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The *Or* Gene Enhances Carotenoid Accumulation and Stability During Post-Harvest Storage of Potato Tubers

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ABSTRACT Provitamin A carotenoids in staple crops are not very stable during storage and their loss compromises nutritional quality. To elucidate the fundamental mechanisms underlying carotenoid accumulation and stability, we investigated transgenic potato tubers that expressed the cauliflower *Orange* (*Or*) gene. We found that the *Or* transgene not only promoted retention of β-carotene level, but also continuously stimulated its accumulation during 5 months of cold storage. In contrast, no increased levels of carotenoids were observed in the tubers of vector-only controls or a yellow-flesh variety during the same period of storage. The increased carotenoid accumulation was found to be associated with the formation of lipoprotein–carotenoid sequestering structures, as well as with the enhanced abundance of phytoene synthase, a key enzyme in the carotenoid biosynthetic pathway. Furthermore, the provitamin A carotenoids stored were shown to be stable during simulated digestion and accessible for uptake by human intestinal absorptive cells. Proteomic analysis identified three major functional groups of proteins (i.e. heat shock proteins, glutathione-*S*-transferases, and carbohydrate metabolic proteins) that are potentially important in the *Or*-regulated carotenoid accumulation. Our results show that regulation of carotenoid sequestration capacity is an important mechanism by which carotenoid stability is regulated. Our findings suggest that induction of a proper sink structure formation in staple crops may provide the crops with a unique ability to promote and/or stabilize provitamin A accumulation during plant growth and post-harvest storage.

Key words: Carotenoids; chromoplasts; *Or* gene; potato; provitamin A loss; staple crops; post-harvest storage.

INTRODUCTION

Vitamin A deficiency is one of the most prevalent nutrient deficiencies in many underdeveloped regions of the world, where it affects an approximately 250 million children under 5 years of age. Between 250,000 and 500,000 of these children become blind each year and about two-thirds of those losing vision die within a year. Mortality would be significantly greater if the costly vitamin A supplementation programs were not available (www.harvestplus.org/). Plant provitamin A carotenoids are the primary dietary precursors of vitamin A. While many fruits and vegetables have high levels of provitamin A carotenoids, staple crops contain low levels of these compounds, which contributes to the global prevalence of vitamin A deficiency (Zhu et al., 2007). To help combat vitamin A deficiency, a global effort is under way to increase provitamin A content in staple crops (www.harvestplus.org/; www.grandchallenges.org/; www.goldenrice.org/), such as the production of Golden Rice (Ye et al., 2000; Paine et al., 2005), high provitamin A cassava (Sayre et al., 2011; Welsch et al., 2010), and yellow maize (Naqvi et al., 2009; Yan et al., 2010).

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Staple crops are harvested during a limited period but consumed throughout the year. Thus, it is necessary to store foods from one growing season to another or for an extended period. The stability of carotenoids in many food crops has been examined and there generally is a loss of carotenoids during storage, especially in staple crops (www.harvestplus.org/). For instance, in studies of commercial high-carotenoid yellow maize lines, it was found that between 30 and 56% of provitamin A carotenoids were lost during long-term storage (Quackenbush, 1963; Dua et al., 1965). Yellow wheat contains significant amounts of carotenoids, but there is a 20–48% loss of both total and provitamin A carotenoids during storage at 20°C (Hidalgo and Brandolini, 2008). Similarly, β-carotene content is reduced from 8.6 μg g^{-1} to trace amounts after 20 d of storage of dried cassava (Nascimento et al., 2009). Degradation of provitamin A carotenoids during food storage is especially problematic for populations at risk of vitamin A deficiency. Thus, the need to both increase provitamin A carotenoid content in staple foods and enhance carotenoid stability during storage is widely recognized.

Chromoplasts are carotenoid-rich plastids found in many fruits, flowers, and storage roots (Waters and Pyke, 2005; Egea et al., 2010). Chromoplasts develop a unique mechanism to accumulate massive amounts of carotenoids by generating carotenoid–lipoprotein sequestering substructures (Bartley and Scolnik, 1995; Vishnevetsky et al., 1999; Walter and Strack, 2011). These substructures are believed to serve as a sink for sequestering excess carotenoids for stable storage and also preventing the end products of the carotenoid biosynthetic pathway from overloading the site of carotenoid biosynthesis to stimulate continuous production (Figure 1) (Li and Van Eck, 2007). Our pioneering studies on the cauliflower orange mutant have demonstrated that biogenesis of chromoplasts to enhance sink strength exerts a robust impact on carotenoid accumulation (Lu et al., 2006; Lopez et al., 2008). Indeed, an increase in chromoplast compartment size and number has been shown to affect cellular carotenoid content (Liu et al., 2004; Kolotilin et al., 2007; Galpaz et al., 2008). Carotenoids are synthesized in the membranes of nearly all types of plastids in addition to chromoplasts (Howitt and Pogson, 2006). Many important staple crops such as wheat, rice, barley, maize, potato, and cassava synthesize and accumulate low levels of carotenoids in amyloplasts in the edible seeds or roots. Induction of chromoplast biogenesis to create an effective provitamin A sequestration and deposition sink appears to represent an effective means to enhance provitamin A synthesis and stability in the edible tissues of staple crops.

