Expression of yeast *Saccharomyces cerevisiae* K2 preprotoxin gene in transgenic plants

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The aim of this work was to investigate expression possibilities of yeast K2 preprotoxin gene in plant *Nicotiana tabacum*.

All experiments were performed using general methods of gene engineering, microbiology and molecular biology.

Plant transformation vector pGA/ADH1-Kil2 carrying *S. cerevisiae* K2 preprotoxin gene under control of yeast ADH1 promoters as well as yeast plasmids pAD/CGT-Kil2D and pAD/CGT-Kil2R (K2 under control of *Cauliflower mosaic virus* CaMV promoter) were constructed. Analysis of expression of K2 gene controlled by CaMV promoter in yeast showed 72–75% stability of plasmid and weak killer activity with suicidal phenotype. Study of peculiarities of functional activity of K2 killer gene in yeast demonstrated that expression of this gene is not strictly dependent on the context of regulatory sequence.

The plant transformation vector bearing K2 type killer preprotoxin gene under transcriptional control of ADH1 promoter pGA/ADH1-Kil2 was introduced into plant *Nicotiana tabacum* via *Agrobacterium* mediated transformation. The transgenic plants possessed active K2 type toxin. Such results allow conclude that promoter of yeast gene ADH1 is transcriptional active in plant as well as preprotoxin can be proceeded in plant cell. Since K2 type toxin can prevent developing of some pathogen fungus it may be adopted in future to construct disease-resistant plants.

**Key words**: *Saccharomyces cerevisiae*, K2 preprotoxin gene, plant *Nicotiana tabacum*, transgenic plants.

**Introduction.** Plants are constantly exposed to a great variety of potentially pathogenic organisms, such as viruses, fungi, bacteria, protozoa, mycoplasma and nematodes, and can be affected by adverse environment conditions (Castro, Fontes, 2005). Although they do not have immune system, plants have evolved a variety of potent defense mechanisms including synthesis of low-molecular-weight compounds (phytoalexins), proteins (chitinases, ureases) and peptides (thionins, defensins, hevein-like proteins, knottin-like peptides) that have antibacterial or antifungal activity (Claude et al., 2001; Becker-Ritt et al., 2007).
Similarly, some bacteria, fungi or mammals synthesize a number of proteins and peptides with antiphytopathogenic properties (Selitrennikoff, 2001; Mandryk et al., 2007). There are discovered a number of yeast (*Saccharomyces cerevisiae, Ustilago maydis, Kluyveromyces lactis*) secreted proteins that are lethal to fungal cells (Magliani et al., 1997; Melvydas et al., 2006) or microbial-originated substances having antibiotic features (Melvydas et al., 2007; Mandryk et al., 2007). These proteins appear to be involved in either constitutive or induced resistance to bacteria or fungi. The mechanism of the action are as varied as their sources and include cell wall degradation of pathogens, membrane channel and pore formation, damage to cellular ribosomes, inhibition of DNA synthesis and inhibition of the cell cycle.

Many different genetic strategies have been proposed to engineer plant resistance to diseases, including producing antibacterial or antifungal proteins of non-plant origin, inhibiting microbial pathogenicity or virulence factors, enhancing natural plant defense and artificially inducing programmed cell death at the site of infection (Mourgues et al., 1998). Unlike classical breedings, genetic engineering allows the modification or introduction of one or more resistant traits into susceptible varieties (Mourgues et al., 1998). These days many genes encoding antibacterial proteins (as lytic peptides from insects, lyzocimes, lactoferrin, *Pichia anomala* killer toxin) have been cloned and expressed in plants (Donini et al., 2005). However, there are some problems associated with the efficiency of expression of foreign peptides and their toxicity. Considerable effort has been made for optimizing production of recombinant proteins. This research includes molecular technologies to increase replication, boost transcription, direct transcription in tissues for protein accumulation, stabilize transcript, optimize translation, target proteins to subcellular locations optimal for their accumulation and engineer proteins to stabilize them (Streatfield, 2007).

