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Full Length Research Paper

Cisgenic inhibition of the potato cold induced phosphorylase L gene expression and decrease in sugar contents

Morteza Kamrani[1], Bahram Baghban Kohnehrouz[1]* and Ashraf Gholizadeh[2]

[1]Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.
[2]Research Institute for Fundamental Sciences (RIFS), University of Tabriz, Tabriz, Iran.

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To decrease the accumulation of reducing and non-reducing sugar in potato tubers stored at low temperature, a single gene silencing vector pARTPhL-IR, harboring a part of starch phosphorylase L gene as inverted repeats with pdk intron within was constructed and transformed into potato (*Solanum tuberosum* L.) cultivars Agria and Marfona. Polymerase chain reaction (PCR) of *npt*II gene and pdk intron indicated that the RNA interference construct was transformed successfully into the genome. Real time RT-PCR analysis of starch phosphorylase L gene in stored microtubers for 90 days at 4°C showed that the expression level of this gene in transgenics ranged from 1.63 to 7.54% of that in the non-transgenic plants. Analysis of sugar content in these plants showed that the total sugar content in transgenic microtubers was significantly reduced compared to the control, up to 35% in line M4. The accumulation of reducing sugars in transgenic lines at 4°C was reduced from 9.13 (in Agria) to 5.57 mg/g fresh weight (transgenic line A5) and from 9.56 (in Marfona) to 6.52 mg/g fresh weight (transgenic line M4), implying that silencing of starch phosphorylase L gene reduced starch breakdown during cold storage conditions.

Key words: Cold sweetening, potato (*Solanum tuberosum* L.), RNA interference, starch phosphorylase L. gene,

INTRODUCTION

Potato (*Solanum tuberosum* L.) is ranked fourth in production of all agricultural commodities in the world and produces more dry matter and protein per hectare than the major cereal crops (Horton, 1980). Storage of potatoes at low temperatures has advantages like natural control of sprout growth, easier maintenance of the high humidity atmosphere required to minimize transpirational losses and reductions in senescent sweetening and losses due to storage rot (Burton, 1969). Dormant potato tubers are metabolically inactive except for slow starch degradation and synthesis of sucrose with energy provided by respiration potato tubers under a phenolmenon known as cold sweetening. These potatoes accumulate both reducing sugars (glucose and fructose) and sucrose when subjected to chilling temperatures (Burton, 1969). Cold sweetening is explained as a shift in the balance between starch degradation and glycolysis, leading to the accumulation of sucrose (Isherwood, 1973), which is then converted into glucose and fructose. The process of starch degradation in potato tubers during cold sweetening despite of starch degradation in mesophyll chloroplasts is principally phosphorolytic rather than hydrolytic in nature (Lin et al., 1988; Morrell and ap Rees, 1986; Zeeman et al., 1998; Zeeman et al., 2004). Starch phosphorylase (EC 2.4.1.1.; α-1,4-glucan phosphorylase) transfers a glucosyl residue from the free non-reducing end of an amylose-like chain to inorganic phosphate forming Glc-l-P. In potato tubers, two starch

*Corresponding author. E-mail: bahramrouz@yahoo.com. Tel: +98-411-3392031. Fax: +98-411-3356003.

Abbreviations: MS, Murashige and Skoog; **YEB,** yeast extract broth; **dsRNAi,** double strand RNA interference; **FW,** fresh weight.

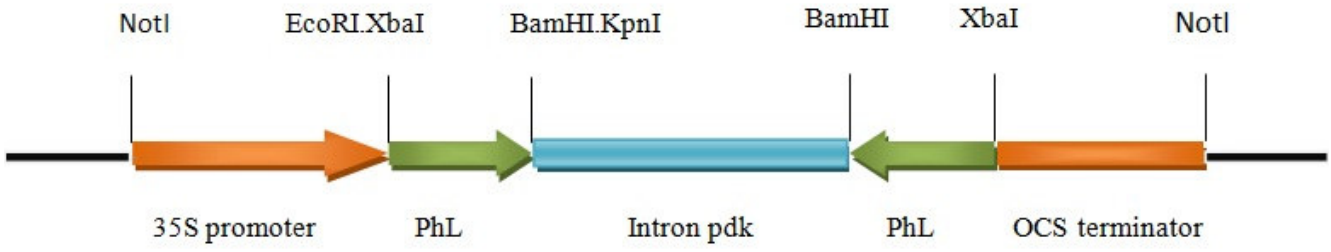

Figure 1. Schematic representation of starch phosphorylase gene-targeting RNA silencing construct.

phosphorylase isozymes, types L and H, have been described and are believed to be responsible for the complete breakdown of starch (Sonnewald et al., 1995). The L type is found in amyloplasts, whereas type H is cytosolic in location.