The *Or* gene from cauliflower represents the only known gene that acts as a bona fide molecular switch to trigger the differentiation of non-colored plastids into chromoplasts (Lu et al., 2006; Giuliano and Diretto, 2007). Our previous studies showed that expression of the *Or* transgene in potato tubers leads to increased carotenoid levels (Lu et al., 2006; Lopez et al., 2008). Interestingly, we noticed that carotenoid levels were increased during cold storage (Lopez et al., 2008). To elucidate the mechanisms underlying carotenoid accumulation and stability during post-harvest storage, we performed a detailed investigation of transgenic potato tubers expressing the *Or* transgene after various months of storage. Proteomic analysis was performed to unveil the potentially important cellular processes that contribute to carotenoid accumulation. Our results suggest that induction of a proper storage sink structure formation offers a potential to develop staple crops with an improved capacity to synthesize and stabilize carotenoids during plant growth and post-harvest storage.

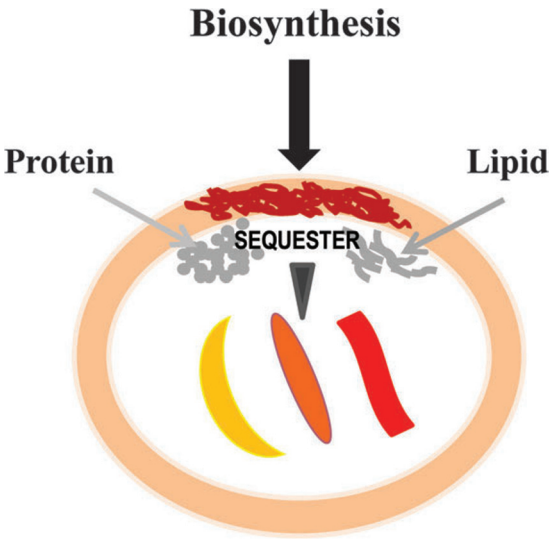

Figure 1. Chromoplast as a Metabolic Sink.
Carotenoids synthesized in chromoplast membranes are sequestered away to form carotenoid lipoprotein sequestering substructures within chromoplasts. These sequestering substructures serve two roles in facilitating high levels of carotenoid accumulation in chromoplasts: one is to stimulate continuous carotenoid biosynthesis and the other is to stably store the synthesized products.

RESULTS

Or Transgenic Tubers Show Continuously Increased Provitamin A Carotenoid Accumulation during Long-Term Cold Storage

Our previous studies have revealed that, while potato tubers from non-transformed and vector-only controls accumulate mainly lutein, expression of the *Or* transgene in tubers leads to enhanced levels of lutein and the production of β-carotene (Lu et al., 2006; Lopez et al., 2008). We noticed that the cross-section of the *Or* transgenic tubers showed a much deeper orange–yellow color following long-term cold storage than at harvest (Figure 2A).

To examine carotenoid accumulation during storage, we performed a time-course study. Mature tubers of vector-only controls, two *Or* transgenic lines (L29 and L93), and a yellow-flesh potato cultivar (91E22) (Brown, 2005) were stored in paper bags in a cold room at 5°C for 0, 1, 3, and

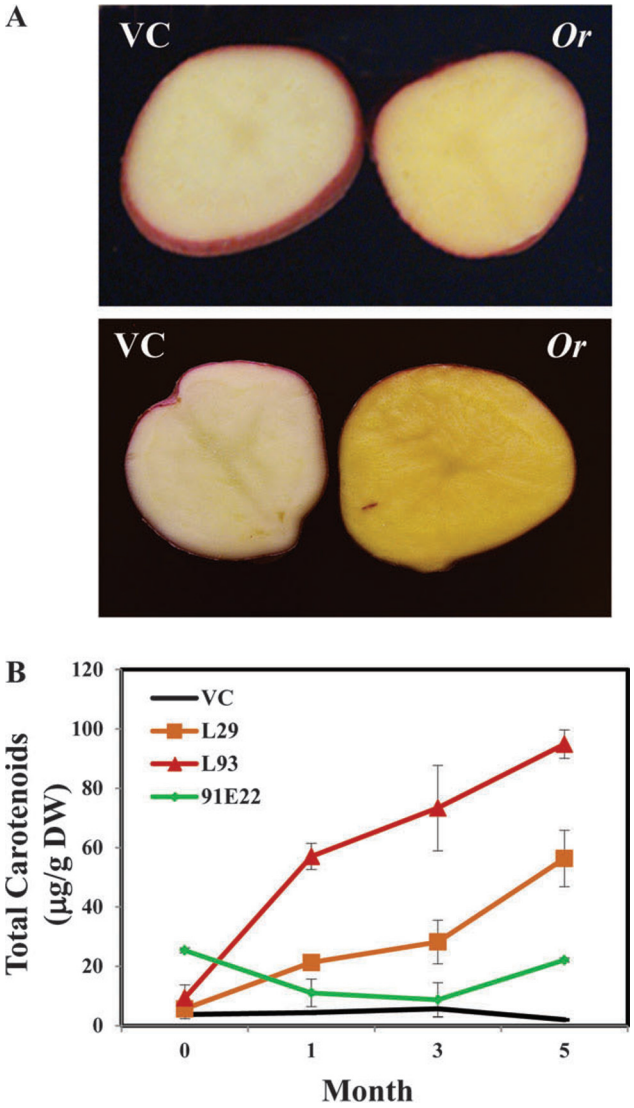

Figure 2. The *Or* Transgene Induces Continuous Carotenoid Accumulation in Potato Tubers during Long-Term Cold Storage.