Previously antiphytopathogenic effect of different yeast species isolated from natural apples and grapes habitats was reported. Specifically, ability of *S. cerevisiae* K2 toxin to kill *Aspergillus* culture was demonstrated (Melvydas et al., 2006). Taking in account widespread appearance of killer yeasts and their attractive features, the aim of this work was to investigate expression possibilities of yeast K2 preprotoxin gene in plant *Nicotiana tabacum* and peculiarities of its functional manifestation depending on different transcriptional regulation.

**Object, methods and conditions.** The yeast expression plasmids pAD4 and pYEX12 (Gulbinienė et al., 2004) and plant vectors pCGT (Jefferson et al., 1987) and pGA482 (Proscievičius, Žukas, 1999) were used for construction of recombinant plasmids pCGT-Kil2 and pGA/ADH1-Kil2 (carrying *S. cerevisiae* K2 preprotoxin gene under control of *Cauliflower mosaic virus* CaMV and yeast ADH1 promoters) as well as yeast plasmids pAD/CGT-Kil2D and pAD/CGT-Kil2R (K2 under control of CaMVpr.). General procedures for the construction and analysis of recombinant DNAs were performed as described by Sambrook et al., (2001). All restriction enzymes (*SalI, SmaI, XbaI, Eam1105I, EheI, EcoI136II*), T4 DNA ligase, bacterial alkaline phosphatase, Klenow fragment and DNA size marker (GeneRuler™ DNA Ladder mix) were obtained from UAB “Fermentas” (Vilnius) and used according to the manufacturer’s recommendations.
The \textit{S. cerevisiae} strain \(\alpha\ 1\) (MAT\(\tilde{\alpha}\) leu2-2 (KIL-0)), sensitive to all killers (Čitavičius et al., 1972), was transformed by plasmids of interest according to (Gietz et al., 2002). Transformants were selected by complementation of \textit{LEU}2 auxotrophy. Killer phenotype-selective indicative medium (MB) (Sherman et al., 1986) was used to test killer toxin production and the immunity of transformants. Transformants were checked for toxin production in a killing zone plate assay following replica-plating of transformants onto a lawn of the sensitive strain \(\alpha\ 1\). Immunity was tested by the streaking the standard K1, K2 and K28 killer strains on the lawn of transformed cells. Stability of the Leu\(^+\) and K2\(^+\) phenotype of transformants was analysed growing cell colonies on non-selective YEPD medium (Sherman et al., 1986) 3 days at 30 °C and plating onto selective media: minimal – in case of LEU2; indicatory MB with layer of \(\alpha\ 1\) – in case of K2 preprotoxin gene.

Three parental crosses were performed to introduce the plant transformation vector pGA/ADH1–Kil2, possessing yeast killer toxin gene under control of yeast promoter ADH1, into \textit{Agrobacterium tumefaciens} GV 3101 containing Ti plasmid PMP90RK (Koncz et al., 1986). Fresh overnight cultures of \textit{Agrobacterium tumefaciens} GV 3101, \textit{E. coli DH}5\(\alpha\) containing vector pGA/ADH1-Kil2 and \textit{E. coli DH}5\(\alpha\) possessing helper plasmid Lelpm7623 were mixed for conjugation on solid YE medium (Draper et al., 1991). \textit{Agrobacterium} possessing vector pGA/ADH1-Kil2 was selected on medium containing 50 mg/L kanamycin, 10mg/L tetracycline and 50 mg/L rifampicin and was used for plant transformation.

Leaf explants of \textit{Nicotiana tabacum} SR1 grown \textit{in vitro} on MS medium (Murashige et al., 1962) were inoculated with overnight suspension culture of \textit{Agrobacterium} and co-cultivated on MSD-4 medium (MS supplemented with 0.1 mg/L naphthaleneacetic acid (NAA) and 1 mg/L benzylaminopurine (BAP)). After 3–5 days leaf explants were washed and replaced on MSD-4 medium supplemented with 400 mg/L cefatoxim to kill \textit{Agrobacterium}. Latter, callus-forming explants were replaced on the same medium supplemented with 50 mg/L kanamycin to select transgenes. Shoots regenerated on survived explants were rooted in MS medium with kanamycin. Plants possessing resistance to kanamycin were rooted and selected as transgenes.