The reducing sugars glucose and fructose, participate in the Maillard reaction with free amino acids during frying resulting in dark-brown-colored fries and chips. These darkened chips and fries are unacceptable to consumers and also may result in greater amounts of acrylamide production which has been linked to many cancers (Chuda et al., 2003; Hogervorst et al., 2007). Ideal reducing sugar content is generally accepted to be 0.1% of the tuber fresh weight with 0.33% as the upper limit (Davies and Viola, 1992).

In this study, we evaluated the effect of potato starch phosphorylase L on starch degradation and accumulation of reducing sugar contents during cold storage conditions using dsRNAi technology.

MATERIALS AND METHODS

Plants and bacterial strains

Potato (*S. tuberosum* L.) *in vitro* plants of cultivars Agria and Marfona were propagated using single-node segments on MS medium (Murashige and Skoog, 1962) containing 2% w/v sucrose and 0.8% w/v agar. The pH of the medium was adjusted to 5.8 before autoclaving and the cultures were incubated under a 16 h light/ 8 h dark cycle at 20°C.

Escherichia coli strain DH5a was used for the construction and cloning of recombinant plasmids. *Agrobacterium tumefaciens* strain EHA105 possessing the required constructs was inoculated on YEB medium and used for gene transfer into potato.

Construction of the RNAi plasmid and plant transformation

The gene construct was made according to standard DNA genetic engineering protocols(Sambrook and Russel, 2001). A DNA fragment of starch phosphorylase L. (coordinates 1975-2322 of accession no. AF143202) for the RNAi construct was amplified by PCR from genomic DNA of *S. tuberosum* cv. Agria and Marfona with specific primer pairs of 5'-AAGAATTCTCTAGACCAAACGACCCCTTAGAGTG-3' and 5'-AAGGTACCGGATCC TGGATGAATCTGGAATTGGA-3' (anchoring *Eco*RI.*Xba*I and *Kpn*I.*Bam*HI sites at 5' ends, respectively), generating a 347 bps DNA fragment that contains a part of the 5' UTR and first exon of phosphorylase L. gene. The amplification product was cloned in pGEMT-easy vector and validated by restriction and sequence analysis. The obtained plasmid was digested with *Eco*RI-*Kpn*I and *Bam*HI–*Xba*I restriction enzymes to produce sense and antisense fragments inserted into the pHANNIBAL plasmid vector (by step cloning to develop RNAi construct, Figure 1). This construct provide required DNA sequences in sense and antisense orientation with a pyruvate dehydrogenase kinase (pdk) intron as spacer between them (Wesley et al., 2001). The entire RNAi construct was then subcloned as a *Not*I fragment into the binary vector pART27 and named pARTPhL-IR, and then introduced into *A. tumefaciens* strain EHA105 by the freeze-thaw procedure (Jyothishwaran et al., 2007). Plant transformation was done according to previously published protocol using leaf and internodal explants (Banerjee et al., 2006). The putative transgenic shoots were produced on regeneration medium containing 50 mg/L kanamycin antibiotic and transferred to microtuberization medium (MS supplemented by 10% sucrose) as nodal explants containing axillary buds.

PCR confirmation of transgenesis

The PCR screening of the putative transgenic lines for cissgenesis event was carried out by avoiding the amplification of endogenous DNA sequences using designed specific primer pairs of 5'-ATGACTGGGCACAACAGACAATCGGCTGCT-3' and 5'-CGGGTAGCCAACGCTATGTCCTGATAGCGG-3' for the *npt*II gene and 5'-TGACAAGTGATGTGTAAGACGAAG-3' and 5'-CAATCCAAATGTAAGATCAATGAT AAC-3' for pdk intron in RNAi construct, generating 612 and 608 bps products, respectively. Amplifications were done in a thermocycler (ASTEC PC-818; Fukuka, Japan). The conditions of PCR were: Pre-denaturation at 94°C for 3 min, followed by 35 cycles consisting of 45 s at 94°C, 45 s at 55°C, 90 s at 72°C, and then extension at 72°C for 5 min. PCR products were separated by electrophoresis in a 1% agarose gel containing ethidium bromide and visualized under UV using Gel Documentation System OptiGo 600/650 (ISOGEN).

