infections or escape transpositions. Whenever RISC pairs imperfectly with a target mRNA, the translation of mRNA is inhibited. The RNAs involved are termed miRNAs. Non-coding genes for miRNAs are present in genomes. This mechanism is called post-transcriptional gene silencing (PTGS; Snustad et al., 2012)

There is another mechanism of RNA-induced silencing at the level of transcription called transcriptional gene silencing (TGS). TGS is a type of heritable silencing and plays a role in epigenetics and it is referred to as RNA-directed DNA methylation (RdDM). RdDM involves siRNAs which guide cytosine methylation at DNA regulatory

sequences. Therefore DNA is inaccessible to transcriptional machinery. The hypothesis of siRNAs involved in DNA methylation is widely accepted. However, recent studies suggested that RdDM may not always be triggered by small RNAs but also long ones. It is also not clear whether single or double stranded RNA are the guide RNA and if they target nascent transcript or DNA. Further studies are necessary to clarify the mechanism. (Dalakouras & Wassenegger, 2013)

Transgene-induced RNAi

Since 90's genetically modified plants have been designed to generate dsRNA to initiate silencing of homologous genes. It was shown that dsRNA will be produced if transgene includes gene sequence in an inverted repeat orientation around a spacer region. Typically, if the trigger sequence in a designed construct forming dsRNA bears homology to the promoter of a target gene, TGS is initiated by methylation of promoter region. To initiate PTGS, the trigger sequence should be homologous to the coding region of a target gene. In such case, the expression of the transgene will result in dsRNA that bears homology to intended mRNA (McGinnis, 2010).

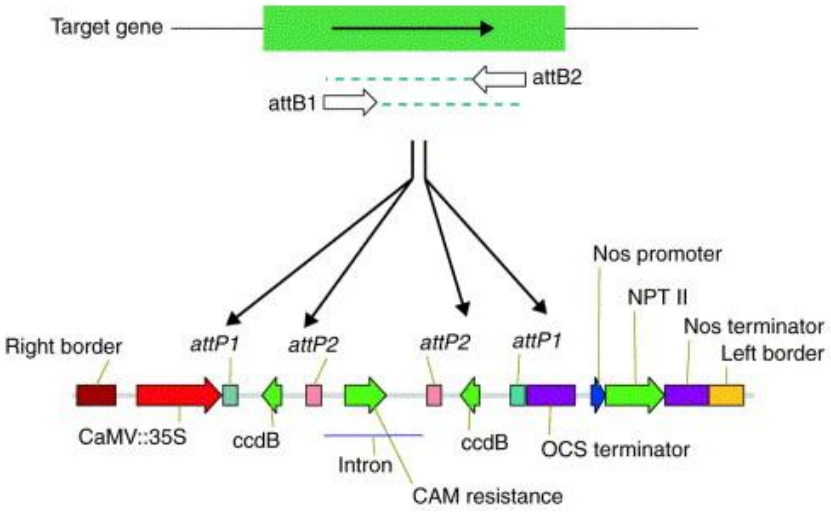


Figure 4: Map and cloning strategy for pHELLSGATE. aatB1, aatB2 – recognition sites, attP1, attP2 sites for recombination of one sense-oriented and one anti-sense-oriented molecule (forming two arms of hairpin), ccdB gene coding for gyrase inhibitor, lethal in standard *E. coli* strains,CAM resistance - chloramphenicol-resistance gene. The scheme taken from Wesley et al., 2001.

In the study from 2001, Wesley and co-workers demonstrated that effective silencing in plants can be done by designing constructs bearing self-complementary "hairpin" RNA (hpRNA; Wesley et al, 2001). These constructs encoding sense/anti-sense sequences, usually 100 to 900 nucleotides long, silence its target effectively in various plants. The

method gives nearly 100 % plants showing the silencing. Researchers designed vector pHELLSGATE (Fig. 4) in which the single PCR product from a targeted gene can be converted into hpRNA and can be transformed into *Agrobacterium tumefaciens*.

Another possibility is to synthesize dsRNA with nucleotide sequence of the gene to be silenced *in vitro*. This step is followed by injection of the syntesized dsRNA into the organism (Snustad & Simmons, 2012). Both approaches are depicted below (Fig. 5).

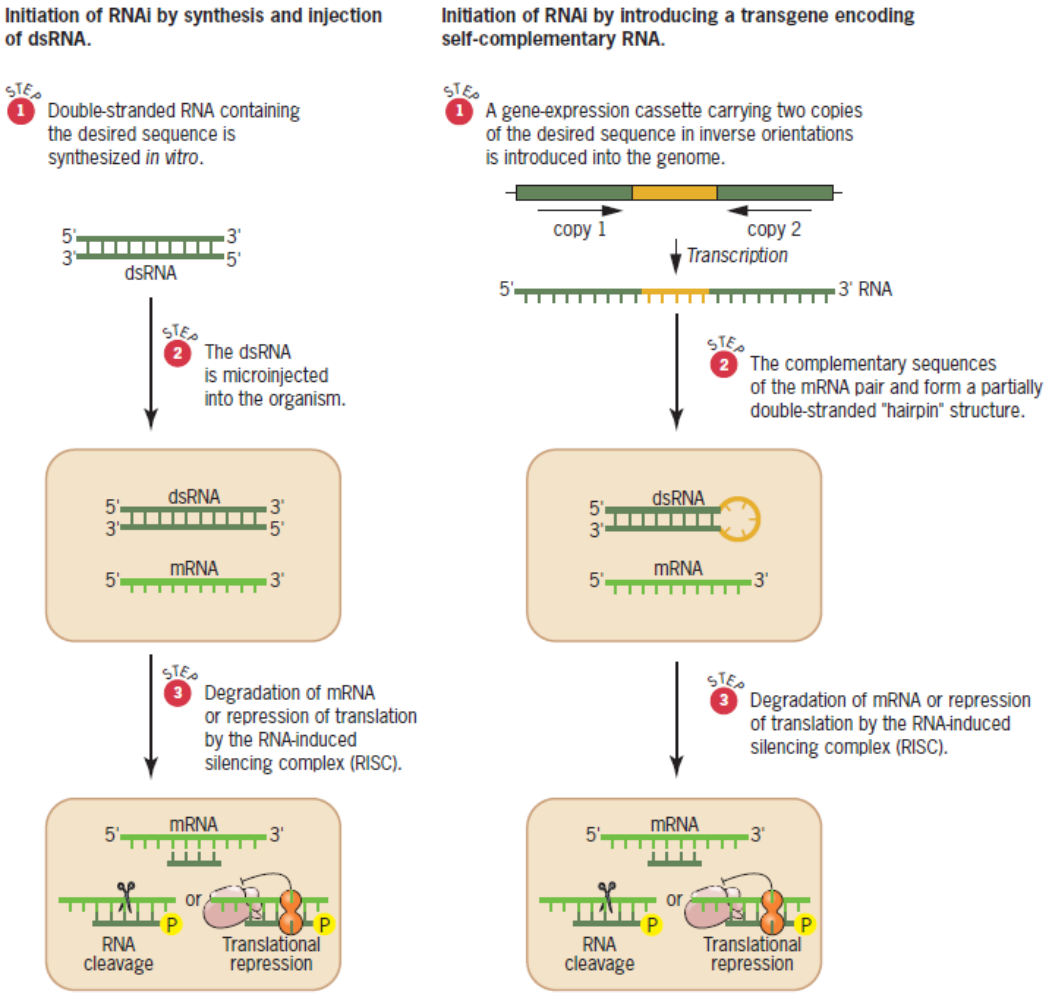


Figure 5: Two ways of initiation of RNAi. Taken from Snustad & Simmons (2012).

Advantages of siRNA-induced silencing

Using transgene-induced RNAi in genomic applications has shown its effectiveness in silencing one or more genes in many organisms, including several agriculturally significant plants such as cotton, soybean, potato, wheat, etc. It has also potential as a great tool for functional genomics. Common projects involve creating knock-down lines with attenuated activity of certain genes. Then, plants are tested for phenotypes to characterize the function of the knocked down gene. In transgene-induced RNAi

mutants, it is sufficient to use only a few copies of the inducing transgene to induce silencing and therefore arbitrary promoter can be used what simplify such projects. Another benefit of the technique is ability to silence a subset of related genes. By using insertional or point mutations, it would require several generations of crosses to generate double or multiple mutants within a gene family (McGinnis et al., 2007). It is also beneficial to design a construct with an inducible promoter. It allows the transgene to be expressed and the gene of interest to be silenced only under specific conditions. Disruption of some genes might be lethal in early phases of development and without the inducible promoter their function could not be studied.

Disadvantages of siRNA-induced silencing

The silencing efficacy varies not only between transgenic events but there is also a great variation between observed phenotypes in knock down lines when PTGS approach is applied.

It was reported that variability in RNA level occurs and therefore also level of silencing varies when using transgene-induced RNAi. Transgene-induced RNAi Arabidopsis mutants were created to silence approximately 25 endogenous genes. The construct was designed to induce PTGS and multiple transgenic lines were screened. There was some variability in silencing efficiency but notably, some of the intended genes were not silenced in any of the assayed lines (Kerschen et al., 2004). In maize lines which were transformed with the same construct the level of silencing differed across silenced lines. For yet undetermined reason, some DNA sequences may be resistant to silencing by this particular approach (McGinnis et al., 2007). Other disadvantage is the variability observed at the phenotypic level. Expected phenotypes do not have to be expressed at the same level for one silenced gene or the phenotype does not need to be expressed at all. Nakatsuka and co/workers used in their study chimeric RNAi technology to down-regulate genes for anthocyanine and flavonoid activity. Targeted enzymes are involved in creating petal color of gential flowers. The expected change in pigmentation occurred but the extent of pigmentation differed between clones (Nakatsuka et al., 2010). There are three possible explanations that such wide range of variability is observed. First of all, target genes might be redundant with other genes and therefore metabolism is not altered. Secondly, the target gene can be silenced only in certain tissues and the amount of mRNA could have been measured only in those tissues where silencing failed. In this case phenotype can be dependent only upon silencing in few specific tissues. Then, desired changes cannot be observed. Alternatively, knock down does not always silence the target gene completely and the

remaining amount of mRNA might be sufficient to maintain WT functionality (McGinnis, 2010). All studies mentioned above represent the efforts to induce PTGS. Similarly, variations in silencing of endogenes were described for TGS approach, too. There is no more consistency than in PTGS technique (Eamens et al., 2008). All possible variables contribute to the variability of silencing at both molecular and phenotypic level. These variables must be considered before designing the experiment especially when the success lies in using these techniques.