(A) Cross-section of transgenic potato tubers without (VC) and with the *Or* transgene (*Or*) stored at 5°C in a cold room for 0 (top panel) and 5 months (bottom panel).

(B) Total carotenoid contents at various time points of cold storage. Values shown are the mean of three replicates. Error bars indicate SD. VC, vector control; L29 and L93, two different *Or* lines; 91E22, yellow-flesh potato cultivar (Brown, 2005).

5 months. Pigments from these stored tubers were extracted and analyzed by HPLC. As shown in Figure 2B, the total carotenoid levels in the *Or* transgenic tubers continuously increased during long-term cold storage. In comparison with the newly harvested tubers, an up to 10-fold increase in total carotenoids was found following 5 months of storage as observed previously (Lopez et al., 2008). Noticeably, the value reported here for L29 line at 0 months was lower than the number from previous study of freshly harvested tubers (Lopez et al., 2008). This discrepancy was mainly due to the fact that the samples used here at 0 months were frozen on the day of harvest. In contrast, total carotenoids in the vector-only control tubers showed no significant changes during long-term cold storage. To investigate whether tubers with high capacity of carotenoid synthesis also showed continuously increased carotenoid accumulation during long-term cold storage, we examined the carotenoid levels of a yellow-flesh cultivar (91E22), which contains a relatively high amount of zeaxanthin (Brown, 2005). Like the vector-only control tubers, the yellow-flesh variety also exhibited no increased level of carotenoid accumulation during the same period of cold storage, suggesting that the continuously increased carotenoid accumulation in the *Or* transgenic tubers was due to the presence of the *Or* transgene.

The vector-only control tubers contained mainly lutein and the pigment elution profiles remained similar at different months of storage (Figure 3A). In contrast, the *Or* transgenic tubers produced β-carotene, which is normally negligible in the controls. Noticeably, the β-carotene peak increased during extended period of storage and could reach a level that was up to 20-fold higher than that first observed in fresh tubers (Figure 3B and 3C). Thus, the *Or* gene not only helped retain the β-carotene level, but also promoted its continuous accumulation during the extended period of storage.

Enhanced Carotenoid Accumulation Is Associated with Increased Amounts of Carotenoid–Lipoprotein Sequestering Structures

Carotenoid–lipoprotein sequestering structures serve as a deposition sink to stimulate carotenoid biosynthesis and sequester excess carotenoids for stable storage within chromoplasts (Figure 1) (Li and Van Eck, 2007). Orange fragments of the sequestering structures were observed in both *Or* transgenic cauliflower and potato (Lu et al., 2006; Lopez et al., 2008). To examine whether formation of sequestering structures was correlated with enhanced carotenoid accumulation during long-term cold storage, we examined the amounts of carotenoid–lipoprotein sequestering substructures using light microscopy. The sharply outlined orange fragments were absent from the tuber extracts of both control and yellow-flesh potato cultivar 91E22 (data not shown). When we examined *Or* transgenic tubers, continuously increasing amounts of those sequestering structures were observed with increased storage time (Figure 4). This increase paralleled carotenoid accumulation in the *Or* transgenic tubers, suggesting that the formation of the sequestering structures plays an important role in sequestering and promoting carotenoid accumulation during long-term storage.

Expression of the *Or* Transgene and Carotenoid Pathway Genes during Storage

To determine whether expression of the *Or* transgene was affected by long-term cold storage, we examined the transcript abundance in the transgenic tubers by qRT–PCR. As shown in Figure 5A, the *Or* transgene was expressed in tubers of both *Or* transgenic lines. Transcript levels of the *Or* transgene