Transcription vanalysis by real-time reverse transcription PCR (RT-PCR)

The PCR positive transgenic lines were transferred to micropropagation medium to produce microtubers. Resulted microtubers were collected and stored in cold conditions for molecular analysis. Real-time RT-PCR analysis was performed to test the effect of the RNAi construct on the endogenous starch phosphorylase L gene transcription products. To this purpose, total RNA was isolated from microtubers, by an RNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions. 1 µg aliquot

Table 1. Sugar contents in non-transgenic potato of Agria (AC), marfona (MC) and transgenic lines of Agria (A), Marfona (M) stored at 4ºC for 90 days.

Line	Total soluble sugar (mg/g FW)	Reducing sugar (mg/g FW)
A1	8.86±0.13^{ij}*	5.90±0.30^{h}
A2	9.67±0.30^{g}	6.31±0.20^{fg}
A3	8.60±0.17^{jk}	5.74±0.15^{hi}
A4	9.65±0.09^{g}	6.24±0.23^{g}
A5	8.53±0.10^{k}	5.57±0.21^{i}
A6	9.28±0.17^{h}	5.81±0.16^{hi}
AC	12.98±0.10^{b}	9.13±0.13^{b}
M1	11.61±0.12^{e}	7.71±0.10^{d}
M2	10.11±0.19^{f}	7.02±0.12^{e}
M3	11.72±0.22^{d}	8.11±0.13^{c}
M4	9.77±0.30^{c}	6.52±0.12
MC	15.73±0.27^{a}	9.56±0.16^{a}

*Duncan's multiple range tests. Values with different letters are significantly different at the 0.05 probability level.

of total RNA treated with RNase-free DNase I (Fermantas) was reverse transcribed using oligo $(dT)_{20}$ Primers and Superscript III (Invitrogen). The resultant cDNA was used for quantitative real-time PCR along with the iQ SYBR Green supermix (Bio Rad) and 100 nM primers. Quantitative real-time PCR was performed in three replicates for each sample on Miniopticon Real-Time PCR Detection System (Bio-Rad) using primer pairs 5'-GCGACCTGAGTTCTTTTGCT-3' and 5'-TAAGGAGCGAATCACGAACA-3' for Starch phosphorylase L transcripts and primer pairs 5'-CACCAAGC CAAAGAAGATCA-3' and 5'-TCAGCATTAGGGCACTCCTT-3' for ubiquitin transcript as a control. The best PCR amplificants were obtained with the following program: Denaturation at 95°C for 30 s, annealing for 30 s at 61°C, and elongation for 30s at 72°C, in a program of 45 cycles in a 20 µl reaction volume. All the expression data were normalized by adjusting the expression level of ubiquitin.

Extraction and evaluation of total sugars and reducing sugars

The transgenic microtubers (250 mg) were homogenized in boiling 80% ethanol and extracted at 70°C for 30 min (Stitt et al., 1989). After centrifuging at 3000 g for 5 min, the pellet was re-extracted twice more in 80% ethanol. Supernatants were bulked and reduced to dryness at 65°C for nearly two hours. The residual matters were re-dissolved in 2 ml distilled water and stored at -20°C for subsequent sugar determinations. Total sugar was quantified by Anthrone method, as described by Hedge and Hofreiter (1962) and reducing sugars was determined by 3, 5-dinitrosalicylic acid method (Lindsay, 1973).

RESULTS

Potato transformation and its validity

Leaf and internodal explants of potato cultivars Agria and Marfona were used for *Agrobacterium* mediated potato transformation. Callus formation was observed within 2 weeks after co-cultivation in all types of explants of the studied cultivars (Figure 2A and B). Regeneration of the shoots was observed on the calli formed from cut sections after 4 to 6 weeks on selective medium (Figure 2C and D). Shoots were formed on 23 and 32 out of 60 explants per cultivar, whereas roots were formed on 16 and 21 explants for Agria and Marfona, respectively. No shoots and roots were observed for control explants (Figure 2E).