Experimental design

Each and every technique has its pros and cons and for using transgene-induced RNAi in genomics applications following must be considered (McGinnis et al., 2010):

- a construct should be stably expressed in the plant throughout the stages of development
- evaluation of stable silencing of desired genes over multiple generations by measuring transgene expression
- additionally, test for expected phenotypes to identify useful lines which can be then propagated
- to consider alternative techniques such as zinc-finger nucleases, virus-induced gene silencing etc.

Zinc finger nucleases

Biotechnology had been limited by inefficient transformation methods which do not enable massive production of insertion mutants or directed mutations in some plant species. Recently, one of the most heavily studied tools for specific gene editing are zinc finger nucleases (ZFNs). ZFNs couple custom-designed zinc-finger proteins (ZFPs) together with the non-specific *Fok*I endonuclease domain. The key component of ZFNs is DNA binding domain which recognizes specific DNA sequence and the nuclease is directed precisely to the desired site to introduce a double-strand break (DSB). DSBs are repaired by endogenous mechanism which introduces changes (deletion or insertion) within the genome and create permanently modified organisms (Durai et al., 2005).

Structure and design of ZFNs

ZFPs are the part responsible for binding of ZFN to DNA. Amino acid sequence contains a repeat of Cys2 His2 fingers (Wolfe et al., 2000). Each finger recognizes 3 bp (base pairs) of DNA. Current studies use up to six ZFPs. Since ZFNs function as dicers, they recognize 36 bp target (Urnov et al., 2010). Each ZFN (dimer) contains a cleavage domain of *Fok*I nuclease. For the successful cleavage, ZFN need to form a heterodimer pair (Miller et al., 2007). A scheme of ZFN with 4 zinc fingers is depicted below (Fig. 6a).

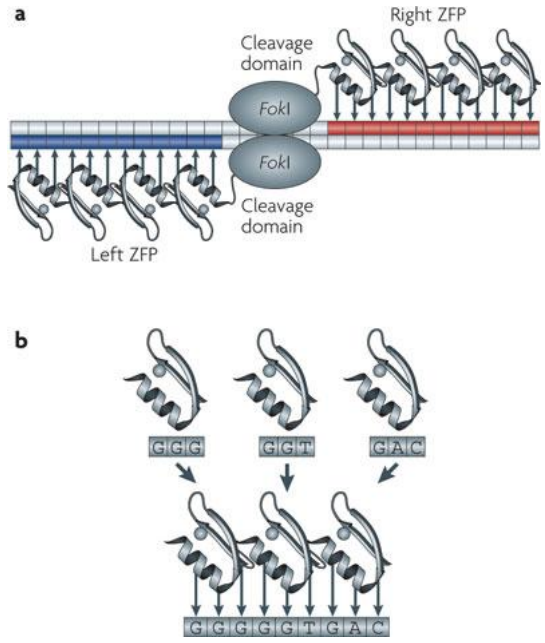


Figure 6: Zinc finger nucleases. a) ZFN scheme with 4 zinc fingers. b) Modular assembly of 3 individual finger proteins. Taken from (Urnov et al., 2010).

Researchers have applied several strategies for making ZFNs. The most conventional method is called modular assembly (Segal et al., 2003). Modular assembly is the identification of fingers for each triplet of the target sequence. Afterwards, they are linked together to form ZFP which recognizes the target DNA sequence. ZFP designed according the modular assembly approach with specificity for the spicific sequence is shown in Fig. 6b. To the date, there have been developed fingers for almost all known 64 triplet sequences (Urnov et al., 2010). Several web-based tools (Mandell & Barbas, 2006, Sander et al., 2007) of zinc finger modules have been made to facilitate the design and assembly of tandem ZFPs by this method. Besides this particular method, several alternative techniques have been developed (reviewed in Urnov et al., 2010). Whatever the design, ZFPs must be first evaluated *in vitro* for their specificity and affinity towards the intended target sequence before the use *in vivo*.

Mechanism

DSB is introduced after the cleavage by ZFNs In the locus of interest. There are two DNA repair pathways in eukaryotic cells:

Non-homologous end-joining (NHEJ)

The broken strand ends are connected by specific protein factors. NHEJ often introduces deletion or insertion in the targeted gene (Lieber et al., 2010).

Homology- directed repair (HDR)

Precise repair of DNA breaks in cells is homologous recombination using the homologous sequence of an unaffected chromatid as its template. Gene targeting uses a heterologous donor molecule (e.g. linearized plasmid) which leads to the addition of a given sequence to the intended locus (e.g. selection gene cassette; Moynahan & Jasin, 2010). Both pathways enable several types of genome-editing by ZFNs (Fig. 7) and lead to generating permanently modified organisms.

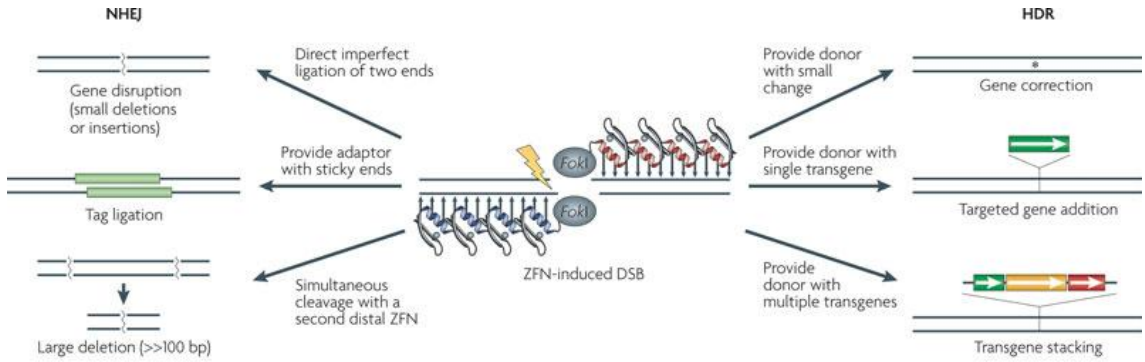


Figure 7: Genome editing by ZFNs using either NHEJ or HDR mechanisms. Taken from Urnov et al., 2010.

Genome editing in plants

Targeted gene modification in plants was considered as almost impossible for a long time (Hanin & Paszkowski, 2003). However, the remarkable progress was made in the use of ZFNs in their recent application for precise editing of model and crop plants (Weinthal et al., 2010). In 2009, a dimer of designed 4-finger ZFNs enabled site specific transgene integration into tobacco cell cultures (10 % of cells were indicated to have the transgene integration precisely in the cleavage site). The donor construct comprised of sequences necessary to complement a non-functional PAT herbicide resistance gene. (Cai et al., 2009). The use of ZFNs was successful also in other plant species. In maize, the addition of an herbicide-tolerance gene was accomplished

similarly as in the study on tobacco cells. The disrupted gene was IPK1, which codes for enzyme catalyzing the last step in phytate biosynthesis in seeds. The transformation was stably transmitted to the next generation and both herbicide tolerance and altered phytate biosynthesis were observed (Shukla et al., 2009).

Advantages

Zinc finger nucleases have become useful reagents for manipulating the genomes of many plants. ZFNs can be designed to specifically recognize long DNA sequences and therefore target specific genes to be replaced or knocked-out. Both ZFPs and recognition domain (Guo et al., 2010) can be optimized to achieve higher specificity. Broadly, ZFP technology enables to target virtually any sequence. When the organism is successfully transformed, genetic modifications are faithfully transmitted to the next generation as it was proved by Shukla and co-workers (2009). A direct delivery of purified ZFN proteins into cells overcame the toxicity and low efficiency occurring when DNA- and mRNA-based delivery methods are used. Moreover, there is no risk of insertional mutagenesis as well as less off-target effects. Thus, the method can be used for precise genome engineering (Gaj et al., 2012). The technology based on ZFNs has been under development for almost 17 years and currently is tested in human clinical trials (Barton et al., 2013). All studies provided convincing results for use in genome editing in various organisms. ZFNs are commercially available by Sangamo Biosciences (Richmond, CA, USA) in partnership with Sigma–Aldrich (St. Louis, MO, USA; Gaj et al., 2013).

Disadvantages

The specificity can be increased by adding more zinc fingers which increase the number of base pairs that the ZFN is bound to. However, too many zinc fingers increase the probability of binding to other parts of genome (Caroll, 2011) and the interaction between multiple zinc-fingers can reduce specificity at the end. After designing ZFPs, the evaluation of its specificity and affinity is performed in vitro. The successful in vitro experiments do not imply the success for application in vivo. In fact, many validated ZFPs failed after tests in cells (Ramirez et al., 2008). There are multiple reasons for the failure. The existence of paralogues and pseudogenes in complex genomes with the identical target sequence can act as an intended target. The target sequence can be in a form of heterochromatin and therefore not accessible for a cleavage (Urnov et al., 2010). In studies on human cells, genotoxicity associated with high expression of ZFNs can lead to cell apoptosis (Cathome & Joung, 2008). For application in medicine, it must be thoroughly tested. The intellectual property, which

belongs to one biotechnological company, makes the price of generating ZFNs high, the price range from 1 000 – 25 000 $ depending on generating premade or custom ZFNs, respectively. Required time to produce and test ZFNs varies from 2-12 months (DeFrancesco, 2011).

TAL nucleases

TAL nucleases represent other chimeric nucleases. They are created by fusion of *Fok*I nuclease and transcription activator-like (TAL) effectors secreted by bacterial plant pathogens from genus *Xanthomonas* (White et al., 2009). The mechanism is similar to ZFN - DSB is introduced after the cleavage and genome can be edited. TAL effector nucleases (TALEN) are alternative technology in biotechnology and one believes, they could replace the use of zinc-finger nucleases in the future (DeFrancesco, 2011).

Structure and design

Each TAL effector consists of transcription activation domain (typical for eukaryots), a motif which ltargets the protein into nucleus and a repeating unit of 34 amino acids (aa) at the central region. This repeating sequence is unique for each effector and is responsible for binding specifically to a certain nucleotide sequence (Gurlebeck et al., 2006). The number of repeats varies and the sequence is almost identical, but in each one, two aa at positions 12 and 13 are different and so called repeat variable di-residues (RVD) (Moscou & Bogdanove, 2009). A schematic picture of a typical TAL effector is depicted in Fig. 8. The recognition of DNA sequence is enabled by RVD – one nucleotide is recognized by RVD of one repeat. Naturally, TAL effectors consist of up to 30 repeats, therefore they can presumably recognize the same number of nucleotides. Theoretically, novel TAL effectors can be designed by the TAL recognition code when for each nucleotide the RVD is known (Boch et al., 2009). FokI nuclease domain can be fused either at the N-terminus or C-terminus.