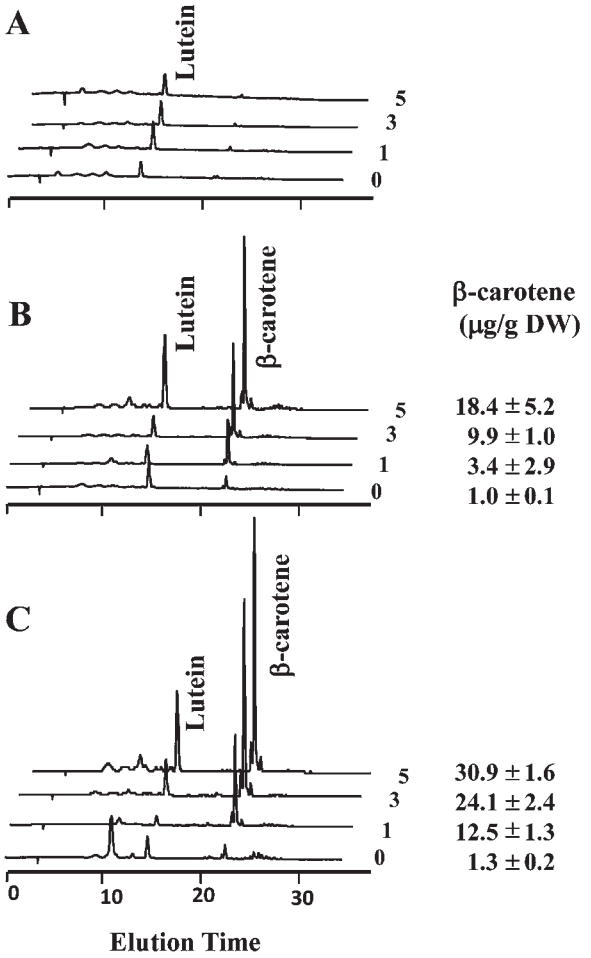

Figure 3. β-carotene Levels in the *Or* Potato Tubers Increases with Extended Period of Cold Storage.

HPLC analysis of carotenoids extracted from vector-only control tubers **(A)**, L29 transgenic *Or* line **(B)**, and L93 transgenic *Or* line **(C)**. The numbers 0, 1, 3, and 5 represent the number of months that samples were stored in cold room. Samples with same fresh weight were used for pigment extraction. Noticeably, the β-carotene peak areas in the *Or* lines (B, C) increased with increased months of storage. The absolute levels of β-carotene for L29 (B) and L93 (C) lines at different months of storage are shown beside the elution profiles.

remained consistent throughout the 5-month period of storage.

There are limited reports on examination of the expression of carotenoid pathway genes during cold storage. However, cold storage has been shown to stimulate expression of the flavonoid biosynthetic pathway genes, leading to enhanced flavonoid accumulation (LoPiero et al., 2005). To investigate whether the *Or* transgene and cold temperature affected the transcript levels of carotenogenic genes for the enhanced carotenoid accumulation in the *Or* tubers, we examined expression of a panel of genes associated with carotenoid metabolism by qRT–PCR. In general, expression of the *Or* transgene did not markedly affect expression of carotenoid metabolic

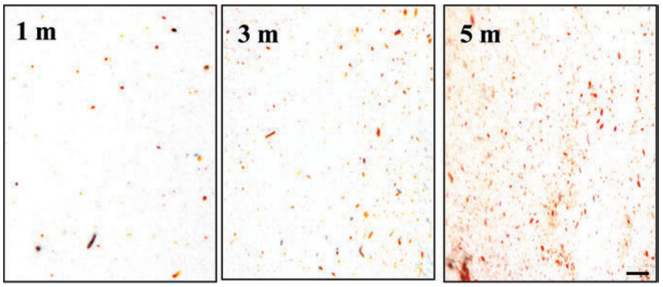

Figure 4. Carotenoid–Lipoprotein Sequestering Substructures.

Frozen samples of tubers following storage for 0, 1, 3, and 5 months in a cold room were ground and the upper suspensions were observed under a light microscope. The orange structures represent fragments of carotenoid lipoprotein sequestering substructures. The image for the fresh sample (0 month) is not shown due to a lack of much observed sequestering substructures. Bar = 10 μm.

pathway genes (Figure 5B). Similarly, long-term cold storage had minimal effect on the transcript levels of these genes, although expression of *DXS*, *PSY1*, *ECYC*, *BCH1*, and *NCED1* were slightly decreased after 3 months of storage.

The *Or* Transgene Enhances Phytoene Synthase (PSY) Protein Stability during Storage

We next examined whether the continuous accumulation of carotenoids in the *Or* transgenic tubers was due to continuous biosynthesis. PSY is known to play a crucial role and serve as a bottleneck in determining carotenoid biosynthesis (Cazzonelli and Pogson, 2010; Cazzonelli et al., 2010). Thus, manipulation of *PSY* expression in many plants has been shown to dramatically enhance provitamin A carotenoid synthesis by directing metabolic flux into carotenoid biosynthetic pathway (Shewmaker et al., 1999; Ducreux et al., 2005; Fraser et al., 2007; Maass et al., 2009).

To investigate whether expression of the *Or* transgene and/or cold storage might enhance or stabilize PSY protein, thus facilitating continuous carotenoid synthesis in the *Or* tubers, we examined PSY protein levels in tubers of control and *Or* transgenic lines at various times during cold storage by Western blot analysis. As shown in Figure 6, PSY level was maximally increased in both control and the *Or* lines after 1 month of storage. While PSY protein abundance in control tubers was reduced during continued storage, PSY protein appeared to be more stable in the *Or* tubers. The amount of PSY in different *Or* lines was correlated with carotenoid content. These results suggest that the *Or* gene may stabilize PSY to facilitate further carotenoid synthesis in the *Or* transgenic tubers during long-term storage. Despite the relatively high level of PSY in the control tubers at 1-month storage, the lack of a dramatic increase in carotenoid content might be due to the absence of a suitable sink for carotenoid accumulation as is the case of white cauliflower compared with the orange curd mutant (Li et al., 2001).

