Gene expression and plant performance in oryzacystatin-I expressing transformed tobacco (*Nicotiana tabacum* L. cv Samsun) plants under abiotic stress.

By

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ABSTRACT

Plant cysteine proteinase inhibitors or also called phytocystatins inhibit the action of cysteine proteinases in plants. These proteinases are involved in many developmental processes by degrading proteins. In this study possible effects of an exogenous oryzacystatin-I (OC-I) expressed in transformed tobacco has been investigated. By challenging OC-I expressing and non-expressing tobacco with drought and heat stress, OC-I transcription and translation were not affected in OC-I expressing plants and plant extracts from stressed plants containing the inhibitor inhibited papain activity *in vitro*. Further, plant growth and photosynthesis was not greatly different under the selected growth conditions in both plant types under stress and non-stress conditions. However, OC-I expressing plants showed slightly lower photosynthetic rate, were shorter and had a higher lower dry mass production under non-stress condition. By applying cDNA Representational Difference Analysis (cDNA-RDA) to detect differentially expressed genes in the two types of plants, a gene coding for the light harvesting chlorophyll *a/b* binding protein gene (*lhcb1*) of photosystem II (LHC II) was

isolated from non-OCI expressing plants. Northern blot analysis showed lower transcript accumulation of the *lhcb1* gene in OCI-expressing plants both under non-stress and stress conditions, which was accompanied by lower chlorophyll content in OC-I expressing plants. Furthermore, plants benefited from OC-I expression by protection of a variety of expressed proteins against degradation. Identification of possible target cysteine proteinases for OC-I in tobacco resulted in the isolation, cloning and characterization of two new papain-like cysteine proteinases from tobacco designated *Nt*CP1 and *Nt*CP2. *Nt*CP1 was expressed only in senescent leaves and it was not induced in mature green leaves upon exposure to drought or heat stress. *Nt*CP1 has therefore a possible potential as a developmental senescence marker in tobacco. In contrast, *Nt*CP2, which was expressed in mature green leaves, has a high similarity to KDEL-tailed cysteine proteinases that are involved in programmed cell death. Both drought and heat decreased *Nt*CP2 transcript abundance in mature green leaves. Overall, this study has provided evidence that expression of exogenous OC-I does not significantly improve plant performance in tobacco in terms of physiological traits under drought and heat stress but provides protection in terms of stability of protein expression by possibly interacting with endogenous tobacco cysteine proteinases. Further detailed studies are suggested on the interaction of endogenous cysteine proteinases and exogenous phytocystatins to elucidate in more detail the type of interaction.

RESEARCH AIM AND OBJECTIVES

Genetic enginnering of plants, which involves the transfer of a sigle or multiple genes of interest to a plant genome, have been widely used both for introduction of desirable traits to plants and for a basic molecular biology study of gene function. A siginificant number of plants that have been transformed with stress tolerance genes have been generated. Evidences, however, suggest that the introduction of such genes into plant genome may not always results in desirable abiotic stress tolerant phenotype. This can partially be attributed to the level of expression of the transgene as well as subsequent stability of the transgene encoded protein under abiotic stress. Undesirable interaction of the introduced transgene with plant nomal function has been also a frequent phenomenon. In this PhD study, it was hypothesized that constitutive overexpression of a rice cysteine proteinase inhibitor transgene (OC-I) in tobacco could confere protection against abiotic stresses, such as drought and heat. The aim of this study was to compare OC-I expressing tobacco plants with non-transformed plants both at physiological and molecular level in order to prove the working hypothesis that OC-I could confer protection against abiotic stresses. The specific objectives were to: (1) study the expression and stability of the OC-I transgene under drought and heat stress, (2) evaluate growth performance of transformed and non-trasformed plants under drought and heat stress, (3) isolate differentially expressed genes between transformed and non-trasformed plants under heat stress by using a technique of representational difference analysis of cDNA (cDNA-RDA) and (4) clone tobacco cysteine proteinases that could be possible endogeous targets of exogenous OC-I.