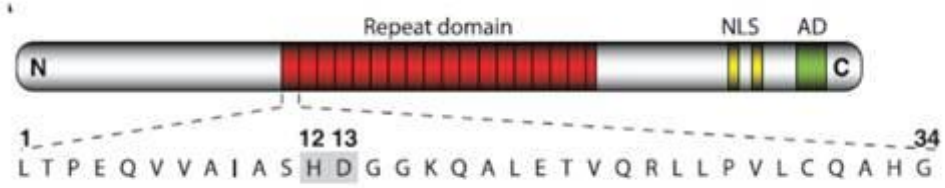


Figure 8: A typical TAL effector. 34 aa direct repeats (red boxes) in the central region, 2 nuclear localization motifs (NLS, yellow bars) and a transcription activation domain (AD, green solid box at C-terminus). Taken from http://www.biodiscover.com/news/product/2263.html.

The fusion of AvrXa7 and PthXo1 TAL effectors from Xanthomonas oryzae pv. oryzae was reported in Li et al., 2011. They generated new type of TAL effector nuclease after fusion of *Fok*I nuclease domain to above mentioned TAL effectors. The activity of such chimeric protein can be proved in yeast cell assay after the transfection into yeast.

Advantages

Simplicity and flexibility has catapulted transcription activator-like effector nucleases together with zinc-finger nucleases to the forefront of genetic engineering. The single base recognition makes TALENs more flexible than ZFNs to design. They can be combined without limitations to recognize any defined DNA sequence (Briggs et al., 2012). Toxicity of TALEN is less of a concern as studies suggest, even when fused with WT FokI than in ZFNs (Szczepek et al., 2007). Custom-designed TALEN arrays are commercially available through Cellectis Bioresearch (Paris, France), Transposagen Biopharmaceuticals (Lexington, KY, USA), and Life Technologies (Grand Island,NY, USA) (Gaj et al., 2013). The cost of TALEN ranges from 400 - 10 000 $ and testing takes few weeks. This is a major advantage over ZFNs (DeFrancesco, 2011).

Disadvantages

TAL nucleases have been discovered later than ZFNs and their studies are still in the beginning. Further research can reveal more details about usage of TAL nucleases (DeFrancesco, 2011). When TALEN are not specific enough, the off-target cleavage may occur within the genome. It might lead to overproduction of DSB. The repair machinery can be overwhelmed which might consequently lead to cell death (Mussolino et al., 2011). It is technically difficult to clone many identical repeat sequences. This issue can be overcome by “Golden Gate” molecular cloning (Cermak et al., 2011) and for example ligation-independent cloning techniques (Schmidt-Burgk et al., 2013). TAL effectors are found only in plant pathogens and might present problem in the use in therapeutics. Unlike ZFNs, TALEN can be strong antigens (DeFrancesco, 2011).

Experimental part

Chemicals

Tris base was purchased from Duchefa (Netherlands). Ascorbic acid was from Lach-Ner (Czech Republic). Sodium bisulfate was purchased from Sigma-Aldrich (USA). Ethanol and hydrochloric acid were from Lachema (Czech Republic). Isopropanol was from MACH (Czech Republic). DNA ladders (1kb; 100 bp Gene Ruler) were purchased from Fermentas (Lithuania). Ethidium bromide was from NeoLab (Germany). Agarose was from Amresco (USA). Mercaptoethanol and sodium hypochlorite solution were from Fluka (Switzerland). EDTA and sodium chloride were purchased from Penta (Czech Republic). MagMAX™-96 DNA Multi-Sample Kit was from Life Technologies (USA). Immomix was purchased from Bioline (UK). 10x loading buffer was from Takara (Japan).

Buffers and solution

Solution for sterilization of rice seeds

- 10 % NaClO in distilled water

Lysis buffer for isolation of genomic DNA (25 ml)

- 0.730 g NaCl; 0.303 g Tris/HCl; 0.470 g EDTA; 0.125 g NaHSO3; 0.025 g ascorbic acid, 25 µl mercaptoethanol

- always freshly prepared

50 x TAE pufr for agarose electrophoresis (1 l)

- 242g Tris base, 100mL of 500mM EDTA (pH 8.0)

Material and methods

Plant material

The seeds of japonica rice *Oryza sativa cv.* Dongjin or Hwayoung (T1 generation) with T-DNA insertion (Jeon et al., 2000; Jeong et al., 2002) were purchased from POSTECH http://www.postech.ac.kr/life/pfg/risd/. Rice T-DNA Insertion Sequence Database (RISD) can be searched for desired knock-out genes using gene code, position or their sequence. Four knock-out lines used in the bachelor thesis are enlisted in the Table 2.

Table 2: Studied rice lines with a specific gene knock-out

Gene	Insertion in	POSTECH code
Os08g33300 - CYP	intron	PFG_2D-11508.L
Os08g35860 - CKX11	exon	PFG_2B-40098.R
Os10g34230 - CKX3	exon	PFG_1B-11519.R
Os06g51350 - IPT10	exon	PFG_3A-15290.R

Growing conditions

The rice seeds from T0 generation were sterilized by vortexing in 10% sodium hypochlorite solution for 20 minutes and then washed with plenty of distilled water. They were imbibed and grown in Petri dishes on filter papers in plant growth chambers MLR-350H/351H (Sanyo, UK) for few days until they germinated and formed shoot and first roots (27°C). Afterwards they were put into soil, watered regularly to keep 2 cm water layer above the soil surface. They grew in plant growth chambers (Fig. 9) under 14 hours night and 10 hours day light conditions at 26 °C. Any assistance or support is possible to get from International Rice Research Institute http://irri.org/. After a month, samples of leaves were collected to isolate genomic DNA (gDNA). After this step, plants might be moved from the growth chamber to the greenhouse. Seeds were collected approximately after 6 months of growth. There was not necessary to use special treatment such as fertilizers. Seeds from T1 generation were grown under the same conditions.

Figure 9: Rice plants in a growth chamber

Isolation of gDNA using MagMAX™-96 DNA Multi-Sample Kit

Ttwo pieces of rice leaf 2 cm long were dried out overnight at 45 °C. Two 5 mm glass beads were put into tubes with samples. Then, samples were homogenized in the mixer mill MM 400 (Retsch, Germany)for 8 minutes at 27 kHz. After this step, gDNA was isolated by following procedure:

1. Add 1 ml of lysis buffer and incubate for 45 min at 65 °C.
2. Centrifuged at 10,000 rpm for 5 min at 4 °C.
3. Add 3 µl of magnetic beads (MagMAX™-96 DNA Multi-Sample Kit) and 70 µl of 100 % isopropanol to 100 µl of sample.
4. Mix and incubate samples at the room temperature (RT) and place them on the magnetic stand for 5 min.
5. Discard the supernatant.
6. Add 70 % ethanol and mix it.
7. Discard the supernatant.
8. Repeat steps 6 and 7 twice to achieve higher purity.
9. Gently pipet up/down with Wash Buffer from the kit.
10. Place on the magnetic stand for 5 min, discard supernatant and place uncapped samples in the fume chamber to air-dry.
11. Add 40 µl of sterile water, incubate for 2 min at RT and transfer the eluate with purified DNA into a new tube.

Samples can be stored at -80 °C to prolong their life-span.

Primer design

Primers were designed in BioEdit to fit either gene or vector to screen individual plants for the vector presence in none, one or both alleles, respectively.

pGA27xx_fw	**5′-CAG GGA GGC AAA CAA TGA AT-3′**
pGA27xx_rev	**5′-GGC AGT TTG CTG CAC TTA CA-3′**
RB_pGA27XX_rev	**5′-AAC CCC AAA GAT GTC CTG CAT TG-3′**
RB_pGA27XX_rev_compl	**5′-TTG GGG TTT CTA CAG GAC GTAA C-3′**
LB_pGA2707_rev	**5′-TCA CAA GCC GTA AGT GCA AG-3′**
LB_pGA2707_rev_compl	**5′-AGT GTT CGG CAT TCA CGT TC-3′**
LB_pGA2715_rev	**5′-CTA GAG TCG AGA ATT CAG TAC A-3′**
LB_pGA2715_rev_compl	**5′-GAT CTC AGC TCT TAA GTC ATG T-3′**
Os06g51350_fw	**5′-CAT GAG GTA CCG AAC GGA CA-3′**
06g51350_fw	**5′-CCT GTC AGT CAA CAA CTG GTC A-3′**
06g51350_rev	**5′-TCC GTT GTG CCT ACA CTG AA-3′**
Os08g33300_fw	**5′-AAA GAG AGC ACT TTA TGC AGC AG-3′**
08g33300_fw	**5′-CAA AAG CTA GGA GGC ACT GG-3′**
08g33300_rev	**5′-TGC TCG GAA AAT ACC TGG AG-3′**
Os08g35860_fw	**5′-TAG TGG TAG CAG CTA GTA GG-3′**
08g35860_fw	**5′-CCG AAT GGC GAG ATC TTC TA-3′**
08g35860_rev	**5′-ACC GAA ACC TCC CTC TTT TG-3′**
Os10g34230_fw	**5′-TGG TGG AAT CAT GTG GAT TG-3′**
10g34230_rev	**5′-TTG TAG GGA AGG CAA TGG AG-3′**
ACTfw	**5′–AGC AAC TGG GAT GAC ATG GAG AAA A-3′**
ACTrev	**5′-CCT GTT CAT AAT CAA GGG CAA CGT A-3′**
01g45110_fw	**5′-GCA TCA TAC GCC AAA CAA TG-3′**
01g45110_rev	**5′- AGT TTA GCA CGG GCT CTG AA-3′**
01g08110_fw	**5′-CAT GAG CTC AAA CTG GCT GA-3′**
01g08110_rev	**5′-GAG ATC CCT GGA CTG GAT CA-3′**

PCR

PCR was always set up to final sample volume of 25 µl. The composition of one PCR sample is written in the Table 3.

