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Stress-induced genome alterations in plants

by

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ABSTRACT

Stress can alter the genomic composition of a plant. Among the stresses that can change the genome is the introduction of foreign DNA molecules via a plant tissue culture process. Representational difference analysis (RDA) was used as a novel technique to isolate and characterize genomic changes that may be associated with transgene insertion. Three different DNA sequences were isolated by RDA as subtraction products from tobacco expressing an exogenous cysteine proteinase inhibitor (cystatin) transgene and showing an conditional phenotype, namely reduced stem elongation, under low light. Subtraction products represent possible DNA sequence differences between wild-type plants and plants derived from a plant tissue culture/gene insertion process. Two methylation-sensitive subtraction products, Hp12 and Hp14, were similar to part

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of the tobacco chloroplast genome and the tobacco 18S rRNA gene, respectively. A third non-methylation sensitive DNA subtraction product, Hi30, had no significant homology to any reported DNA sequences. Screening a genomic library derived from a cystatin expressing plant, the Hi30 sequence could be localized in regions with homology to known repetitive DNA sequence families. Flanking regions of the Hi30 subtraction further revealed homology to DNA sequences of ribosomal RNA genes and to general cloning vectors. A variety of both wild-type and plant tissue culture/gene insertion derived tobacco plants were further screened by PCR for the presence/absence of the RDA subtraction products. Amplification products showed both changes in the copy number and DNA sequence variability. However, these changes could not clearly differentiate between the two types of plants. Due to the homology of one subtraction product to rDNA, plants were also screened for possible changes in the rDNA repeat unit. A similar pattern for the rDNA transcribed regions using Southern blot analysis were found regardless if wild-type or plants derived from a plant tissue culture/gene insertion process were used. However, generally less rDNA was present in plants derived from a plant tissue culture/gene insertion process. Overall, only minor genome changes could be identified with RDA in plants derived from a stressful tissue culture/gene insertion process, which could however not be clearly associated with to the genetic modification process.

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Research Objectives

There is evidence that stress can alter the genomic composition of the plant. The introduction of foreign DNA molecules into the plant genome to produce genetically modified plants might be among such stresses. Plant transformation involves a plant tissue culture process requiring plant growth regulators, antibiotics to regenerate and select from an explant and the transfer of an exogenous transgene. In this PhD project, the potential of the technique of Representational Difference Analysis (RDA) was therefore evaluated to possibly isolate and characterize genome changes that might have occurred as a direct result of the plant tissue culture/gene insertion process in tobacco plants. In particular (1) the evaluation of the RDA technique to identify genome changes in *in vitro* propagated genetically modified tobacco, (2) the identification and characterization of such possible genome alterations associated with plant transformation, (3) the location of such genome differences in the plant genome and (4) the general occurrence of such genome changes in a variety of both genetically modified and non-modified wild-type plants were studied. The experiments carried out in this PhD project were therefore focused on three primary objectives. These were (1) the evaluation of the usefulness to isolate genome changes in tobacco, which were possibly induced by a stressful event such as plant tissue culture/gene insertion, (2) the isolation and characterization of possible genomic variations associated with plant tissue culture/gene transfer in genetically modified tobacco, (3) genome localization of possibly altered genome sequences and (4) the possible detection of such changes in a variety of genetically modified tobacco plants. An additional avenue, the relationship of an unusual plant phenotype, which was observed in the transgenic lines, to transformation and exogenous gene expression, was also pursued in a preceding study.

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Thesis Composition

Chapter 1 of this thesis presents an introduction into stress in plants and what is currently known about stress-induced changes of the plant genome with a primary emphasis on changes in the DNA sequence. This first chapter summarizes research results so far obtained and includes the techniques that have been applied for characterization of genome changes and the advantages and disadvantages of these techniques. **Chapter 2** is an outline of a preceding physiological/biochemical study with genetically modified plant material carrying and expressing a cysteine proteinase inhibitor gene. This study formed the basis and objectives for a detailed analysis of the tobacco genome analysis *by Representational Difference Analysis* (RDA) to characterize genetically modified plants derived from an *in vitro* propagation/gene insertion process. **Chapter 3** focuses on the application of (RDA) on genetically modified tobacco. This chapter outlines the results obtained from application of this subtractive technique executed on genomic DNA digested with methylation-sensitive and non-sensitive restriction enzymes including the isolation and cloning of subtraction products. This chapter further outlines the results of their analysis using DNA sequencing and bio-informatics tools. **Chapter 4** focuses on experiments carried out identifying the location of subtraction products in genetically modified tobacco plants by screening a constructed genomic library with cloned subtraction products. This chapter also outlines the results of the application of a two-step PCR method with biotinylated primers to identify and characterize tail-end flanking regions of one of the RDA subtraction products. **Chapter 5** describes the results obtained from the screening of different types of tobacco lines with DNA primers designed from analyzed subtraction products with the emphasis to identify a possible genetic marker for transformation in genetically modified plants. **Chapter 6** the **Summary and Perspective** outline the scientific achievements made by this thesis and also the failures are mentioned and discussed, and an overview about possible future research

