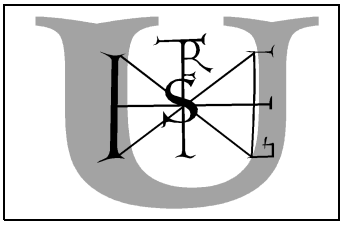

SZENT ISTVÁN EGYETEM

Improving the nutritional value of tubers by overexpressing the serine acetyl-transferase gene

PhD thesis

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Gödöllő

2008

PhD school: Plant Sciences

Field of science: Plant cultivation and horticulture

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BACKGROUND, AIMS

Humans and monogastric livestock are incapable of synthesizing ten of the twenty amino acids essential to life. The inability to synthesize the ten missing 'essential' amino acids can be compensated through a diet rich in plant matter. It therefore follows that the nutritional quality of a plant crop grown for human consumption should not only be measured by its' energy producing sugar/starch; yet also by the amino acid composition of its' storage proteins. Improving the nutritional value of crop plants is a major focus of current biotechnological research. In developing eatable crops with a high amino acid composition within their storage proteins, biotechnological research currently focuses on:

-Overexpression of the key enzyme of the amino acid's pathways.

-Repression of the key enzyme (antisense techniques).

-Expression of protein enriched in the limited amino acids by foreign, manipulated or synthesised genes.

-A combination of the approaches above.

The potato is the most important non-cereal food crop used for animal feed. The amino acids limiting its nutritive value are the sulphur containing amino acids: cysteine and methionine. These are the only amino acids containing sulphate-the reduced form of sulphur. They are not only an important substrate of protein biosynthesis but also precursors to various other metabolites such as glutathione, phytochelatins, S-adenosilmethionin, ethylene, polyamines, biotin. As methyl donors they are involved in numerous cell processes. In plants methionine and cysteine are the initial product of sulphate assimilation.

Synthesizing of cysteine from sulphide and *O*-acetyl-L-serine (OAS) is catalysed by *O*-acetyl-serine (thiol) lyase (OAS-TL). OAS is formed by serine acetyl-transferase (SAT) from acetyl-coenzyme A and serine. In plants, SAT and OAS-TL form a complex that plays an important role in the regulation of both enzymatic activities. The most important steps of cysteine biosynthesis are

similar in bacteria and plants. In addition, the bacterial isoforms of SAT and OAS-TL are active in plants. Both enzymes are targeted into compartments with active protein synthesis: mitochondria, chloroplast and cytosol. According to the model proposed for *Arabidopsis thaliana*, the feedback inhibition of the cytosolic SAT isoform by cysteine plays a central role in regulating the level of OAS in the cytosol. Progress in this field has led to the cloning of genes that play pivotal roles in cysteine formation.

Evidence that SAT plays an important role in regulating cysteine biosynthesis has been obtained through the overproduction of SAT in transgenic tobacco, *Arabidopsis* and potato plants.

The molecular biological gene transfer - so called genetic modification - is getting widespread use in plant breading too. These GM plants will inevitably encourage new agricultural markets for the future thus ensuring that green-biotechnology becomes the unavoidable basic platform towards environmentally safer agriculture.

The vector used for gene transfer in genetically modified plants contains not only the 'useful' gene, but also a marker gene for selection - usually a gene responsible for antibiotic or herbicide resistance.

According to these our main aim was to improve the nutritional value, namely its cysteine and maybe methionine content, of potato by marker-free transformation.

MATERIALS AND METHODS

Bacterial and plant materials

Escherichia coli strain was used for the cloning steps with *Agrobacterium tumefaciens* containing pGV2260 strain being used for

transformation. Transgenic lines were obtained by marker-free tuber disc transformation. The in vitro plants were vegetatively propagated from shoot cuttings and were grown in a lightroom.

Molecular biological methods

The nucleic acid purification and manipulation were done by the generally used methods in accordance with protocols supplied by manufactures.

Oxidative-, osmotic-, metylglyoxale treatment

The leaf discs cut out from wild type and transgenic plants were floated in different concentrations of H2O2, NaCl, or methylglyoxale; with distilled water being the control. The chlorophyll content was measured photometrically.

Assay of serine acetyl-transferase activity

This method is based on the disulfide interchange between HS-CoA released from acetyl-CoA during the SAT catalysed reaction, and DTNB. The production of TNB was monitored at 412nm using a spectrophotometer.

Determination of cysteine and glutathione levels

Both cysteine and glutathione levels were determined by reverse-phase HPLC.