Table 3: PCR sample

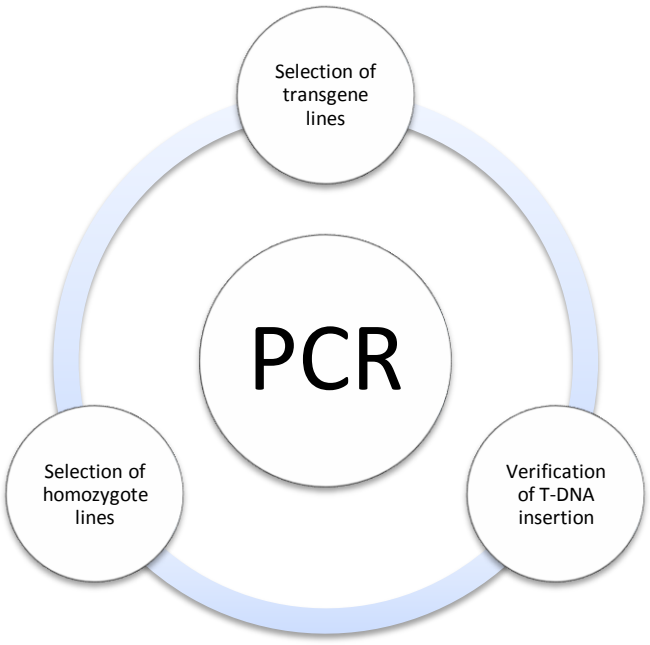
water	9,5 µl
immomix	10,5 µl
primer fw	2 µl
primer rev	2 µl
template	1 µl
sample	25 µl

PCR was set up in the thermocycler as shown in Table 4. Annealing temperature 55°C was below Tm of all used primers. The final number of cycles during PCR was 43.

Table 4: Thermocycler set-up

Steps	Temperature	Duration	#
Initialization	95°C	10 min	
Denaturation	95°C	30 s	42 cycles
Annealing	55°C	30 s	
Elongation	72°C	1 min	
Final elongation	72°C	10 min	
Final hold	4°C	30 min	

PCR experiments were performed according the following scheme:



Agarose gel electrophoresis

Gel electrophoresis (GE) was employed to check whether anticipated DNA fragments were generated. The size of PCR products was compared with a DNA ladder. 1 % agarose in TAE buffer was used in all separations. Electrophoresis ran 20 min under 120 V. The gel was stained in ethidium bromide and afterwards visualized under UV light.

Results and discussion

Selection of Gene knock-out lines

Four different gene knock-outs (Table 2) of three types of enzymes involved in cytokinin metabolism were chosen to study:

- IPT, tRNA isopentenyl transferase (EC 2.5.1.75, Takei K, et al., 2001. Fig. 11);
- CYP450, cytochrome P450 monooxygenase (EC 1.14.13.X, Takei et al., 2004);
- CKX, cytokinin dehydrogenase (EC 1.5.99.12, Galuszka et al., 2001. Fig. 10).

Cytokinins are plant hormones that play an important role in regulation of development and in environmental responses of plants. Metabolic activity of targeted enzymes is depicted below (Fig. 10 and 11). There are two genes coding for tRNA IPT in rice genome (OsIPT1 and OsIPT10; Sakamoto et al., 2006). Seeds with disrupted ORF were available only for IPT10. Cis-zeatin is a major cytokinin form in cereal species (Gajdošová et al., 2011); however its origination is not clearly explained. One of the hypothesized biosynthesis pathway for cis-zeatin is degradation of specific tRNA, which is previously altered by tRNA IPT activity. Studying levels of cis-zeatin metabolites in OsIPT10 knock-out line can elucidate possible role of OsIPT10 gene in cytokinin (cis-zeatin) biosynthesis.

Based on the homology to Arabidopsis genes, two rice cytochrome P450s were identified as a putative cytokinin specific hydroxylation enzyme (Takei et al., 2004; Fig. 10). Both characterized Arabidopsis enzymes are able to hydroxylated isopentenyladenine only to transposition to form trans-zeatin. High level of cis-zeatin in cereal species implies specificity of rice cytokinin specific cytochrome P450 also to cis-position. Studying knock-out line of this gene can shed more light into the hypothesis.

Rice genome comprises in total eleven eleven CKX genes (Ashikari et al., 2005).

CKX3 gene encodes cytokinin dehydrogenase which clusters together with maize ZmCKX6 in phylogenetic three (Vyroubalová et al., 2009).Both enzymes contain several specific amino acid motifs which are not common for other CKX enzymes throughout whole plant kindgdom (Zalabák et al., unpublished results). Moreover, recombinant ZmCKX6 do not possess standard CKX activity which is measurable in vitro. Both genes have very high expression levels contrary to other CKX genes in many tissues what dispute their characterization as pseudogenes. OsCKX11 has predicted cytosolic localization. Each till now sequenced plant genome revealed

existence of only one unique gene encoding cytosolic CKX enzyme (Frébort et al., 2011). Studying knock-out line can reveal physiological function of this specific type of CKX enzymes.

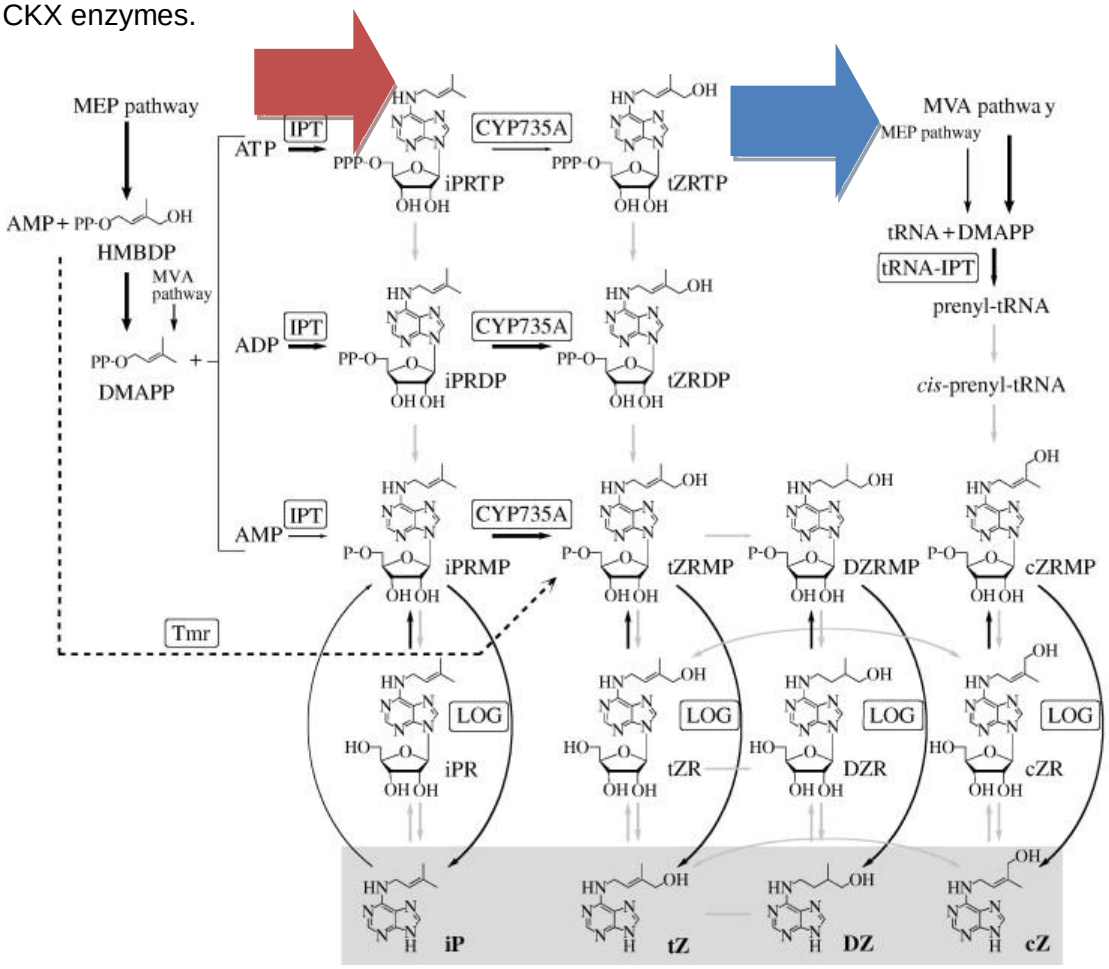


Figure 10: CK biosynthetic pathways in higher plants. The red arrow shows the pathway of CYP735A. The CK-nucleotides are converted into the corresponding tZ-nucleotides by CYP735A. The blue arrow shows the pathway of tRNA-IPT. The tRNA-IPTs catalyze prenylation of tRNA that leads to the production of cZRMP. (Taken from Kamada-Nobusada & Sakakibara, 2009)

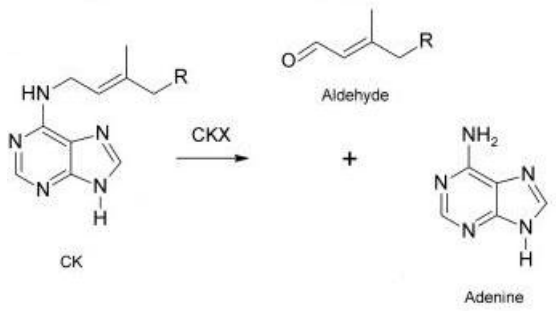


Figure 11: Cytokinins are irreversibly degraded by cytokinin dehydrogenase (CKX). (taken and edited from Zalabák et al., 2011).

