SZENT ISTVÁN EGYETEM

Isolation and characterisation of the subunit of the SNF1 protein kinase complex from *Solanum tuberosum.*

Thesis

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Introduction

Potato (*Solanum tuberosum*) is one of the most important crop plant. Potato is used as a stock by the food, alcohol, and pharmaceutical industry.

Potato tuber is an underground storage organ that is also can be used for vegetative propagation of potato. Potato tuber contains high amount of starch (12-24%) and the protein content is also significant (0,7-4,6%).

Administration of high concentration of sucrose the in vitro tuberisation system leads the single node stem segments to develop tuber. Administration of lower concentration of sucrose (2%) does not lead tuber formation at all. It was concluded that sucrose is the primary signal for tuberisation. The in vitro tuber induction system was studied at the molecular level. Number of genes has been isolated with high level of expression in the potato tuber. Some of them encoded enzymes involved in starch synthesis (sucrose synthase, AGP-ase, GBSS). The others are considered to be encoded storage proteins (patatin, proteinase inhibitors of different kinds). Genes for starch synthesis and storage proteins are also regulated by administration of high concentration of sucrose, which means that in the leaves of potato the signaltransduction pathway triggered by the high concentration of sucrose is also present. Nevertheless the regulation of patatin promoter was studied in heterologous systems. These studies told to us that patatin promoter was also strictly regulated in the leaves of tobacco and in the leaves and the root of *Arabidopsis thaliana* by high concentration of sucrose proving that the signaltransduction pathway triggered by the high concentration of sucrose is also conserved in higher plants.

We know the primary signal that triggers potato tuber development and the abundant genes expressed in potato tuber, however we have no information about the signaltransduction pathway regulates tuber development and starch accumulation.

The yeast SNF1 kinase is one of the best known protein kinase The SNF1 gene takes part in the derepressing of the glucose repressible genes, involved in glycogen storage, regulates different metabolic enzymes at the post transcriptional level. SNF1 kinase is a key regulator of carbon metabolism in the yeast *Saccharomyces cerevisiae.*

The most typical characteristic of tuber development is the accumulation of starch. Since in the budding yeast the key regulator of carbon metabolism is the SNF1 kinase, we suspected that plant SNF1 kinases might be involved in starch accumulation during tuber development. Therefore we decided to isolate and characterize SNF1 kinase and its interacting proteins from *S. tuberosum.*

Materials and Methods

Library screening

Yeast transformations were performed by the lithium acetate method. Strain YRG-2 was transformed with pLL32 to Trp prototrophy. The resulting strain was used in the interaction trap screening with an oriented cDNA library in vector pAD-GAL4. The library was established from epidermal fragments of *Solanum tuberosum* L. cv. Désirée. After 4-6 days of incubation His^+ colonies were screened for β-galactosidase activity using a filter lift assay. Quantification of β-galactosidase activity was done by the ONPG assay. Positive colonies were picked from SD-Trp-Leu-His plates and grown overnight in SC-Trp-Leu liquid medium. DNA prepared from these cultures was transformed into DH5α and selected for ampicillin resistance bearing the AD library plasmid.

Molecular biology techniques

Plasmid constructs were generated by standard methods. Restriction enzyme digestions, ligations and PCR reactions have carried out by the manufacturers instructions.

Isolation of the 5`-end of the StubGAL83 cDNA

Isolation of the 5`-end of StubGAL83 cDNA was performed by two rounds of nested PCR using the entire two-hybrid cDNA library as a template. Primer GALT (position 168-187 on the resulted full length StubGAL83) 5'-GAC CAT CAA GTC AGC CGA AG-3' was used for the first round PCR, and GALK (position 139-158 on the resulted full length cDNA StubGAL83) 5'-AGG TCA GAG CGT GGT GAT CT-3' for the second PCR reaction as gene-specific primers. For a non-specific primer, Gal4AD primer 5'-TTC GAT GAA GAT ACC-3' was used. After the second PCR reaction the longest PCR product was cloned into an *Eco*RV digested pBluescript and sequenced

DNA sequencing

Sequencing was completed by using the USB kit Sequenase version 2.0 and partly by the company MWG-Biotech, Germany.

Nucleic acid isolation and hybridization

Total RNAs were extracted with the SDS-phenol method followed by a LiCl precipitation. Hybridization of filters was carried out in Church buffer.

Genomic DNA was isolated by the method of CTAB. Hybridization was performed in 50 mM Tris pH7.5, 1 M NaCl, 1% SDS, 10% dextran sulfate, 100 μg/ml denatured salmon sperm DNA at 65 °C for overnight. Washing of the filter was carried out first in destilled water for 2 min at room temperature then in 2×SSC, 1% SDS for 30 min at 65 °C.

Protein expression and in vitro binding assay

GST and TRX protein expression and binding assay was carried out according to the manufacturers instruction with the following modifications: i, Bacterial pellets were resuspended in cold ST (30 mM Tris pH 8.0, 120 mM NaCl) with 100 μg/ml lysosyme. ii, The TRX fusions were eluted with ST containing 20, 50, 300, 500 mM imidazole. iii, Western blot analysis was carried out against S-Tag using the S-Tag Western blot kit (Novagen).

Results

We have found that that the already known plant SNF1 kinases are showing 70-85 % homology to each other. The homology is more striking within the kinase catalytic domain, therefore we designed degenerated primers according to the alignment of the tobacco NPK5, rye RKIN1, ATKIN1 of Arabidopsis, and the barley BKIN12 SNF1-like kinases. We used these degenerated primers in a PCR reaction containing a 5 days old potato tuber plasmid cDNA library. The 400 bps long PCR product was sequenced and was used to screen a tuber phage library to isolate the full-length cDNA clone. The protein encoded by the full-length cDNA clones 512 aa long (StubSNF1) and showed 89% homology to tobacco NPK5 and 73% homology to potato PKIN1, an other SNF1-like protein kinase from potato.