RESULTS

Development of serine acetyl-transferase overexpressing plants by marker free transformation

Cloning of E. coli SAT gene into a vector applicable for marker free transformation

The potato cv. White Lady is a Hungarian potato cultivar resistant to several viral strains found in Hungary. Our aim was to further improve the value of this line by increasing methionine content in tubers. To increase SAT activity of the potato cv. White Lady, the same chimeric *SAT* gene consisting of the constitutive CaMV 35S promoter-*cysE-rbcS* transit peptide sequence was used as in Désirée before by Harms et al. (Plant J., 2000, 22: 335-343). For the marker free transformation method, we recloned the chimeric gene into the binary vector pBinMF.

The marker free transformation of Solanum tuberosum cv. White Lady

The vector from *E. coli* was transferred into *A. tumefaciens* by conjugation, and then used to infect forty in vitro, White Lady tuber slices. Six weeks after placing the discs on regeneration medium, one hundred and ten, 3-5 cm length shoots were excised and placed on rooting medium.

The selection of SAT transgenic potato lines and the expression study of SAT

PCR reaction analysis identified two promising transgenic lines designated as SAT1 and SAT2. Expression of the SAT gene in the leaves of the two transgenic lines was compared to that of the transgene in Désirée lines (Plant J., 2000, 22: 335-343) and showed much higher SAT transcript level in the White Lady than in the Désirée transgenic lines.

Analysis the presence of vector backbone in transgenic lines

We concluded that the SAT1 and SAT2 lines are possible backbone free because the npt III and trfA as vector genes were not able to be detected by PCR analysis.

The molecular analysis of the SAT overexpressing potato lines

Analysis of SAT activity in the leaves of transgenic lines

The analysis of SAT activity in the leaves of transgenic lines showed an 80-fold increase in SAT activity relative to controlled plants. SAT activity was much higher than such measured in Désirée plants. The SAT enzyme activity correlated with the amount of *SAT* mRNA.

Analysis of expression and activity of SAT in the tubers of transgenic potato lines

However, the detected mRNA level was lower in tubers than in transgenic plant leaf samples with an additional loss in comparitative storage. Although not performing as strong as the transgenic plants, the transgenic tuber lines still performed better than the controlled plant tubers.

The SAT enzyme activity showed correlation with the mRNA level, with SAT activity being in the fresh SAT1 and SAT2 tubers being 20 times higher than that found in the controls.

Thiol content in SAT overproducing White Lady plants

The measurement of thiol content by reverse phase HPLC found a comparative 1.4-1.5-fold increase in the level of cysteine and glutathione in transgenic leaves to that of the control. However, after calculating the standard deviation of the separated measurements, only the SAT2 line showed significant differences to the control White Lady plants. The thiol content of the SAT expressing tubers was also affected. The amount of cysteine was significantly higher in the SAT2 tubers compared to controls, with glutathione content being higher in SAT1 and SAT2 tubers than that of the control group. The total protein content was not affected.

The effect of SAT overexpression on the stress resistance

The alteration of the chlorophyll content was analysed in leaf discs treated with different concentrations of H_2O_2 and NaCl. There was no notable chlorophyll content change between the SAT overexpressing plants and the untransformed control group. However, glutathione content was higher in the SAT lines, yet could not reach levels sufficient enough as to improve resistance to osmotic or oxidative stress. The same conclusions were drawn from the methylglyoxal test.

The effect of SAT overexpression on gene expression

The expression of 116 genes, mainly of carbohydrate metabolism and amino acid biosynthetic pathways were analysed by reverse northern analysis. No repeatable significant differences were detected in the SAT1 and SAT2 lines compared to that of the controls. So the overexpression of the SAT gene had no effect on the expression of genes we analysed.

Tuber yield and sprouting behaviour of the SAT overexpressing lines

No phenotypical difference was shown between the transgenic and control plants. Visual assessment found no morphological difference in shape, colour, size or yield distribution between the tuber groups either. Analysis of sprouting behaviour found that SAT transgenic plants break dormancy and produce sprouts at the same time and rate as the control.

Novel results

1. To develop SAT overexpressing potato plants, the chimeric *SAT* gene consisting of the constitutive CaMV 35S promoter-*cysE*-*rbcS* transit peptide sequence, was cloned into the pBinMF vector applicable to marker-free transformation, and then transferred into *Agrobacterium*

tumefaciens. After tuber transformation, two independent marker and backbone free transgenic lines designated as SAT1 and SAT2 were isolated by PCR.