T-DNA insertion

Location of T-DNA insertion after sequencing (Jeong et al., 2006) was guaranteed by the provider with 20 % probability of incorrect mapping. Expected location of T-DNA insertion from RISD with available knock-out lines (Fig.12a-d):

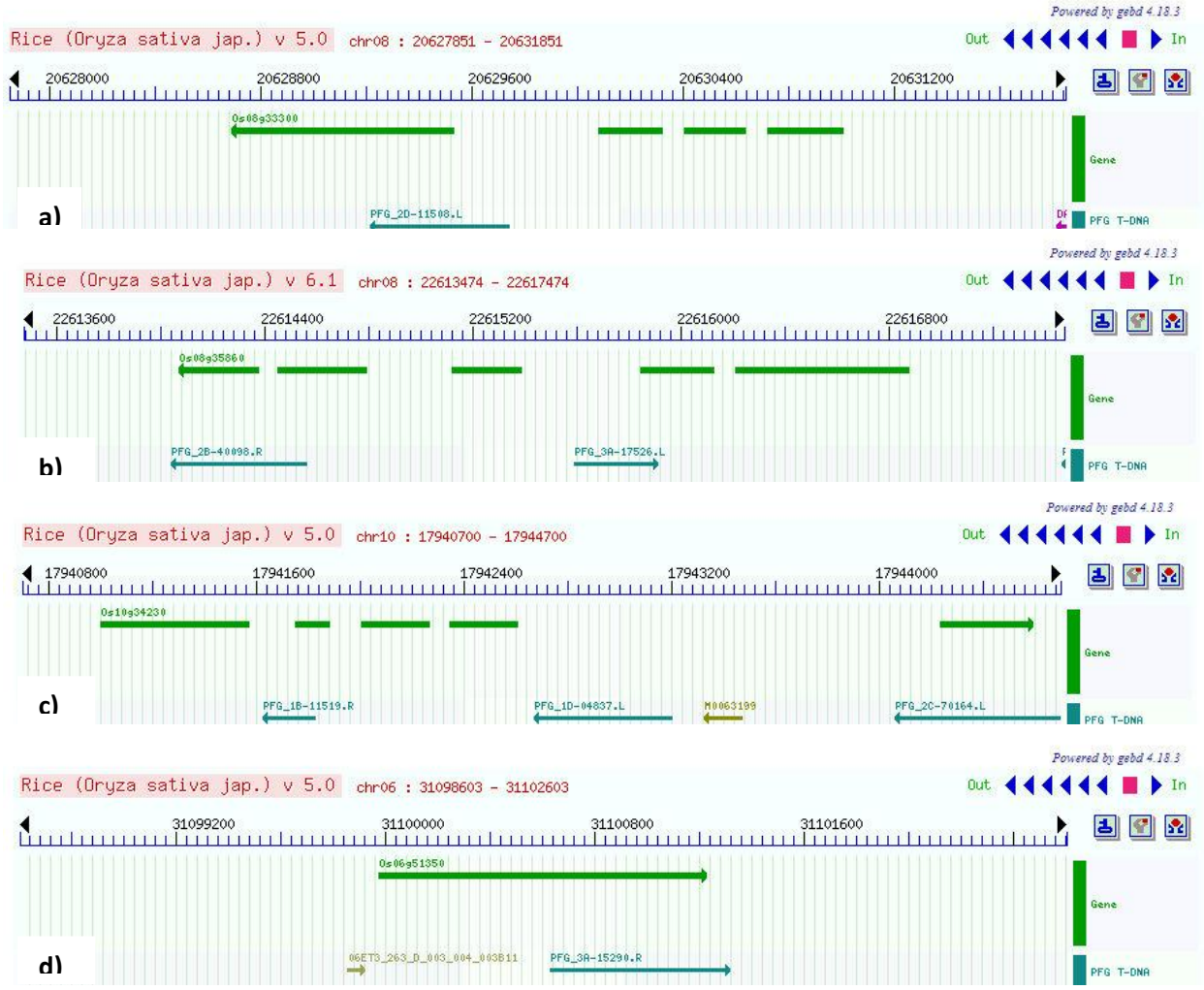


Figure 12: a) CYP450 - Os08g33300, chromosome 8, vector pGA2772; b) CKX11 - Os08g35860, chromosome 8, vector pGA2707; c) CKX3 - Os10g34230, chromosome 10, vector pGA2717; d) IPT10 - Os06g51350, chromosome 6, vector pGA2715. Exons are depicted as green lines with an arrow representing the direction of transcription, the beginning of blue arrow indicates position of T-DNA insertion.

Vectors present in the rice genome are pGA2707 (GUS trapping vector), pGA2717 (GUS and GFP trapping vector), and activation tagging vectors pGA2715 (Fig. 13) and pGA2772. pGA2707 resembles the binary vector pGA2715 except its lack of enhancer elements (Jeong et al., 2002). pGA2772 is modified pGA2715 containing pUC18 vector backbone (Jeong et al., 2006). pGA2717 contains the promoter-less green

fluorescence protein (gfp) gene (Ryu et al., 2004).

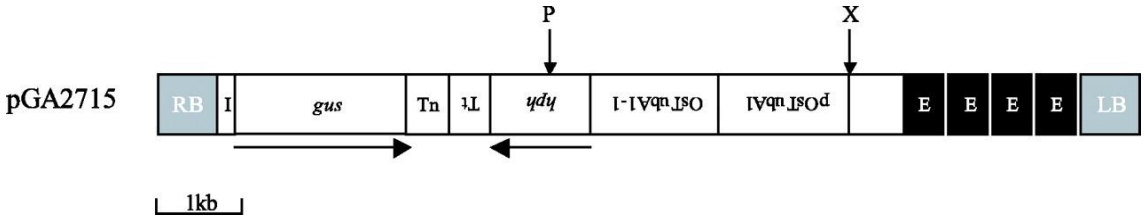


Figure 13: Map of the T-DNA region of vector pGA2715 - RB right border; LB left border; I, OsTubA1 intron 2 carrying three putative splicing acceptor and donor sites; gus, gus reporter gene;Tn, nos terminator; Tt, OsTubA1 terminator; hph, hygromycin phosphotransferase gene; OsTubA1-1, the first intron of OsTubA1; E, enhancer element of CaMV 35S promoter; P, PstI site; X, XhoI site (taken from Jeong et al., (2002)).

Rice seeds of four different knock-out lines (T1 generation) and WT obtained from POSTECH database were germinated in a growth chamber and afterward grown in soil under short day conditions. The lines with a potential knock-out were following:

OsCKX11 (15 seeds) , OsCKX3 (15 seeds) , OsIPT10 (15 seeds) and OsCYP450 (11 seeds) and several appropriate wild-type plants.

The everyday care included regular watering to keep plants flooded at least with a few cm of water above the soil surface. Every pot with a plant was visibly labeled to differentiate all lines. As soon as rice had few leaves, one leaf of each rice plant was collected to isolate DNA. If leaves were cut too early, it might be non-viable for the organism. It was not desire as one of the aims was to propagate enough seeds of T1 generation. From the collected leaves samples, gDNA was isolated using MagMax method. Concentrated DNA was measured on UV/VIS spectrophotometer Agilent 8453. Average concentration was around 0,150 µg/ml. Matured seeds were collected approximately after 6 months of growth and then stored in plastic bags at a dry place.

Seed germination rate

Sterilized seeds from T0 generation were put on a filter paper in Petri dishes and kept in a growth chamber. After 3 days, the seed germination rate was determined (Table 5).

Planting rice and labeling plants

Several seeds did not germinate at all as mentioned above. In several cases, seeds were contaminated by mold. Each individual plant had own code and each line had own color. The code labels each plant, isolated DNA and seed progeny whether it was

collected. During the early stage of growth, some plants died before leaf samples were collected. The list of rice codes is in the Table 6. Table 6 summarized all independent lines, which gave seeds and were thus evaluate in T2 generation.

Table 5: Seed germination rate T1 generation

Rice line	Number of seeds	Germinated seeds	Seed germination rate/%
CYP450	11	8	72.7
CKX11	15	10	67.7
CKX3	15	10	67.7
IPT10	15	12	80
WT	11	9	81.8

Table 6: The list of plants in T1 generation

CYP450	CKX11	CKX3	IPT10	WT
13B	11A	A	07	02A
124	11B	B	08A	02B
	12	C	08B	
		03	09	
		05	106	
		118	115	

Screening of T1 generation plants by PCR

The seeds purchased from POSTECH were collected from T0 generation plants after using the Agrobacterium-mediated transformation method. In T0 generation, there is possible to have either WT or heterozygotes. In T1 and further generations, homozygotes can be selected (Fig. 14).

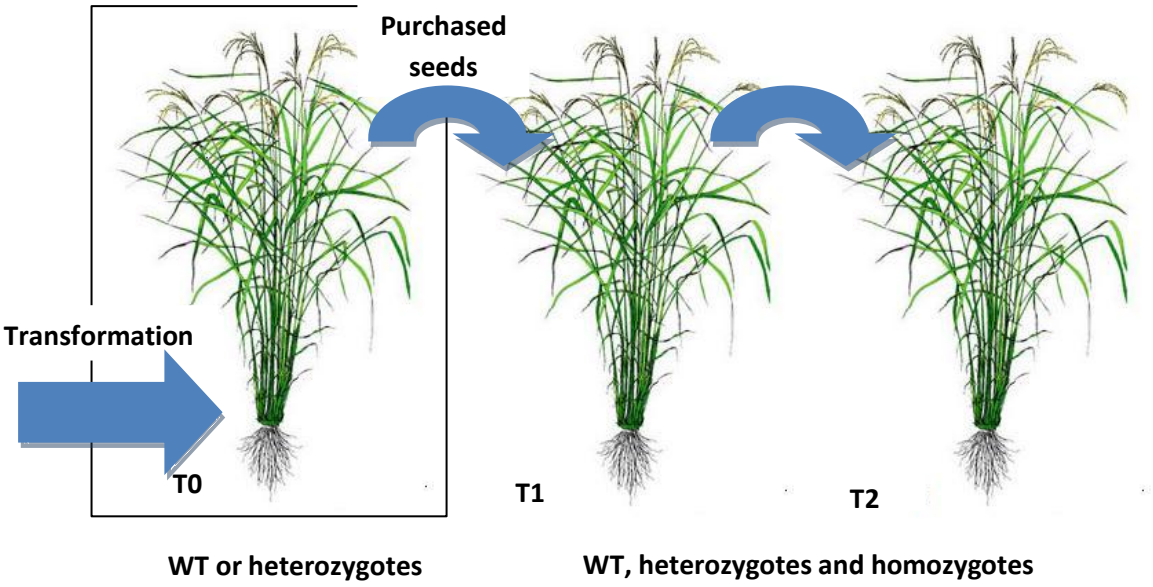


Figure 14: Scheme of rice generations used in experimental part of this bachelor thesis.

Three different approaches were employed in PCR reactions.

- Differentiate between transformed and WT plants. To do so, it was necessary to use primers to prove presence of inserted vectors in DNA. When primers for vector were applied at WT plant gDNA, no amplicons will be present. The difference in gels, whether DNA of WT or heterozygote is in the sample, is shown in Figure 15A.
- Second type of PCR was done, when primers complementary to target genes and flanking T-DNA insertion were applied on transformed plants. With the T-DNA insertion, the sequence is too long to be amplified, therefore we will not observe any amplification if gDNA from homozygote plants is used as a template. The difference between homozygote and heterozygote is depicted in Figure 15B.

