Transgenic tobacco plants expressing fungal copper and zinc transporter genes show enhanced acquisition of copper and zinc

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Abstract

Copper (Cu) and zinc (Zn) are micronutrients essential for growth and differentiation of all organisms, but are often deficient in human diet. In the present study, transgenic tobacco plants expressing two fungal genes - namely a copper transporter (tcu-1) and a zinc transporter (tzn1) gene from Neurospora crassa were developed and the plants showed enhanced acquisition of Cu and Zn respectively compared to wild-type plants.

Introduction

Copper (Cu) and zinc (Zn) are essential micronutrients, indispensable for life of all organisms, Zinc and to a lesser extent Cu are normally deficient in human diet, especially in the diet of the population of developing countries. Phyto-fortification – enrichment of edible parts of crop plants with essential elements can be achieved, by forthcoming plants using conventional plant breeding methods or by transgenic technology. Although, plants have the inherent potential to take up these metals, their ability can be further enhanced by development of transgenic plants, with potential genes from heterologous sources. In the present work, a copper transporter (tcu-1) and a zinc transporter (tzn1) gene from fungus Neurospora crassa were cloned in binary vector pCAMBIA1301 (CAMBIA, Brisbane, Australia) [1,2]. The resulting plasmid has hpt II as a plant selectable marker and uidA as the reporter gene, apart from tcu-1/tzn1. Plasmids were finally introduced individually into Agrobacterium tumefaciens EHA 105 cells, using an electroporator 2510 (Eppendorf, Hamburg, Germany).

Experimental

Cloning of Cu and Zn transporter from Neurospora crassa and development of transgenic plants

Based on Cu and Zn accumulation studies in N. crassa, a Cu transporter gene referred as tcu-1 (NCU00830.2) and a Zn transporter gene tzn1 (NCU07621.3) of N. crassa having higher affinity for Cu and Zn, respectively, were chosen for the present work. The complete cDNA of the tcu-1 and tzn1 were cloned in plant binary vector pCAMBIA1301 (CAMBIA, Brisbane, Australia) [1,2]. The resulting plasmid has hpt II as a plant selectable marker and uidA as the reporter gene, apart from tcu-1/tzn1. Plasmids were finally introduced individually into Agrobacterium tumefaciens EHA 105 cells, using an electroporator 2510 (Eppendorf, Hamburg, Germany).

Transgenic Nicotiana tabacum L. cv Havana 425 plants were developed using Agrobacterium tumefaciens, according to the procedure described earlier [1].
Stable integration of tcu-1/tzn1 in the genome of transgenic plants was confirmed by Southern blot hybridization. Expression of tcu-1/tzn1, as well as other transgenes in plants, was confirmed by reverse-transcription PCR (RT-PCR) using Affinity Script Multiple Temperature cDNA Synthesis kit (Stratagene, La Jolla, CA, USA)[1,2]

Copper/Zinc acquisition in transgenic (T₃) plants grown in hydroponics

Initially, ten independently transformed and confirmed T₀ tobacco lines in triplicate, (multiplied by micropropagation of shoots for developing same clones) along with control, were grown in Hoagland’s liquid medium spiked with different concentrations of Cu/65Zn. Copper content was estimated by GBC 932 B+ Atomic Absorption Spectrophotometer (GBC, Melbourne, Australia) using air-acetylene flame. 65Zn estimation was done through gamma ray spectrometry [1].

Results and Discussion

Cloning of tcu-1/tzn1 and development of transgenic tobacco plants

A high affinity copper transporter gene tcu-1 and zinc transporter gene tzn1 were cloned from Neurospora crassa and introduced into plant expression vector pCAMBIA1301. Transformation of tobacco was conducted by Agrobacterium tumefaciens-mediated co-culture method, using leaf discs and transgenic plants selected on medium containing hygromycin B.

In Southern blot analysis with genomic DNA, all transgenic lines selected for the study showed a single fragment, while control plant did not show any signal, suggesting stable integration of single copy of tcu-1/tzn1 in selected transgenic lines as well as absence of homologous DNA sequence in control plants (Fig. 1). Results of RT-PCR using total RNA from transgenic plants confirmed that tcu-1/tzn1 gene (as well as hptII and uidA) expressed in all selected transgenic lines, while there was no expression in control plants (Fig. 2).