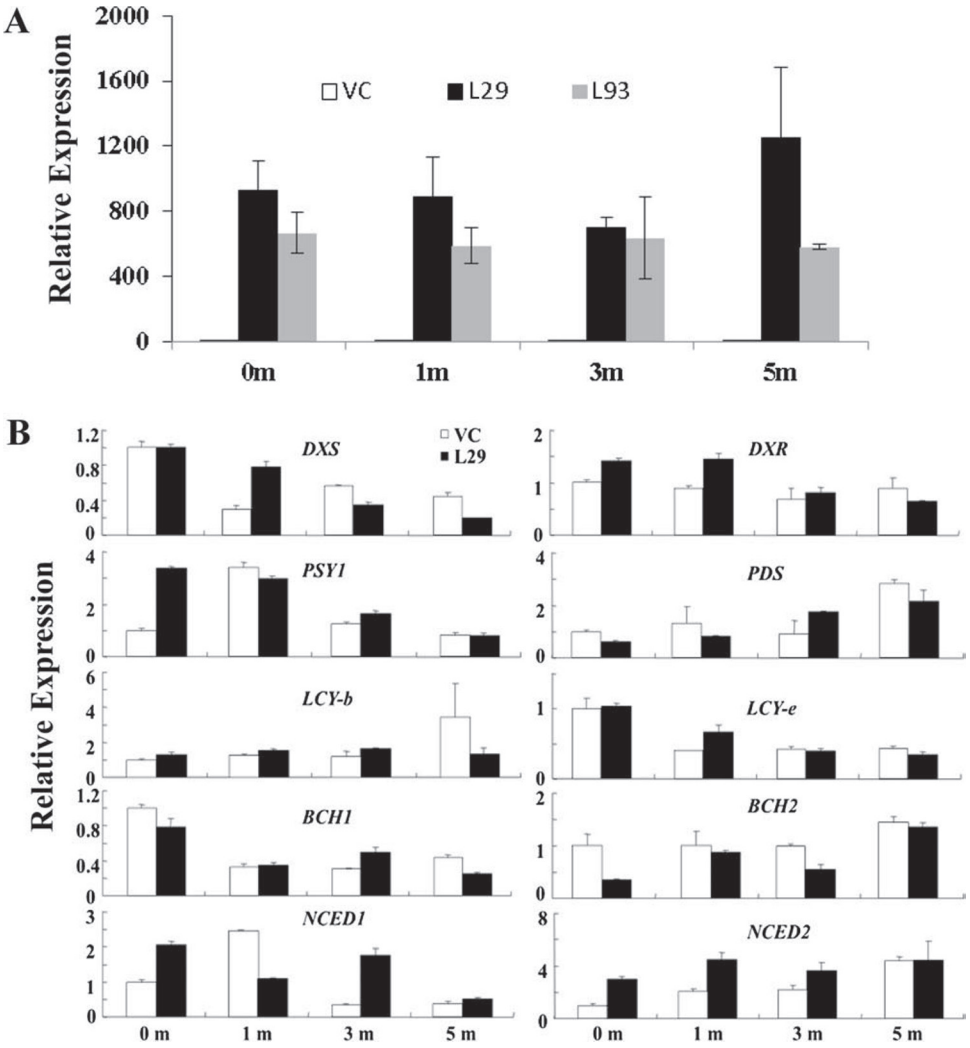

Figure 5. Expression of *Or* and Carotenoid Metabolic Pathway Genes Is Not Dramatically Changed in Transgenic Potato Tubers during Long-Term Cold Storage.

(A) Transcript levels of *Or* at various time points of cold storage.

(B) Transcript levels of selected carotenoid metabolic pathway genes at various times of cold storage. *DXS*, 1-deoxy-D-xylulose-5-phosphate synthase; *DXR*, 1-deoxy-D-xylulose-5-phosphate reductase; *PSY1*, phytoene synthase 1; *PDS*, phytoene desaturase; *LCY-b*, lycopene β-cyclase; *LCY-e*, lycopene ε-cyclase; *BCH*, β-carotene hydroxylase; *NCED*, 9-cis-epoxycarotenoid dioxygenase.

qRT–PCR was performed in triplicate with two biological repeats. Error bars represent SD. The expression of individual genes in vector control at 0 months was set 1.