THESIS COMPOSITION

Chapter one reviews the current knowledge about plant responses to drought and heat stress. This chapter in particular covers the present knowledge on genes that have been identified and investigated to respond to drought and heat and have also been used to enhance stress tolerance. Further, this chapter provides in greater detail an overview about previous and current research on the different types of plant proteinases and proteinase inhibitors, their action and location in plants and their involvement in plant stress reactions. **Chapter two** reports on the characterization of transformed tobacco, which expresses an exogenous rice cysteine proteinase inhibitor (OC-I) gene. In particular, the chapter deals with detection of inhibitor integration into the plant genome and expression of the inhibitor in transformed tobacco under drought and heat stress. **Chapter three** compares, by measuring a variety of physiological parameters, plant performance of OC-I expressing and non-expressing tobacco plants under drought and heat stress and combination of both stresses to evaluate any benefit for plants of exogenous OC-I expression. This chapter reports about studies that have been carried out in the greenhouse and in environmentally controlled growth chambers. **Chapter four** presents results of the isolation of gene sequences differentially expressed between OC-I expressing plants and non-expressing plants in response to heat treatment by applying the technique of c-DNA Representational Difference Analysis (cDNA-RDA). In particular, results of expression of a sequence coding for a chlorophyll-binding protein under heat stress are reported. Finally, this chapter also deals with results obtained for pigment production and protein expression patterns in OC-I expressing and non-expressing tobacco under stress and non-stress conditions using spectro-photometry for pigment content determination and two-

dimensional gel electrophoresis (2DE) for detection of expressed proteins. **Chapter five** describes the cloning and detailed characterization of two new papain-like cysteine proteinases from tobacco leaves. This chapter also presents the expression patterns of these proteinases in response to drought, heat and combination of both stresses. **Chapter six** summarizes the new aspects of the study. This chapter specifically focuses on how the study has contributed to an advanced understanding of the consequences of exogenous OC-I expression in tobacco and in particular the benefits gained from OC-I expression but also its limitation. Finally, this chapter also outlines possible future research activities including the isolation and characterization of endogenous cysteine proteinases that might interact with expressed exogenous inhibitors.

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ABBREVIATIONS AND SYMBOLS

ABA	Abscisic acid
ABRE	ABA-responsive element
APX	Ascorbate peroxidase
AREB	ABRE-binding proteins
BBTI	Bowman-Birk trypsine inhibitor
bp	Base pair
CaMV	Cauliflower Mosaic Virus
CO_2	Carbondioxide
COR15A	Cold-Regulated 15A
DRE/CRT	Dehydration-responsive element/C-repeat
DREB1/CBF	DRE/CRT binding protein
E-64	T*rans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
ESTs	Expressed Sequence Tag sequencing"
g	Gram
HR	Hypersensitive
HSF	heat shock factors
HSPs	Heat shock proteins
IPG	Immobilized pH gradient
IPM	Integrated pest management
JA.	Jasmonic acid
kDa	Killo Dalton
KIN	Cold-inducible
LEA	Late embryogenesis abundant proteins
LHC II	light harvesting chlorophyll a/b binding protein of photosystem II
LSU	Large subunit
M	Molarity
MeJA	Methyl jasmonate,
mL	Milliliter
MPSS	Massively Parallel Signature Sequencing
nm	Nanometer
OC-I	Oryzacystatin-I
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell death
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PhyCys	Phytocystatin
PI	Proteinase inhibitor
PMSF	Phenylmethylsulphonyl fluoride
RACE	Rapid amplification of cDNA ends
rbcL	Gene coding for large subunits of Rubisco

rbcS	Gene coding for small subunits of Rubisco
rd29A	Responsive to dehydration rd29A
RDA	Representational Difference Analysis
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RWC,	Relative water content
SAG	Senescence associated gene
SAGE	Serial Analysis of Gene Expression
SD	Standard deviation
SDG	Senescence down-regulated gene
SDS	Sodium dodecyl sulphate
SE	Standard error
SO_2	Sulfur dioxide
SSU	Small subunit
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween
TMV	Tobacco mosaic virus-i
U	Unit
UTR	Untranslated regions
UV	Ultra violet
VPE	Vacuolar processing enzyme
Z-phe-arg-AMC	Benzyloxycarbonyl-phenylalanine-arginie aminomythylcoumarin
α -AI-1	α -amylase inhibitor 1
µg	microgram
µl	Microlitre
µM	Micromolar
%	Percentage
°C	Degree Celsius
2DE	Two-dimensional gel electrophoresis
m	Metre

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