All transformants appeared morphologically normal in comparison with untransformed plants. The validity of the putative transgenics was investigated by PCR amplification of genomic DNA using specific primers. The resulted PCR products (608 bp for pdk intron and 612 bp for *npt*II gene) from transformants and failure of amplification in control plants demonstrated that both the RNAi cassette and *npt*II gene were successfully integrated into the genome of the plant (Figure 3). All of PCR-positive *in vitro* plants were subsequently transferred to micro-tuberization medium to produce microtubers (Figure 2F).

Real-time RT- PCR analysis of phosphorylase L gene suppression

An RNAi-based approach was used to produce transgenic potato lines with reduced levels of starch phosphorylase L gene transcription. Figure 4 shows the estimation of the amounts of starch phosphorylase L transcripts by Real-time RT-PCR of total RNAs isolated from the transgenic and non-transgenic microtubers. Quantification of the relative-fold change in mRNA levels of the transgenic lines was calculated using the $2^{-\Delta\Delta Ct}$ value (Livak and Schmittgen, 2001). The $2^{-\Delta\Delta Ct}$ value reflected the relative transcript of the target gene in the transgenic lines versus those in the control. The expression level of starch phosphorylase L in transgenic lines ranged from 1.63 to 7.54% of that in the control plants. This indicates that overexpression of intron-containing hairpin RNA for starch phosphorylase L gene under the CaMV 35S promoter successfully induced silencing of starch phosphorylase L gene in both cultivars, Agria and Marfona (Figure 4).

Analysis of sugar content in transgenic lines

To determine sugar content in transgenic microtubers, they were first stored for 90 days at 4°C and then assessed for sugar components. The obtained results show that total and reducing sugars in transgenic lines were decreased significantly compared to control lines stored at the same conditions (Table 1). As pointed out in Table 1, the accumulation of the total sugar in transgenic microtubers ranged from 8.53 to 9.67 and 9.77 to 11.72