Provitamin A Carotenoids in the *Or* Tubers are Bioaccessible

High amounts of provitamin A carotenoids accumulated in carotenoid–lipoprotein sequestering structures in the *Or* transgenic tubers. To test whether they were accessible for absorption, we examined the efficiency of partitioning of carotenoids into mixed micelles during simulated oral, gastric, and small-intestinal digestion of boiled *Or* transgenic tubers. The quantity of carotenoids incorporated into micelles during simulated digestion was proportional to their content in the starting plant material. The extent of micellarization during the small-intestinal phase of digestion was 17–26 and 10–15% for lutein and β-carotene, respectively (Figure 7A), as has been previously reported for lutein and β-carotene in other studies with carotenoid-rich vegetables (Garrett et al., 2000). To confirm that carotenoids within the mixed micelles were available for uptake by absorptive intestinal cells, diluted micelle fractions generated during small-intestinal digestion were incubated with highly differentiated monolayers of Caco-2 human intestinal cells for 4 h. While cellular lutein content was similar in cultures exposed to diluted micelle fractions generated during simulated digestion of L29 and L93, β-carotene accumulation in Caco-2 cells incubated with micelles generated during digestion of boiled L93 exceeded that from L29 (Figure 7B). These results showed that cellular uptake of micellar β-carotene was directly proportional to

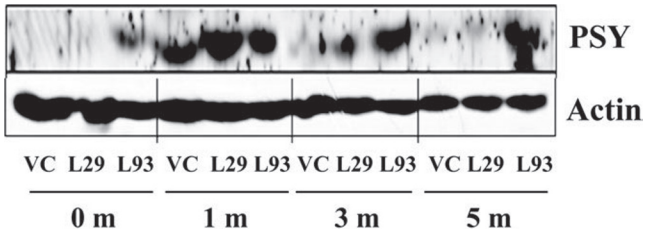

Figure 6. Expression of *Or* Transgene Enhances Phytoene Synthase Protein Stability during Cold Storage.

Western blot analysis of PSY level at various time points of cold storage. Protein extracts from potato tuber samples were resolved on 12% SDS–PAGE, electrotransferred onto membrane, and probed with anti-PSY or anti-Actin polyclonal antibodies (Maass et al., 2009). PSY and Actin signals were detected using ECL system. Actin levels were used as protein loading control.

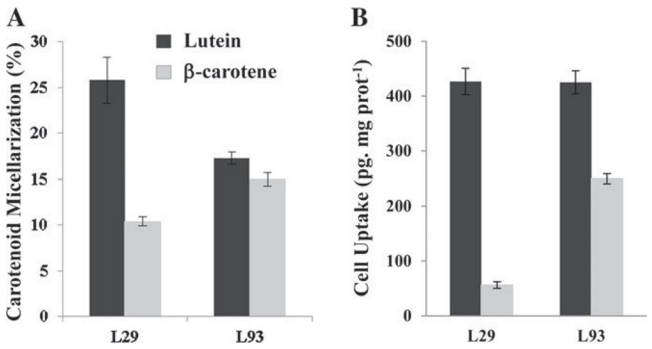

Figure 7. Bioaccessiblity of Carotenoids from Boiled *Or* Tubers Stored at 5°C for 5 Months.

(A) The efficiency of transfer carotenoids into micelle fractions during simulated digestion of the boiled tubers of the *Or* transgenic lines after 5 months of storage. Data are mean ± SD, $n = 5$.

(B) Uptake of carotenoids transferred to mixed micelles during simulated digestion of the *Or* transgenic lines. Data are means ± SD, $n = 3$.

the amount of β-carotene present in micelles and thus *Or* tubers. These data suggest that the additional complement of provitamin A carotenoids in the *Or* tubers was bioaccessible to the cells. As the predictive value of the *in vitro* digestion method for bioavailability of carotenoids has been validated (Reboul et al., 2006), we propose that expression of the *Or* gene in plant foods has the potential to increase vitamin A status.

Proteomic Analysis of Proteins Altered by the *Or* Transgene during Long-Term Storage

The observation that β-carotene content in the *Or* transgenic tubers increased markedly during long-term cold storage suggests that the *Or* transgene may regulate other processes that work in concert with *Or* to facilitate the accumulation of high levels of carotenoids. To identify the cellular processes important for the *Or*-regulated carotenoid accumulation, we conducted a comparative proteomic analysis of protein expression between the *Or* transgenic and control tubers at 0 and 5 months of cold storage. Isobaric Tags for Relative and Absolute Quantitation (iTRAQ)-based shotgun proteomic approach was used to analyze the differentially expressed proteins in the potato tuber samples. In our proteomic experiments, phenol-based extraction protocol was employed that typically has high extraction efficiency. Either OffGel isoelectric focusing or high pH reverse phase chromatography as the first dimension separation of a two-dimensional strategy was used to reduce the complexity of samples for deep coverage. Both approaches are known to provide extremely high-resolution first-dimension separation. Further, an LTQ-Orbitrap Velos operating in the HCD mode set to acquire three full MS/MS scans per second provided ultra-high resolution and accurate mass for confidence of identification.