**Results.** In the previous study it was determined that cDNA of the \textit{S. cerevisiae} K2 preprotoxin gene expressed under control of constitutive ADH1 promoter confer both killer and immunity phenotypes. Also, functional activity of K2 killer gene in yeast was demonstrated, gene expression was not strictly dependent on the context of regulatory sequence (Gulbiniene et al., 2004). Therefore, we decided to investigate expression of K2 gene controlled by ADH1 promoter in plant \textit{Nicotiana tabacum}. For this purpose recombinant plasmid pGA/ADH1-Kil2 (K2 expression controlled by yeast ADH1 promoter) based on plant vector pGA482 was constructed (Fig. 1).
K2 preprotoxin gene surrounded by ADH1 promoter and terminator was isolated from pYEX12 plasmid by the using XbaI restriction enzyme and inserted into pGA482 linearized by identical endonuclease. It is interesting to point out that obtained plasmids are suitable for plant transformation but not transformable into yeast, therefore can’t be replicated in *S. cerevisiae*.

To detect possibility of yeast killer toxin gene to be expressed in plant it was introduced into tobacco *N. tabacum* SR1 via Agrobacterium-mediated transformation. Callus formation on leaf explants started two weeks after co-cultivation with *Agrobacterium* GV 3101 containing plasmid pGA/ADH1-Kil2. Since plasmid pGA/ADH1–Kil2 possessed gene *nptI*, it was used for transgenes selection. Replacement of callus forming explants on kanamycin containing MSD-4 medium allowed growing and regeneration of transgenes. Co-cultivation with *Agrobacterium* during transformation slightly reduced viability of leaf explants. Control (without *Agrobacterium* treatment) callus formed 85.7 % of tested explants, after transformation procedures callus formed 77.2 % explants. Almost half of callus obtained after transformation was able to grow on medium with kanamycin. However, later only 9 out of 56 canamycin-resistant calluses survived and regenerated shoots. All of them were rooted on medium with canamycin and selected as transgenes (Fig. 2).

Explants were analysed for production of active K2 toxin either by the placing of small leafs directly onto yeast λ′1 layer or after the grinding of tested leafs in liquid nitrogen and spotting of biomass on medium inoculated with sensitive to killer toxins yeast (Fig. 3). Appearance of small clear lysis zones around leafs (Fig. 3 b) allowed conclude that yeast ADH1 promoter is transcriptional active in plant as well as preprotoxin can be preceded in plant cell. However, production of K2 toxin regulated by specific to yeast ADH1 promoter is barely detectable in plants and required stronger regulation by using more specific promoter as CaMV. Also, different processing of protein in plant comparing to yeast can decrease killer production and activity.
In order to analyze expression of K2 preprotoxin gene controlled by CaMV promoter in plants recombinant plasmids pAD/CYT-Kil2D and pAD/CYT-Kil2R possessing possibility to analyze this gene expression in yeast was created (Fig. 4).

For construction of abovementioned plasmids at first pCGT-Kil2D and pCGT-Kil2R constructs was obtained, by introducing of 1196 bp K2 preprotoxin gene (digested with SalI endonuclease and blunted with T4 DNA polymerase) into vector pCGT linearized by SmaI and EcorI restriction enzymes. Next, EamI11051 – EheI fragment bearing CaMV promoter, K2 preprotoxin gene and poliA nos terminator (from pCGT-Kil2D and pCGT-Kil2R respectively) inserted into pAD4 plasmid, resulting in pAD/CYT-Kil2D and pAD/CYT-Kil2R (Fig. 4). New constructs were transformed into
It was determined that stability of these plasmids (monitored by maintaining the Leu+K2+ phenotype) under both non-selective and leucine-selecting conditions reached 72–75%.