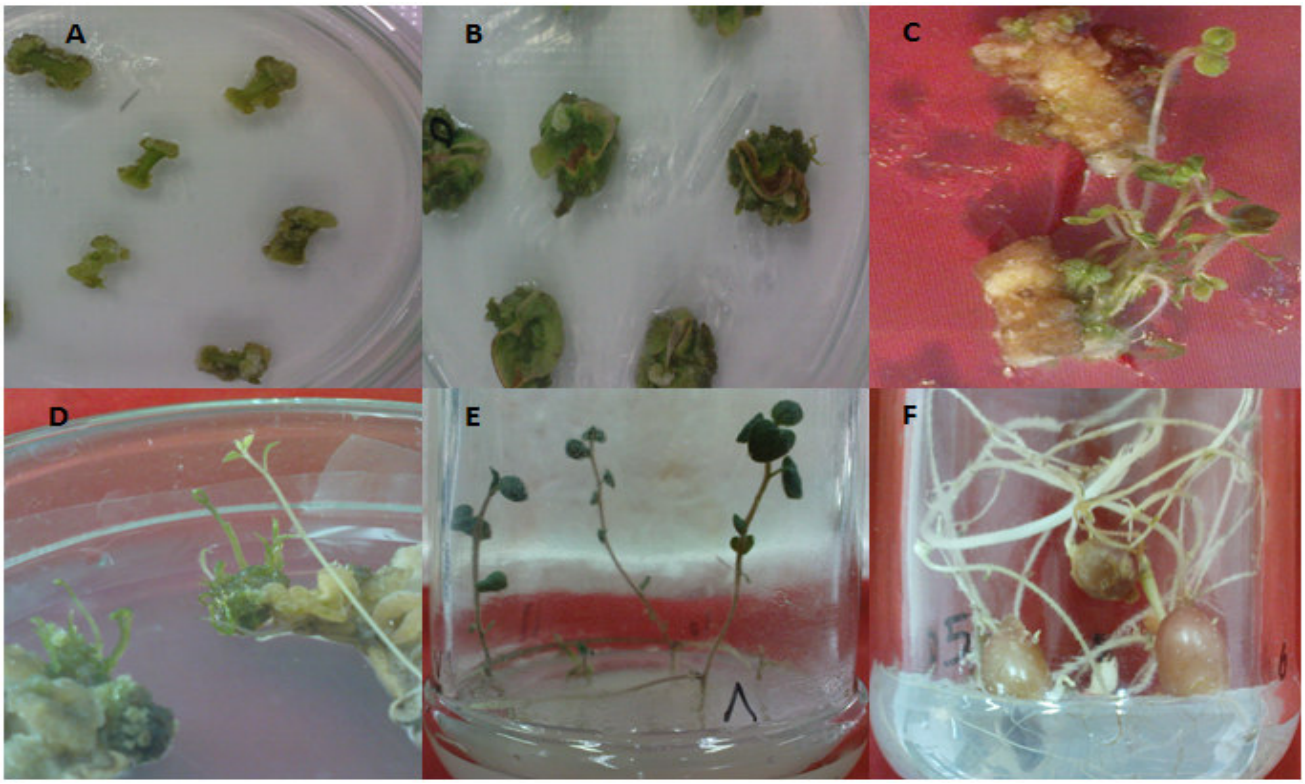

Figure 2. *In vitro* regeneration of putative transgenic potato plants and microtubers. (A, B) Callus formation from internode and leaf explants. (C, D) Shoot regeneration from calli in the selective medium. (E) Rooting of transgenic shoots on MS medium containing kanamycin. (F) Microtuber production in transgenic lines.

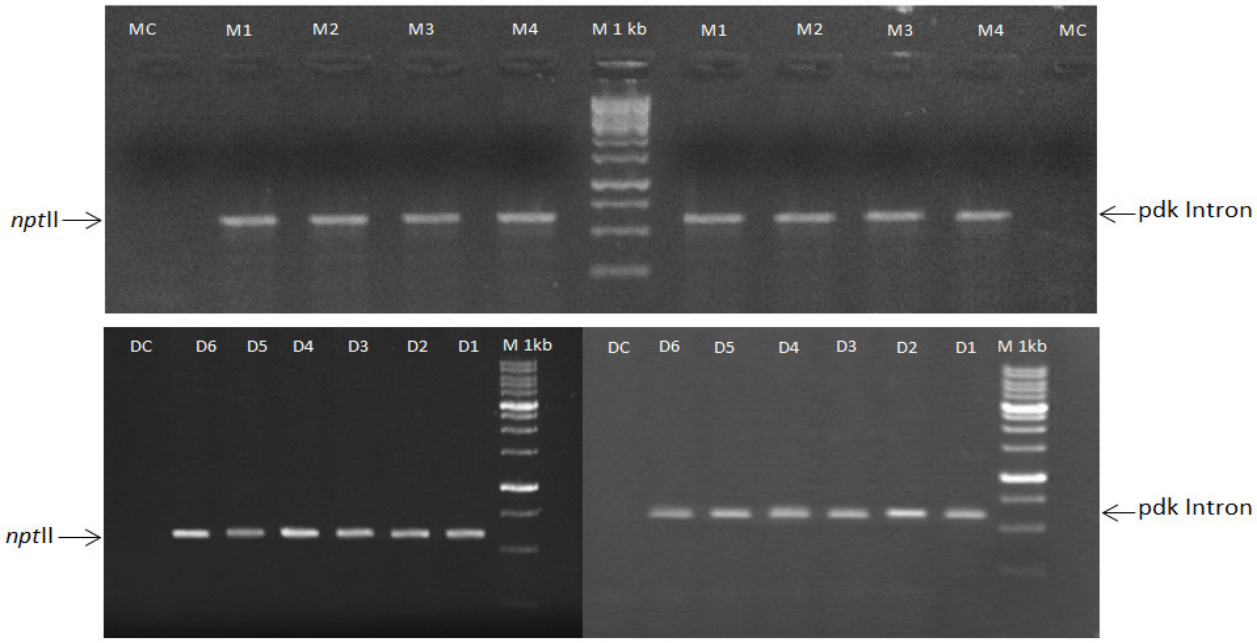

Figure 3. PCR analysis of putative transgenic lines. PCR with *npt*II primers results in an expected 612 bp product and with the specific primers for the pdk intron results in an expected 608 bp product. Lane M1-M4, putative Marfona transgenic lines; Lane MC, non-transgenic Marfona; Lanes A1-A6, putative Agria transgenic lines; Lane AC, non-transgenic Agria; Lane M 1 kb, 1 kb BLUE DNA marker.