A total of 4463 unique proteins on the basis of 18 429 unique peptide identifications resulting from 403 083 MS/MS spectra were confidently identified from two replicate analyses. Of the unique proteins identified, 3129 proteins were quantified on the basis of their iTRAQ ratios (Supplemental Table 1). Cold storage is known to alter protein expression in potato tubers (Yang et al., 2011). To identify those proteins specifically affected by *Or* but not cold storage, we compared protein expression between 0 and 5 months of storage for the control and *Or* tuber samples, and identified those differentially expressed in the *Or* transgenic tubers, but not in vector-only controls. While 96 proteins were identified with iTRAQ ratio $\geqslant \pm 1.5$-fold in control tubers between 0 and 5 months of storage, a total of 312 proteins with iTRAQ ratio $\geqslant \pm 1.5$-fold were identified in the *Or* tubers (Supplemental Tables 2 and 3). Analysis of the differently expressed proteins between the control and *Or* samples revealed that there were 71 common proteins, which were likely associated with cold storage alteration. The presence of the *Or* transgene affected the abundance of 241 proteins in the *Or* tubers (Figure 8A). These proteins were categorized according to their biological processes using the blast2GO program (Conesa et al., 2005) as shown in Figure 8B. A large number of these proteins were associated with metabolic process, biosynthetic process, and response to stress and stimulus. Among the 241 *Or*-regulated proteins, 47 and 194 were up- and down-regulated, respectively. The majority of the up-regulated proteins were involved in response to stress and stimulus, as well as metabolic process. Most of the down-regulated proteins were associated with metabolic process and biosynthetic process.

A subset of proteins that were up-regulated in the *Or* transgenic tubers are listed in Table 1. Three major functional groups of proteins were found among the *Or* up-regulated proteins. These include six heat shock proteins (HSPs) that are a family of chaperones and stress-responsive proteins (Sun et al., 2002; Wang et al., 2004), four glutathione-*S*-transferases that are known regulators of metabolic process and defense-response (Dixon et al., 2010), and five proteins (i.e. two starch-associated proteins R1, fructose 1,6-biophosphatase, 5′-AMP-activated protein kinase, and 4-alpha-glucanotransferase) that are involved in starch and sugar metabolism and signaling. In addition, thioredoxin h was up-regulated in the *Or* transgenic

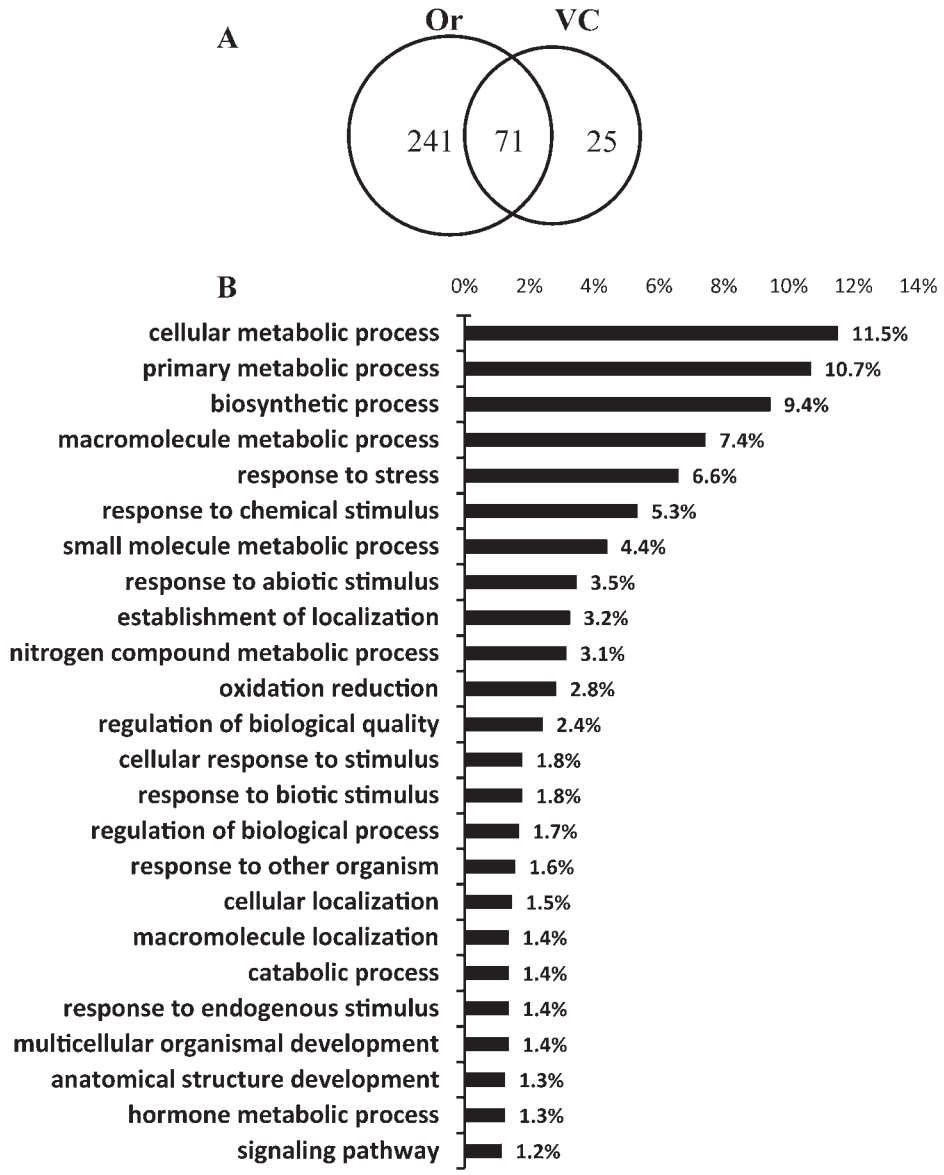

Figure 8. Number of Differential Expressed Proteins and the Functional Annotation of the *Or* Affected Proteins.
(A) Venn diagram showing the number of proteins with iTRAQ ratio ⩾ ±1.5-fold change between 0 and 5 months of storage and the number of overlapping proteins between *Or* and vector control (VC) samples.
(B) Functional categories of proteins whose expressions were specifically affected by the *Or* transgene.