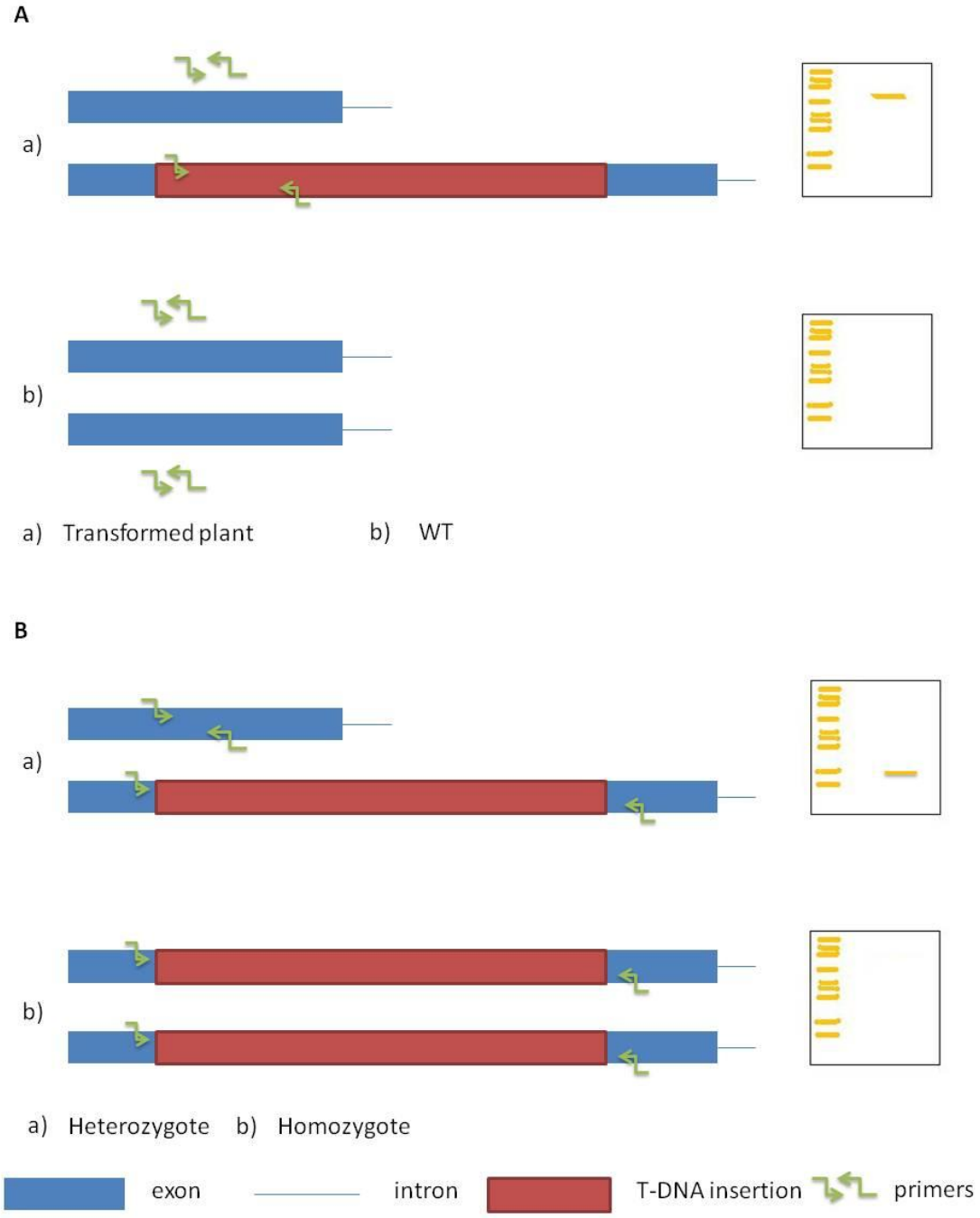


Figure 15: Expected results of performed PCR experiments. A) Selection of transgenic (knock-outed) lines; B) Selection of homozygote lines.

Agarose gel electrophoresis

After PCR, all samples were analyzed by agarose gel electrophoresis (1 % agarose in TAE buffer). If not specified, GeneRuler 1 kb Plus DNA Ladder was used as a marker (Fig. 16).

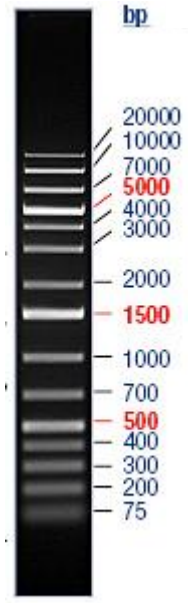


Figure 16: GeneRuler 1 kb Plus DNA Ladder

Testing of designed primers

Designed primers were checked if working on 02A DNA sample (WT). One gel example is in Fig. 17. In the line 4, the product size was estimated to 442 bp, but band migrated to around 900 bp. Therefore, the combination of primers 01g45110_fw and 01g45110_rev does not work properly. In the line 7, primers for actin gene were used as a positive control if PCR reaction mixture works. Visible bands under 100 bp are remaining primers (Fig. 17).

Table 7: Primers used in PCR with estimated size of the product.

Line	Primer combination - gene	Estimated size
2	08g35860_fw; 08g35860_rev - CKX11	520 bp
3	Os10g34230_fw; 10g34230_rev – CKX3	778 bp
4	01g45110_fw; 01g45110_rev – trans glycosylation	442 bp
5	06g51350_fw; 06g51350_rev – IPT10	397 bp
6	01g08110_fw; 01g08110_rev – trans glycosylation	836 bp
7	ACTfw; ACTrev - actin	550 bp
8	08g33300_fw; 08g33300_rev – CYP450	484 bp

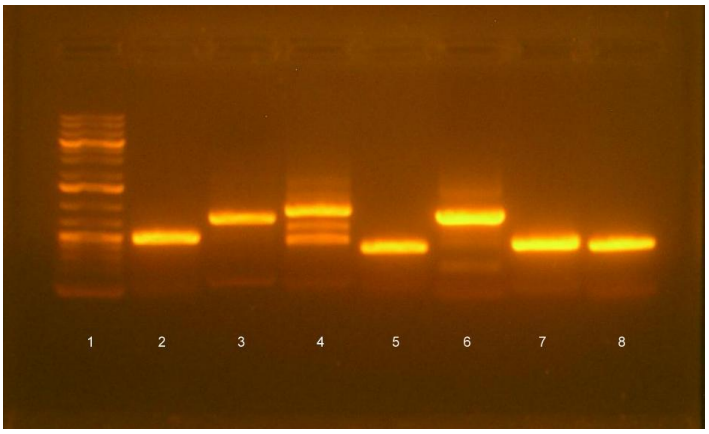


Figure 17: Evaluation of primers. Primers used are in Table 7. For amplification was used 02A gDNA sample (WT). In line 7, primers for a house-keeping gene (actin) were used.

Selection of transgenic lines T1 generation

To select transgenic lines, all DNA samples were screened (Fig. 18 and 19). The following combination of primers for T-DNA insertion was used:

pGA27xx_fw and pGA27xx_rev estimated size of the band is 403 bp

No band indicates no presence of T-DNA insertion.

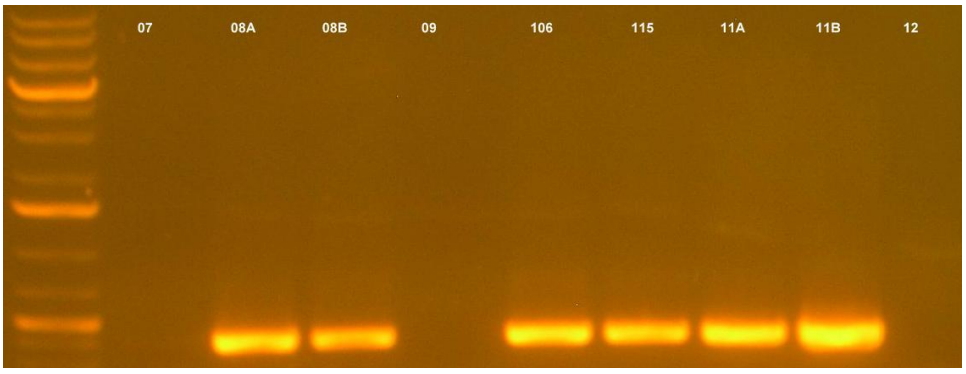


Figure 18: Selection of transgenes lines (IPT10 and CKX11)

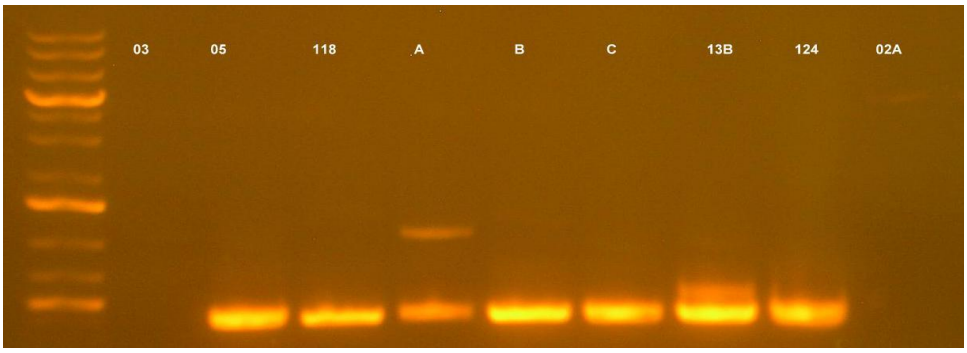


Figure 19: Selection of transgene lines (CKX3, CYP450). DNA template of WT is in the last line.

Selection of homozygote lines

All selected transgenic lines were screened for the homozygous lines (Fig. 20 and 21). The primers amplifying gene (WT allele) summarized in Table 7 were used to distinguish between heterozygote and homozygote. If there is no amplification and simultaneously sample gives positive amplification for T-DNA vector, plant can be considered as homozygote.

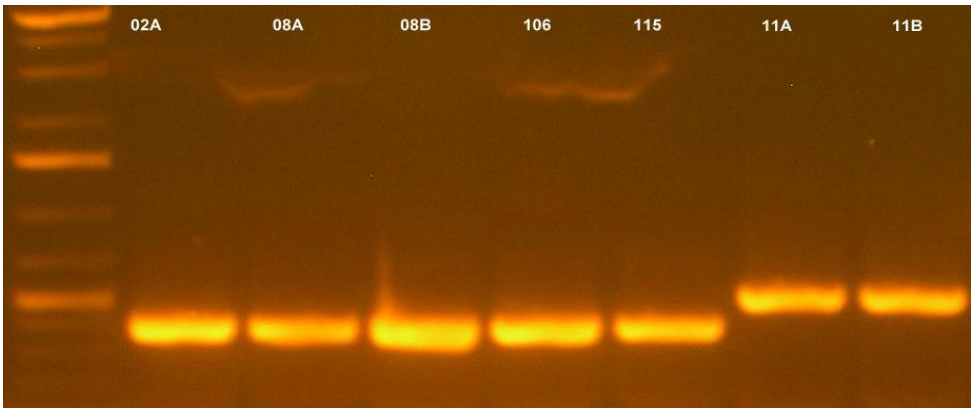


Figure 20: Selection of homozygote lines (IPT10 and CKX11). DNA template of WT is in the first line.