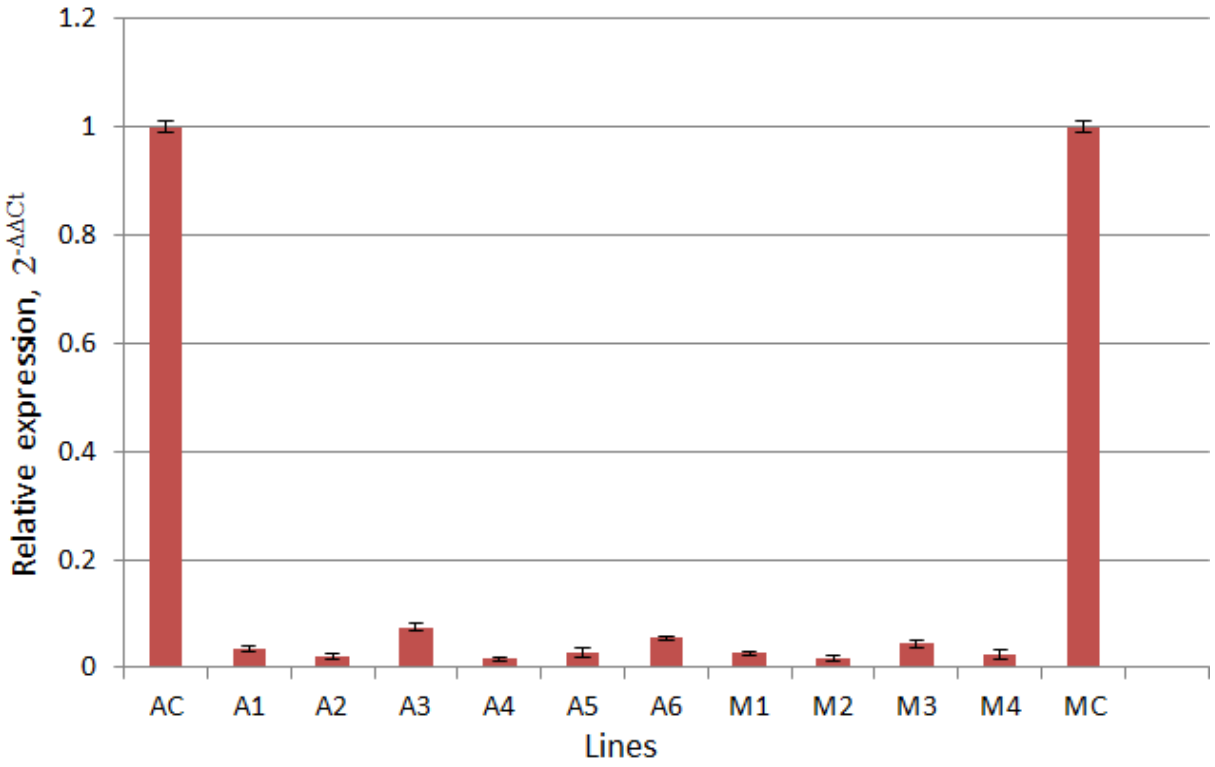

Figure 4. Real-time RT-PCR based transcription analysis of the starch phosphorylase L gene in transgenic potato lines.

mg/g FW for Agria and Marfona, respectively. According to our results, the best performing transgenic lines were devoted to the Agria-A5 with the 35% decreased and Marfona-M4 with the 38% decreased at total sugar content compared to non- transgenic plants. Also, the accumulation of reducing sugars (including fructose and glucose) in transgenic Agria and Marfona microtubres, were varied from 5.57 to 6.31 and 6.52 to 8.11 mg/g FW, respectively (Table 1). The data produced here shows the significant differences between two cultivars for both total and reducing sugar contents. The coefficient of determination of the total and reducing sugars was calculated as R^2 =0.913 demonstrating a positive relationship between them (Figure 5).

DISCUSSION

The starch phosphorylase catalyzing the reversible conversion of starch and inorganic phosphate into glucose-1-phosphate plays an important role in the phosphorolytic degradation of starch in plants (Sowokinos et al., 1997). However, the complete loss does not cause a significant change at the overall accumulation of starch during the day, or its remobilization at night in the leaves of *Arabidopsis* (Zeeman et al., 2004) and potatoes (Sonnewald et al., 1995) as well. Inspite of plastidial isoform of α-glucan phosphorylase in leaves, Claasen et al. (1993) suggested that an increase in starch phosphorylase activity acts as a triggering event in the sweetening of potato tubers during cold storage.