**Fig. 4.** Map of the yeast plasmids pCGT-Kil2D and pAD/CGT-Kil2D

It is important to point out that both α 1[pAD/CGT-Kil2D] and α 1[pAD/CGT-Kil2R] transformants (K2 gene placed in direct or reverse orientation to promoter sequence) shows weak killer activity and were sensitive not only to wt K2 toxins as well as to their own product (partial suicide phenotype) (Fig. 5).

**Fig. 5.** Testing of killer and immunity functions of α 1[pAD/CGT-Kil2D] yeast

**Discussion.** Since plants are exposed to many different pathogenic organisms it is important to construct transgenic cultures possessing defence abilities. Resistance
mechanism can be realized by self-production of antibacterial or antifungal proteins.

Yeast *Saccharomyces cerevisiae* K2 toxin, bearing antipathogenic properties, was selected for introducing into *Nicotiana tabacum* cells. Our investigation showed that transgenic plants possessed active K2 type toxin. Such results allow conclude that promoter of yeast gene ADH1 is transcriptional active in plant as well as preprotoxin can be proceeded in plant cells. However, toxin activity was weak and it is not clear reason of that: possible incorrect transcriptional regulation (non-specific to plants ADH1 promoter), plant reaction to the toxin formation inside the cell, different protein secretion mechanisms of yeast and plants. Also it was detected, that the functioning of foreign toxin protein may influence the plant vitality as the root system of transformants was faintly developed and formation of the callus was delayed. One of the possibilities to increase K2 killer production in plant is to use desirable promoter (exp. *Cauliflower mosaic virus* CaMV promoter) for transcriptional regulation. Given the capability of K2 type toxin to prevent developing of some pathogen fungus this feature may be adopted in future to construct disease resistant plants. It is reliable, that this research will provide important insights into more general aspects of foreign gene functioning in transgenic organisms.

**Conclusions.** 1. New plant transformation vector pGA/ADH1-K2 with K2 type killer preprotoxin gene under transcriptional control of yeast-specific ADH1 promoter was successfully introduced into plant *Nicotiana tabacum* via *Agrobacterium* mediated transformation. K2 toxin activity in transgenic plants was demonstrated.

2. Expression of K2 gene controlled by plant-specific CaMV promoter (pCGT/ADH1-Kil2) in yeast shows production of defective killer protein with reduced killer activity and immunity functions.

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**References**


Mielių Saccharomyces cerevisiae K2 preprotoksino geno raiška transgeniniuose augaluose

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Santrauka

Šio darbo tikslas buvo patikrinti mielių Saccharomyces cerevisiae kilerinio K2 tipo preprotoksino geno raiškos galimybės augaluose.

Eksperimentai atlikti pasinaudojant pagrindiniais genų inžinerijos, mikrobiologijos ir molekulinės biologijos metodais.

Sukonstruotos rekombinantinės plazmidės: augalų transformacijos vektorius pGA/ADH1-Kil2, turintis mielių alkoholdehidrogenazės ADH1 promotoriumi reguliuojamą S. cerevisiae K2 preprotoksino geną, bei mielių plazmidės pAD/CGT-Kil2D ir pAD/CGT-Kil2R (K2 raiška kontroliuojama žiedinių kopūstų mozaikos viruso CaMV promotoriumi). Atlikus išsamią CaMV promotoriumi reguliuojamo kilerinio geno raiškos analizę mielėse, parodytas 72–75 % siekiantis plazmidinis stabilumas bei nustatyta, kad produkuojamas balytmas pasižymi silpnomis kilerinėmis savybėmis ir nesugeba išlaikyti visaverčio imuniteto nei K2 tipo kileriams, nei savo paties produkuojamam toksinui. K2 geno funkcionavimo mielėse tyrimai atskleidė, kad šio geno raiška nėra griežtai priklausoma nuo reguliacinės sekos konteksto.


Reikšminiai žodžiai: Saccharomyces cerevisiae, K2 preprotoksino genas, augalas Nicotiana tabacum, transgeniniai augalai.