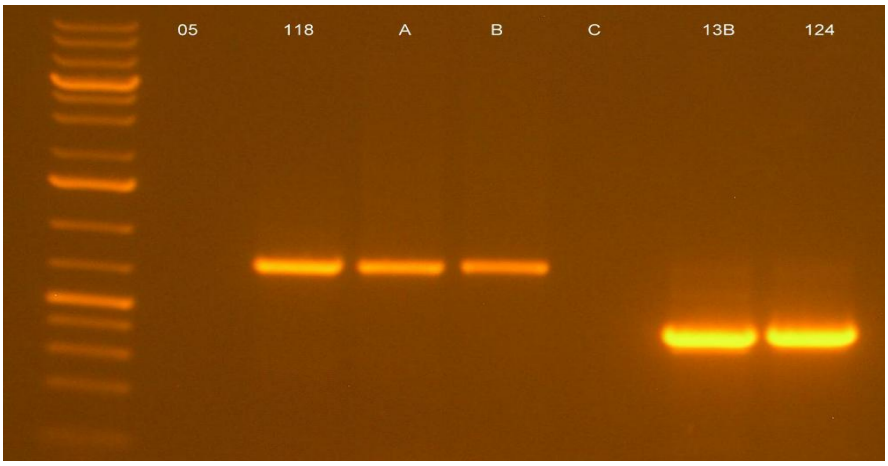


Figure 21: Selection of homozygote lines (CKX3 and CYP450). Samples 05 and C are homozygous.

Confirmation of the presence of T-DNA insertion in the desired gene

T-DNA sequence could be inserted and present in the genome 4 different ways – every gene has 2 alleles, and also the vector can be inserted forward or backwards. Therefore, several combinations of primers were designed to prove the presence of the

T-DNA insertion in given genes. Depending on the direction of the T-DNA insertion, following combinations were found working:

IPT10: 06g51350_fw and RB_pGA27XX_rev_compl

CKX11: none

CKX3: Os10g34230_fw and RB_pGA27XX_rev_compl

CYP450: 08g33300_fw and RB_pGA27XX_rev

The presence of T-DNA insertion was proved within the gene in all independent plants of IPT10 line.As shown in Fig. 22, in the first and third line, the PCR was ran with non-working combination of 06g51350_fw and RB_pGA27XX_rev. The gel for CKX11 DNA samples is not shown, because none of the designed combination worked. Hence, T-DNA insertion is most probably not in the OsCKX11 gene as was predicted in POSTECH database and the obtained transgenic line (PFG_2B-40098.R) is wrongly annotated. The transcription of IPT10 and CKX3 is in the direction of ATG to TAG and CYP450 in the opposite direction.

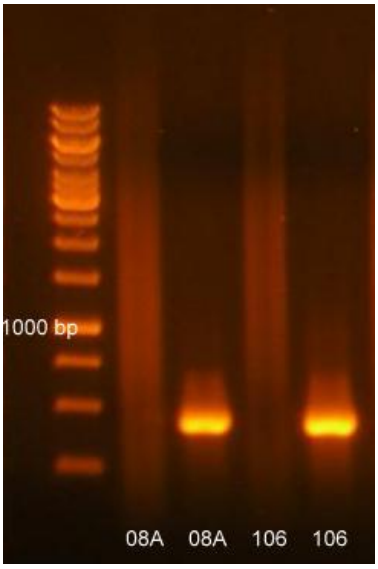


Figure 22: Presence of the T-DNA insertion in IPT10 DNA samples (08A and 106). Further description is in the text above the picture.

In the Fig. 23, there is shown the gel after PCR with DNA of CKX3 and CYP450 samples. The line without band (DNA sample C) is the combination of Os10g34230_fw and RB_pGA27XX_rev primers, which doesn't work.

The line without band (DNA sample 124) is the combination of Os08g33300_fw and RB_pGA27XX_rev, which doesn't work.

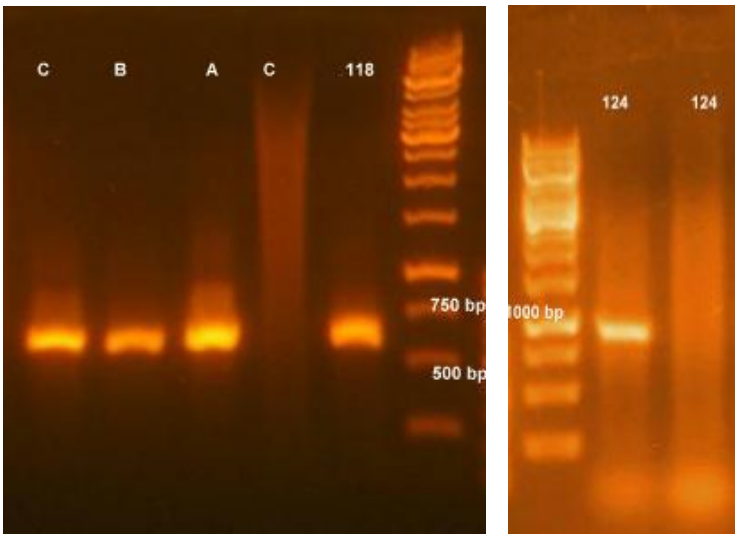


Figure 23: Presence of the T-DNA insertion in CKX3 (A, B, C, 118) and CYP450 (124) DNA samples. Further description is in the text.

In the table 8, there is the complete overview of found results. In T1 generation, we were able to select two independent homozygous lines for OsCKX3 knock-out (sample 05 and C).

Table 8: Overview of T1 generation. Selected homozygote lines are samples 05 and C (both OsCKX3 knock-out).

Gene	Code	WT	Heterozygote	Homozygote	Note
IPT10	07	X			
	08A		X		
	08B		X		
	09	X			
	106		X		
	115		X		
CKX3	03	X			
	05			X	
	118		X		
	A		X		
	B		X		
	C			X	
CKX11	11A				T-DNA insertion is not within the gene
	11B				T-DNA insertion is not within the gene
	12	X			
CYP	13B		X		
	124		X		

Seeds collection from T1 generation

Seeds were collected approximately after six months. The number of seeds from each plant was in a range of 50-80 seeds. The exception was CKX3 B, which had only 20 seeds. In general, all plants had also many empty seeds. Seeds were stored at a dry place and labeled by same code and color as the original T1 generation plant.

T2 generation

From the collected seeds, following lines were chosen to grow:

CKX3: 05; 118; A; B; C

CYP450: 124

IPT10: 08A

20 seeds from 05, 118 and A, 10 seeds from B, C, 124 and 08A were sterilized and germinated and grow the same way as the seeds from T0 generation.

In the Table 9, seed germination rate is listed for each line.

Table 9: Seed germination rate T2 generation

Rice line	Number of seeds	Germinated seeds	Seed germination rate/%
OsCKX3			
05	20	14	70
118	20	15	75
A	20	19	95
B	10	0	0
C	10	7	70
OsCYP450			
124	10	6	60
OsIPT10			
08A	10	6	60

Planting rice of T2 generation and labeling plants

As was shown above, several seeds did not germinate. In fact, none of the seeds from line B germinated at all. In several plates mold contamination was found. Each individual plant had own code and each line had own color. The code labels for each

rice from which DNA was isolated is enlisted in Table 10. Again, several plants died before leave samples I were collected

Table 10: The list of plants analyzed on molecular level in T2 generation

05	118	A	C	124	08A
05-1	118-6	A-1	C-6	124-1	08A-1
05-2	118-7	A-4	C-7	124-2	08A-2
05-3	118-8	A-5	C-8	124-4	08A-3
05-4	118-11	A-6	C-9		08A-4
05-5	118-12	A-7	C-10		
05-8	118-13	A-10	C-11		
05-9	118-14	A-11			
	118-15	A-12			
		A-13			
		A-14			
		A-15			
		A-16			
		A-17			
		A-19			
		A-20			

Screening of T2 generation plants by PCR

As discussed in the papers (Jeon et al., 2000 and Jeong et al., 2002), the vector remains stably inserted across several generations. After the gDNA isolation from leaves, all gDNA samples were screened for T-DNA insertion and homozygote/heterozygote status.

Presence of T-DNA insertion in T2 generation

gDNA samples from all plants in T2 generation were screened for the presence of T-DNA insertion (Fig 24-27). The following combination of primers for T-DNA insertion was used:

pGA27xx_fw and pGA27xx_rev estimated size of the band is 403 bp

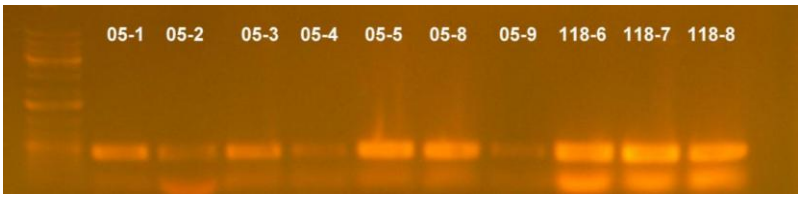


Figure 24: Selection of transgene lines in CKX3 DNA samples (05- 1-5, 8 and 118- 6-8)

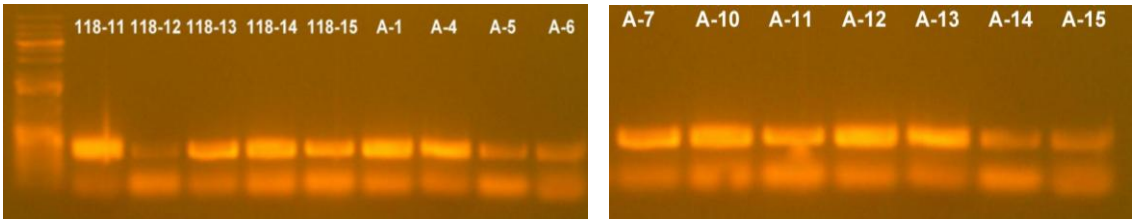


Figure 25: Selection of transgene lines in CKX3 DNA samples (118- 11-15 and A- 1, 4-7, 10-15)