RNAi technology has been proven as an efficient interfering tool with gene expression in various plant systems (Wesley et al., 2001). Inhibition of this cold induced phenomenon in potato tubers has been investigated to some extent via both heterologous expression of the *glgC16* gene for ADP glucose pyrophosphorylase (AGPase) as the key regulating enzyme in starch synthesis (Stark et al., 1992), and cisgenesis by suppression of starch phosphorylase L gene under the tuber specific granule-bound starch synthase (GBSS) promoter (Rommens et al., 2006), endogenous acid invertase gene (Chi et al., 2008), vacuolar invertase gene (Bhaskar et al., 2010) and acid invertase gene (Jingsong et al., 2010). In this study, a cold induced key enzyme involved in starch degradation was silenced under a strong constitutive CaMV 35S promoter. Apart from a significant decrease in total and reducing sugar contents of cis-genic microtubers, not only was it deduced that the CaMV 35S promoter is running the transcription at lower temperatures, but also it was revealed that the RNAi system is working well in potato tubers during 4°C

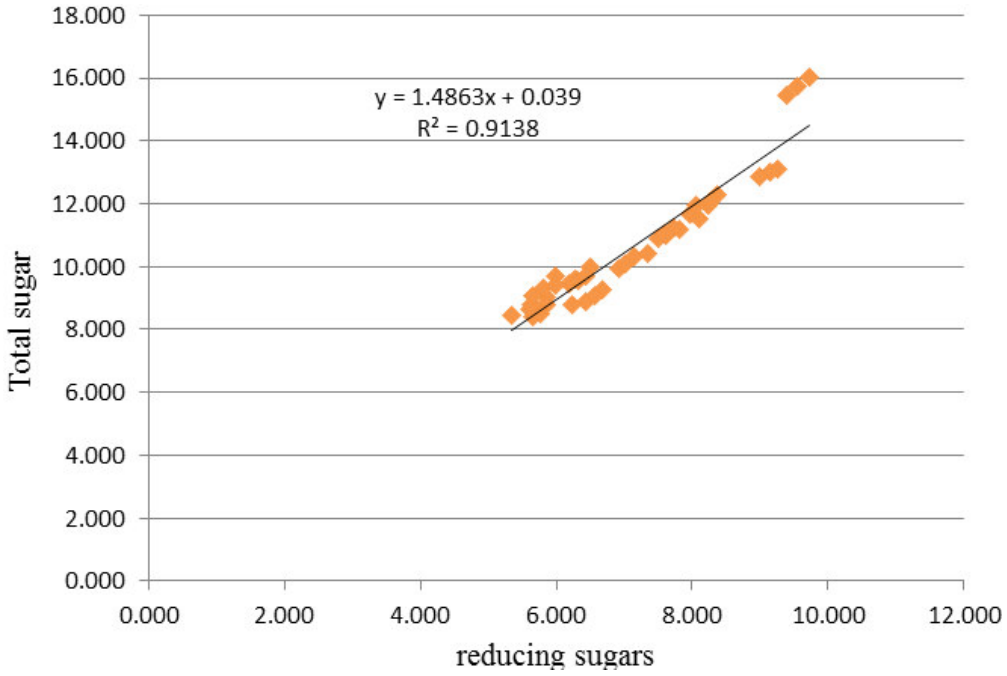

Figure 5. Relationship between reducing sugars and total sugar content in transgenic microtubers stored at 4°C.

storage storage conditions. The functioning nucleotide sequences in RNAi cassette for targeting the endogenous transcripts comprised the 284 bp of 5'-untranslated region and 63 bp of the first exon of starch phosphorylase L gene. Whether the silencing was more affected by 5'-untranslated region or 63 bp of the first exon need to be examined in detailed.

Analysis of RNAi transgenic microtubers by real time RT-PCR showed that the transcription levels of starch phoshphorylase L gene was decreased. As shown in Figure 2, suppression of starch phosphorylase L gene prevents starch degradation, resulting in a decrease in microtubers total and reducing sugars. Significant difference between 'Agria' and 'Marfona' transgenic microtubers for total and reducing sugars could be considered as different genetic background which appeared as pre-existing sugar content in control plants (12.99 mg/g FW in 'Agria' and 15.73 mg/g FW for 'Marfona'). This genotypic variation has been reported by previous works by transferring the bacterial gene encoding 6-phosphfructokinase into two potato cultivars (Navratil et al., 2007).

Regarding the biochemical nature of the sugar contents here, it is documented that the positive correlation between total and reducing sugars indicates that suppression of starch phosphorylase L gene decreases starch degradation and causes to reduce the accumulation of reducing (glucose, fructose) and non-reducing sugars (sucrose) in tubers stored at 4°C.

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