To identify protein(s) interacting with SNF1, a potato leaf epidermal fragment yeast

two-hybrid cDNA library was screened with StubSNF1. From a total of about 600 000 trasformants tested, 24 His^+ yeast colonies were isolated. 8 out of them were proven to be blue in the beta-galactosidase filter lift assay. After partial sequencing, 5 different cDNAs were identified. Three cDNAs were overlapping and showed homology to the yeast GAL83/SIP1/SIP2 gene family. The longest cDNA, designated StubGAL83, was 1026 bps in size. The other two overlapping cDNAs (ΔStubGAL83) were the same in size (516 bps) and resulted in fact from two independent transformation events of one plasmid present in the primary cDNA library. DNA sequence analysis showed that they were 100% identical to the corresponding part of the StubGAL83 .

Filter lift assay was carried out to confirm the positive interaction between StubSNF1 and StubGAL83. cDNAs encoding StubSNF1 and StubGAL83 or ΔStubGAL83 were cloned into both pBD and pAD vectors. Co-transformation experiments were carried out and interactions could be detected when StubSNF1 was present in the bait or pray vector with StubGAL83 or □StubGAL83 provided in the opposite vector. In contrary, interaction was not detected in the presence of StubSNF1 or StubGAL83 pairwaised with empty vectors.

To confirm the direct interaction between StubSNF1 and StubGAL83, bacterially expressed fusion proteins were used for *in vitro* binding experiment. StubSNF1 was expressed as a GST fusion protein. The isolated and re-immobilised GST-StubSNF1 retained the provided TRX-StubGAL83 protein while in control experiments with recombinant proteins GST-StubSNF1 vs.TRX, GST vs. TRX-StubGAL83 and GST vs. TRX, no retention was detected (Figure 1B). These results suggest that StubSNF1 directly binds to StubGAL83. To recontsruct the full-length cDNA the missing 5'-end was PCR amplified and fused to StubGAL83 cDNA isolated in yeast two-hybrid system. The full length StubGAL83 cDNA consists of 1071 nucleotides in length, the longest open reading frame encodes a putative protein of 289 amino acids. Database searching revealed that the protein encoded by the StubGAL83 is homologous to the yeast GAL83/SIP1/SIP2 proteins to the FOG1 protein of *Kluveromyces lactis*, to the mammalian AMPK beta subunits and to an *Arabidopsis thaliana* ORF encoding a 289 aa. protein.

In yeast GAL83/SIP1/SIP2 proteins and in the mammalian AMPK beta subunit, two distinct well-conserved domains were identified. The first internal domain, called KIS domain (Kinase Association Domain), is responsible for the interaction with SNF1. The C-terminal ASC (Association with the SNF1 Complex) domain interacts with SNF4. Sequence alignment revealed that both the KIS and the ASC domains are also conserved in StubGAL83 suggesting these domains might be involved in similar interactions described in yeast and

mammals. Amino acid sequence alignment of the predicted StubGAL83 protein with the yeast and mammalian homologs showed that StubGAL83 contains the two well conserved region characteristic for all proteins aligned .

We subunit have mapped the domain involved in the interaction between StuSNF1 and StubGAL83, and we found that the C-terminal regulatory domain of the StubSNF1 binds to the StubGAL83 protein, just like in the case of the corresponding yeast proteins.

The SNF1 complex in yeast is a heterotrimetric protein of three subunits, i.e. catalytic (SNF1 kinase), the adaptor (GAL83/SIP1/SIP2) and the activator subunit (SNF4). By the help of the yeast two-hybrid screening we have identified the StubGAL83 protein that possibly corresponds to the adaptor subunit of the yeast SNF1 kinase complex. Furthermore, a *Phaseolus vulgaris* cDNA was isolated from encoding a protein similar to the yeast SNF4. These findings led us to speculate that SNF1 in plants may also function as a heterotrimeric complex. To test this hypothesis interaction of StubGAL83 with the yeast SNF4 was investigated in the yeast two-hybrid system These data suggest that plant SNF1 protein kinase may be a heterotrimeric complex similar to that found in yeast and mammals.

Novel findings of the thesis

1. We have isolated StubSNF1, a new SNF1-like kinase from potato tuber.
2. We have isolated StubGAL83, the first plant SNF1 kinase interacting protein by the help of the yeast two-hybrid system.
3. StubGAL83 shows significant homology to the yeast SIP1/SIP2/GAL83 and AMPK β protein. Our result proved that plant SNF1 kinases may function in complexes to those detected in the yeast and mammals
4. It was found that the same domains take part in the interaction between StubSNF1 and StubGAL83 than in the case of the corresponding yeast and mammalian proteins.
5. We established the conserved interaction between the potato StubGAL83 and the yeast SNF4 suggesting that plant SNF1 protein kinase may be a heterotrimeric complex similar to that found in yeast and mammals.

Conclusions and suggestions

We have isolated StubSNF1, a new plant SNF1 protein kinase from potato. Then we used StubSNF1 in the yeast two-hybrid system to isolate StubSNF1 interacting protein(s). A

protein showing homology to the yeast GAL83/SIP1/SIP2 proteins and in the mammalian AMPK β subunit. We began the initial characterisation of the potato SNF1 complex. Having isolated two out of three subunits of the possible plant SNF1 complex let us establish StubSNF1 and StubGAL83 antisense and overexpressing potato plant to unravel the function of the SNF1 kinases in potato.

Publications made the thesis from

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