2. Both selected transgenic lines had higher *SAT* mRNA levels not only in their leaves, but also in their tubers than that of the SAT expressing Désirée transgenic lined obtained by Harms et al. (Plant J., 2000, 22: 335-343).

3. In concert with higher mRNA levels, an 80-fold increase of SAT activity in the leaves as well too, 15-20-fold increase within fresh tubers was detected compared to that of the control plants. The activity in tubers decreased when stored.

4. Reverse phase HPLC found that the altered SAT activity resulted in the increase of cysteine and glutathione content. No difference was found between the soluble protein content of the SAT overexpressing and control plants.

5. Instead of the increased thiol content in the transgenic plants no stress resistance to H_2O_2, NaCl or methylglyoxal could be presented in them.

6. The reverse northern study analysing the expression of 116 genes - mainly of carbohydrate metabolism, nitrogen and sulphur - concluded that they were not influenced by SAT overexpression.

7. The yield, shape, size, and sprouting behaviour of tubers were not influenced by SAT overexpression.

CONCLUSIONS AND SUGGESTIONS

Potato is the most important non-cereal food crop. The amino acids limiting its nutritive value are the sulphur containing amino acids: cysteine and methionine. Several studies have shown that SAT plays an important role in regulating cysteine biosynthesis.

To increase cysteine content in potato, a chimeric *E. coli SAT* gene consisting of the constitutive CaMV 35S promoter-*cysE-rbcS* transit peptide sequence was introduced into Désirée plants by Harms et al. (Plant J., 2000, 22: 335-343).

The aim of our study was the same; to increase the nutritional value of potato variety White Lady. To achieve this goal, the same chimeric *SAT* gene was used as by Harms et al.; however a marker free method was applied for transformation so as to allow further transgene pyraminding and thereby develop an acceptable and safe cultivar for potential market application.

Two independent transgenic lines, designated as SAT1 and SAT2 were isolated. Both showed a four-fold increase SAT activity in their leaves that of the SAT-expressing Désirée transgenic lines SAT26 and SAT48; with an 80-fold increase compared to non-transformed control plants. The molecular reasoning between the two cultivars expressing SAT gene activity might be attributable to having used the genetically different White Lady instead of Désirée, and/or the transgenes' position in the potato genome.

SAT activity was also detectable in tubers. Such was found to be 20 times higher in the fresh SAT tubers than in the control ones. Although there was a decrease in storage, it was still 5 times more active than the non-transformed tubers. The difference between the SAT activity measured in leaves and tubers could be explained by the altered activity of the constitutive 35S promoter in different organs.

The increased SAT activity in the transgenic tubers seemed to sufficiently increase not only the cysteine, but glutathione content as well. This is an

important improvement when compared to the results obtained by Harms et al. This also supports the hypothesis that under normal conditions, when sulphur supply is not limiting the reaction, the very low endogenous activity of SAT is one of the factors limiting cysteine biosynthesis.

Our results show that increasing cysteine content stimulates glutathione biosynthesis with higher glutathione content resulting. Similar results were found in tobacco and poplus.

Since glutathione holds an important role in the elimination of reactive oxygen species, we analysed the stress resistance of the SAT leaves. However, they didn't show resistance to different concentration of H_2O_2, NaCl or methylglyoxale. These results may be explained in the level of glutathione, which was 1.4-1.5 times greater than in the control leaves

Reverse northern analysis found SAT overexpression, to have no effect on the key enzymes directing the main metabolic pathways.

The plants showed normal wild-type phenotype, with no difference in the shape, colour, size, yield, or sprouting behaviour of the SAT tubers compared to the wild types obtained.

The results presented here support the view that SAT overexpression can increase the cysteine content in plants without interrupting the expression of the main metabolic pathways or the yield and sprouting behaviour which is crucial from the agricultural industries perspective.

The SAT overexpressing plants presented in this study are good starting points from which further research into improving the nutritional value of the potato can continue.

The free cysteine is lost in food processing or cooking. Such losses are minimised if amino acids are in a stable protein and a component of a crop. Thus, the strategy is not only to manipulate the amino acid metabolism, but simultaneously overexpress a sink protein – which in our case is a cysteine rich protein like the methallotioneins. The tuber specific expression would be

maintained by an organ specific promoter like the patatin promoter. Patatin is an abundant storage protein in tuber. Channelling the excess cysteine to a cysteine rich protein would further improve the nutritive value of potato. Moreover, synthesis of this protein would decrease both the level of free amino acid and feedback control of cysteine of the cysteine sensitive SAT enzyme. The result would be increased storage of cysteine at levels much higher than the free-cysteine levels found in our experiments.

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