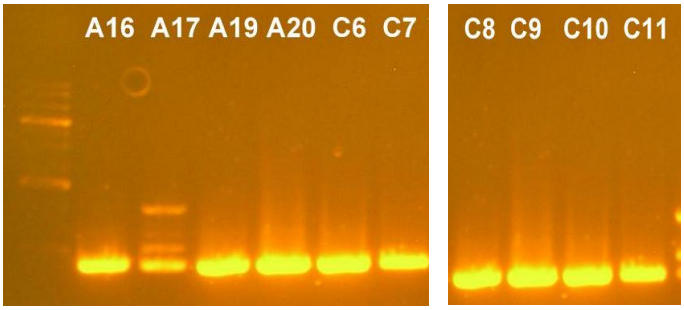


Figure 26: Selection of transgene lines in CKX3 DNA samples (A- 16, 17, 19, 20 and C- 6-11)

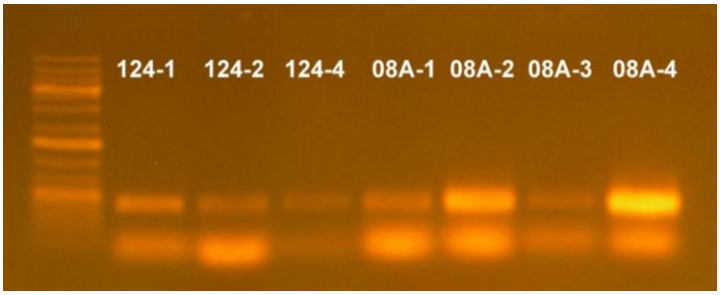


Figure 27: Selection of transgene lines in CYP450 (124- 1, 2, 4) and IPT10 (08A- 1-4) DNA samples

Selection of homozygote lines in T2 generation

All lines from T2 generation were screened for the homozygous lines (Fig. 28-31). The following primers for each concrete gene were used:

IPT10: 06g51350_fw and 06g51350_rev estimated size of the band is 397 bp

CKX3: Os10g34230_fw and 10g34230_rev estimated size of the band is 778 bp

CYP450: 08g33300_fw and 08g33300_rev estimated size of the band is 484 bp

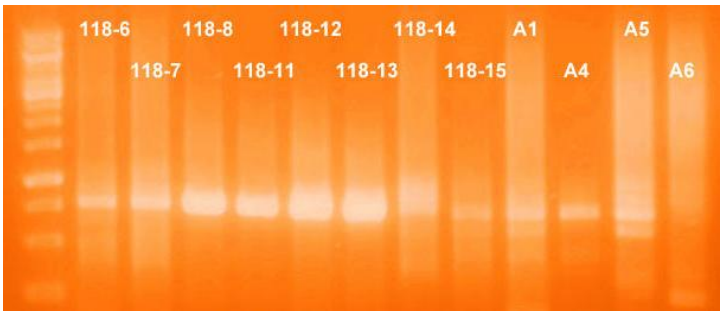


Figure 28: Selection of homozygote lines (CKX3). The line with DNA samle A6 is considered negative.

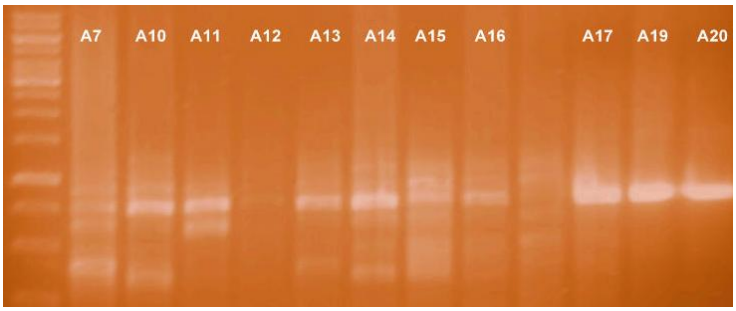


Figure 29: Selection of homozygote lines (CKX3). The line with DNA sample A12 is considered negative. The line between A16 and A17 was a mistake in pipeting.

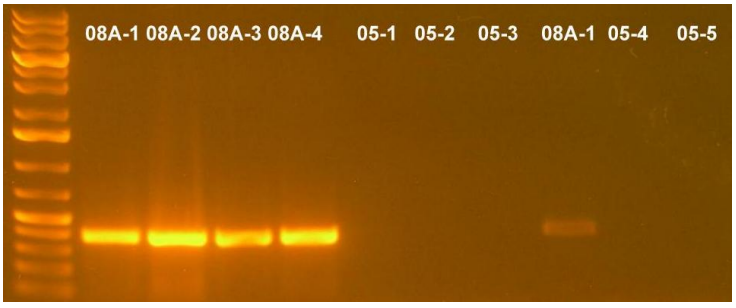


Figure 30: Selection of homozygote lines (IPT10 and CKX3). The sample 08A-1 was applied twice on the gel by mistake.

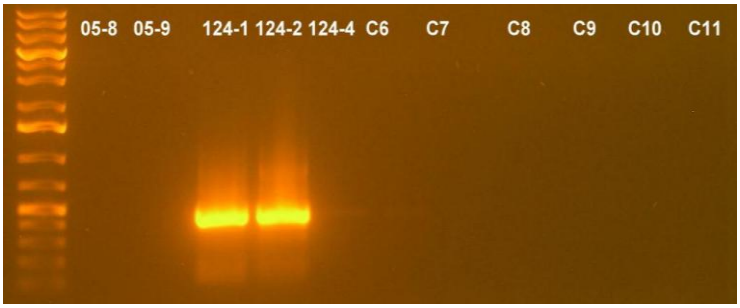


Figure 31: Selection of homozygote lines (CYP450 and CKX3).

In the Table 11, there is the complete overview of found results. PCR results done on plants from T2 generation confirmed homozygote status of OsCKX3 knock-out line 05

and C. Furthermore, two more homozygote lines A-6 and A-12 were detected after segregation in T2 generation and one line 124-4 for OsCYP450 knock-out.

Table 11: Overview – T2 generation. Selected homozygote lines are: A6 (Fig. 28), A12 (Fig. 29), all 05 and C lines (Fig. 30 and 31) and 124-4 (Fig. 31).

Gene	Code	Heterozygote	Homozygote	Note
CKX3	05		X	all lines homozygous
	118	X		all lines heterozygous
	A-1	X		
	A-4	X		
	A-5	X		
	A-6		X	
	A-7	X		
	A-10	X		
	A-11	X		
	A-12		X	
	A-13	X		
	A-14	X		
	A-15	X		
	A-16	X		
	A-17	X		
	A-19	X		
	A-20	X		
	C		X	all lines homozygous
CYP450	124-1	X		
	124-2	X		
	124-4		X	
IPT10	08A	X		all lines heterozygous

Conclusion

For the study, there were chosen 4 potential knock-out lines of rice with T-DNA insertion in genes participating on cytokinin metabolism – two genes encoding cytokinin dehydrogenase - CKX3, CKX11, one encoding cytokinin specific cytochrome P450 hydroxylase - CYP450 and one encoding tRNA isopentenyl transferase -IPT10. The rice seeds were planted in T1 and T2 generations, respectively and all lines were screened to select homozygote lines for further phenotypic studies.

The seeds in T1 generation had the germination rate in a range from 67.2-81.8 %. Planted rice seedlings had a high mortality rate when transfer into the soil most probably due to mold contamination and moisture decay. There were selected 13 independent transgenic lines from 17 surviving plants in T1 generation in total. Rate of transgenic plants corresponds to expected segregation ratio of wt-homozygous-heterozygous plants in T1 generation (1:1:2; 75% of transgenic). Afterwards, transgenic lines were tested for heterozygous and homozygous status. In all tested lines, two independent homozygotes were selected for OsCKX3 knock-out already in T1 generation – line numbered as 05 and C. The integration and orientation of T-DNA insertion was tested in all intended loci (Os06g51350 for OsIPT10; Os08g35860 for OsCKX11; Os10g34230 for OsCKX3 and Os08g33300 for OsCYP450). All transgenic lines had the insertion in expected locus except to OsCKX11 line, which was most probably wrongly annotated T-DNA insertion line in POSTECH database. Matured seeds of T1 generation were harvested and some of them were transferred into subsequent generation.

For planting T2 generation plants, following lines were chosen - CKX3: 05, 118, A, B, C; CYP450: 124 and IPT10: 08A. The seed germination rate was in a scale between 60-95 %, but in case of CKX3 B, none of the seeds germinated. It was not probably due to the lethal effect of disrupted OsCKX3 gene as other two independent homozygous OsCKX3 lines have standard germination rates. 67 rice seedlings of T2 generation were planted but only 43 reach maturity. High mortality rate was caused most probably due to obstacles during the cultivation. The T-DNA insertion was confirmed in all 43 plants proving the stability of T-DNA insertion as previously shown in other studies. PCR test for homozygocity confirmed two previously indicated lines for OsCKX3 knock-out (05 and C) and revealed another three - homozygous lines, two for OsCKX3 (A-6 and A-12) and one for OsCYP450 (124-4). At the time of printing the thesis, plants reached the inflorescence status and gave progeny (T3 seeds). In both generations, 5 albino individual plants were observed in expected OsIPT10 knock-outs,

nevertheless none of them was viable and tested for T-DNA insertion and zygosity status. This observation might indicate that OsIPT10 disruption can have significant effect on rice plant physiology.

Seeds of two selected homozygous lines for OsCKX3 are stored and will be used for detailed phenotypic observation beyond the scope of this thesis.

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Abbreviations

bp	base pairs
CaMV	cauliflower mosaic virus
CHS	chimeric chalcone synthase
CK	cytokinin
CKX	cytokinin dehydrogenase
CYP450	cytochrome p450
Ds	transposable element
DSB	double-strand break
dsRNA	double stranded RNA
HDR	Homology- directed repair
hpRNA	“hairpin” RNA
IPT	isopentenyl transferase
miRNA	microRNA
NHEJ	Non-homologous end-joining
PTGS	post-transcriptional gene silencing
RdDM	RNA-directed DNA methylation
Ri plasmid	root-inducing plasmid
RNAi	RNA interference
RVD	repeat variable di-residues
Spm	transposable element
ssRNA	single stranded RNA
siRNA	short interfering RNA
TAL	transcription activator-like

TALEN	transcription activator-like effector nucleases
T-DNA	transferred DNA
tZ	trans-zeatin
TGS	transcriptional gene silencing
Ti plasmid	tumor-inducing plasmid
Tos17	transposon *Oryza sativa* 17
Vir region	virulence region
WT	wild-type
ZFN	zinc-finger nuclease
ZFP	zinc-finger protein