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activities is provided. Finally, in the **Annex** the methods and sequence data used in this study are described.

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ABBREVIATIONS AND SYMBOLS

BAP	-	6-Benzylaminopurine
bp	-	Base pair
°C	-	Degrees Celsius
dH_2O	-	Distilled water
DNA	-	Deoxyribonucleic acid
E.coli	-	*Escherichia coli*
f.w	-	Fresh weight
g	-	Grams
gus	-	ß-glucoronidase reporter gene
h	-	Hour
H_2O	-	Water
kDa	-	Kilo dalton
kPa	-	Kilo pascal
L	-	Litres
LB	-	Luria Bertani
M	-	Molarity
mg	-	Milligrams
ml	-	Millilitres
mM	-	Millimolar
MS	-	Murashige and Skoog
NaOAc	-	Sodium acetate
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
ng	-	Nanograms
nm	-	Nanometre
NPT II	-	Neomycin phosphotransferase II
OC-I	-	Oryza cyctatin I
PAGE	-	Polyacrylamide gel electrophoresis

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pfu	-	Plaque forming units
pH	-	Log hydrogen ion concentration
RNA	-	Ribodeoxynucleic acid
sdH_2O	-	Sterile distilled water
SDS	-	Dodecylsulphate sodium salt
sec	-	Seconds
SSC	-	Sodium chloride; Sodium citrate
T-DNA	-	Transfer DNA
U	-	Unit
µg	-	Micrograms
µl	-	Microlitres
µM	-	Micromolar
UV	-	Ultra violet
%	-	Percentage

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Electropherogram from direct sequencing of DNA template derived from tobacco cultivar '*Samsun/UK*' (NT1) showing the end of the DNA fragment when sequenced from the opposite side compared to the sequence in Figure 5.2. Black arrow indicates the decrease in peak heights. Grey arrows indicate Hi30L primer site. A similar electropherogram was also seen for DNA template derived from tobacco cultivar '*Samsun/San1191*' (San3).

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Alignment of DNA regions amplified by PCR with primers Hi30R and Hi30L using tobacco genomic DNA from different tobacco plants as template. Hi30 represents the sequence of the original Hi30 subtraction product isolated from genetically modified plants. Sequence data shown are from the 200 bp amplification product from transformed tobacco ('*Samsun/UK*') without a gene insert (T-C1 and T-C2); tobacco cultivar '*Samsun/San1191*' (San2 and San3); NT1 and NT2 sequence data from non-modified wild-type tobacco DNA; 300 bp amplified product using '*Samsun/San1191*' (San3B) and wild-type tobacco '*Samsun/UK*' (NT1B) DNA as template. Underline areas indicated Hi30L and Hi30R primers, Gap=(-), Identical base pairs aligned with Hi30=(.).

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Electropherogram from sequencing of DNA template derived from a genetically modified tobacco plant ('*Samsun/UK*') containing the *gus* gene insert (G1). Arrow indicate an example of a double peak.

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of the Hi30 subtraction product aligned at different locations with the original flanking sequence represented in A. (T-C1 and T-C2) Tobacco plants (*'Samsun/UK'*), which went through a transformation process but not carrying a transgene. (NT2 and NT3) Genomic DNA-derived from non-modified wild-type individual tobacco plants of cultivar *'Samsun/UK'*. (T1 and T2) Amplification of genomic target DNA derived from genetically modified tobacco plants (*'Samsun/UK'*) carrying the OC-I coding sequence. (San1 and San2) DNA from non-modified tobacco cultivar *'Samsun/San1191'*. (NT1) Genomic DNA-derived from the non-modified wild-type individual tobacco plant used in the RDA procedure. Primer 3 used in the amplification of the original flanking sequence (A) are underlined as well as the Hi30R and H30H30 primer sequences, Gap=(-), Identical base pairs aligned with A=(.).

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