Acquisition of copper/zinc by plants grown in hydroponics

When the performance of different T₃ transgenic lines in hydroponic solution with basal Cu level (ie 0.32 μM) was compared, all the transgenic lines showed higher Cu content in shoots with C-2-1 and C-2-4 plants showing up to 2.5 times higher Cu accumulation compared to control plants. Similarly, accumulation of Cu in roots was
higher in most of the transgenic lines compared to control. Among them the best performing line C-2-4 showed 2.8 times higher accumulation of Cu in roots from solutions than control plants.

When the effectiveness of heterologous Cu transporter for Cu acquisition in tobacco plants exposed to a broad range of Cu concentrations (0.32μM, 3.2μM, 9.6μM and 96μM), using three better performing transgenic lines (C-1-1, C-2-1 and C-2-4) were tested, they showed higher accumulation of Cu in both shoots and roots compared to control plants at all Cu concentrations tested. Line C-2-4 showed the highest uptake of Cu from solution at all four concentrations of Cu tested. It accumulated 2.8 times more Cu in roots and up to 2.5 times more Cu in shoots compared to control plants.

When studies on uptake of Zn by control and ten independently transformed and confirmed transgenic plants grown in hydroponics spiked with 65Zn was conducted, transgenic plants showed significantly enhanced uptake of Zn compared to control (Fig. 3). When the accumulation of Zn in root and shoot biomass was studied, roots showed higher accumulation of Zn compared to shoots. Roots of transgenic tobacco line Z5 showed up to 11 times more Zn acquisition compared to control. Levels of Zn in shoot biomass of all transgenic lines were also significantly higher than the control. These results demonstrated that transgenic tobacco plants with tzn1 could take higher Zn at all Zn concentrations compared to control. Accumulation of Zn was higher in roots compared to shoots.

For further studies, best performing transgenic line Z5 along with control tobacco plants were exposed to different concentrations of Zn. Zinc uptake by Z5 transgenic and control tobacco plants increased with increase in concentration of Zn, with the roots of Z5 plants showing significantly higher levels of Zn (~2.7, 4, 6.5, 8, 5.5 times, respectively, at 0.7, 7, 70, 350 and 700 μM Zn concentrations) compared to control plants (Fig. 4). Shoots of Z5 transgenic plants exposed to different concentrations of Zn also showed enhanced Zn acquisition compared to control plants. Although the levels of Zn in roots and shoots of Z5 transgenic tobacco plants were significantly higher at all Zn concentrations compared to control (Fig.4a,b), accumulation of Zn was higher in roots compared to shoots.

Fig. 3: 65Zn accumulation in transgenic and control plants exposed to 65Zn in hydroponics at the end of 10 days. (a) roots (b) shoots.

Fig. 4: Zinc accumulation by control and transgenic plants Z5 grown in hydroponics spiked with different concentrations of Zn (unlabelled ZnCl2) at the end of 10 days. (a) Roots (b) Shoots.
Bio-fortification of crop plants for enhanced accumulation of mineral elements is one of the priority areas of research and transgenic technology can be used to enhance the levels of Zn and Cu in plant tissues. Over-expression of zinc and copper transporters can enhance the acquisition of Cu and Zn in plants. Another strategy to improve Cu/Zn acquisition in plants, is to introduce efficient heterologous Cu/Zn transporters in plants. In the present study, we cloned a high affinity copper transporter gene (tcu-1) and a zinc transporter gene from the fungus *N. crassa* and introduced them into tobacco plants. Transgenic tobacco plants (T₀) expressing tcu-1 and tzn1 were used for studies on Cu and Zn acquisition from solution. All the transgenic lines tested, showed higher levels of Cu/Zn acquisition compared to control plants and the levels of Cu/Zn in shoot biomass of transgenic lines were higher than wild-type plants. Although the shoots of transgenic plants accumulated higher levels of Cu/Zn compared to control plant, roots retained a high proportion of Cu/Zn which was taken up.

The present work perhaps is the first study, where heterologous Cu/Zn transporter genes were successfully transferred and expressed in a model plant-tobacco to enhance Cu/Zn acquisition. Development of transgenic tobacco with heterologous Cu/Zn transporter genes, which was shown to take up high levels of Cu/Zn from solution and retaining high levels of Cu/Zn in roots, may find use in biofortification of Cu/Zn in crop plants such as carrot and cassava, where root is the edible part. To enhance Cu/Zn transport to shoots and seeds of crop plants, other genes involved in translocation of these essential elements to shoots, need to be co-transferred along with copper/zinc transporter genes.

References