tubers during storage. Thioredoxin h has been shown to regulate starch mobilization, as its overexpression enhances the activity of starch debranching enzyme, pullulanase, for starch degradation (Cho et al., 1999). The fact that a large number of these three functional groups of proteins were among the *Or* up-regulated proteins suggests the important roles of chaperones, stress and metabolic regulators, and carbohydrate metabolism in the *Or*-regulated carotenoid accumulation.

DISCUSSION

Provitamin A carotenoids are not very stable in staple crops and a large quantity of them can be lost during long-term storage. While it is crucial to produce staple crops with enhanced provitamin A content, developing food products with reduced loss of provitamin A carotenoids during storage and/or other pre-consumption activities is essential to maximize the nutritional quality of foods.

The principal causes of the loss of provitamin A and other carotenoids during storage of foods are enzymatic and/or non-enzymatic oxidation of carotenoid molecules, although isomerization of *trans*-carotenoids to the *cis*-forms also contributes to the loss of food provitamin A activities (Rodriguez-Amaya, 2003). Factors such as light, storage temperature, oxygen, and the presence of antioxidants are all known to affect the extent of carotenoid loss (Namitha and Negi, 2010).

Table 1. List of Up-Regulated Proteins Identified with Significant Change in *Or* 5M/0M.

Sequence name	Accession	Protein name	Ion Score	*Or* 5M/0M ave ± SD	*WT* 5M/0M ave ± SD	GO annotation
SGN-U268702	gi|60459397	Glutathione-S-transferase	228	3.11 ± 0.52	1.10 ± 0.07	Defense response/auxin-mediated signaling pathway
SGN-U269225	gi|40646966	Chloroplast small heat shock protein class I	631	2.94 ± 0.4	1.25 ± 0.17	Response to stress
SGN-U269762	gi|40646966	Chloroplast small heat shock protein class I	1362	2.90 ± 0.23	1.23 ± 0.16	Response to stress
SGN-U273330	gi|225448536	Eukaryotic translation initiation factor 2 subunit 1	450	2.88 ± 0.44	1.12 ± 0.09	Translation
SGN-U269761	gi|40646966	Chloroplast small heat shock protein class I	728	2.86 ± 0.36	1.25 ± 0.17	Response to stress
SGN-U269135	gi|225429975	Phosphorylase superfamily	120	2.78 ± 0.46	1.10 ± 0.15	Metabolic process
SGN-U287373	gi|4836473	17.6 kD class I small heat shock protein	1693	2.78 ± 0.03	1.11 ± 0.06	Response to stress
SGN-U270650	gi|4928460	Thiol-disulfide exchange intermediat	151	2.60 ± 0.14	1.01 ± 0.07	Protein thiol-disulfide exchange/cell redox homeostasis
SGN-U272800	gi|225461924	Fasciclin-like arabinogalactan protein	205	2.51 ± 0.15	0.90 ± 0.06	Cellular component
SGN-U271995	gi|225439775	Glutathione S-transferase	220	2.46 ± 0.09	0.85 ± 0.06	Response to cadmium ion
SGN-U268891	gi|407944	Epoxide hydrolase	117	2.42 ± 0.56	1.11 ± 0.16	Metabolic process
SGN-U268700	gi|417542	Glutathione S-transferase	62	2.07 ± 0.08	1.05 ± 0.01	Defense response/auxin-mediated signaling pathway
SGN-U271467	gi|3492854	Small heat shock protein 23.6	194	1.94 ± 0.02	1.05 ± 0.09	Response to stress
SGN-U269840	gi|13124867	Starch-associated protein R1	2299	1.90 ± 0.38	1.17 ± 0.14	Starch catabolic process/cold acclimation/phosphorylation
SGN-U272923	gi|11561806	101 kDa heat shock protein	1731	1.89 ± 0.30	1.24 ± 0.14	Response to stress/protein folding and unfolding
SGN-U269599	gi|2764992	Plasma membrane polypeptide	672	1.85 ± 0.28	1.24 ± 0.16	Defense response to bacterium/response to cold
SGN-U278326	gi|225436942	Dienelactone hydrolase family protein	55	1.80 ± 0.11	1.21 ± 0.10	Response to salt stress
SGN-U296746	gi|224068648	Ascorbate peroxidase	707	1.71 ± 0.13	1.10 ± 0.06	Oxidation reduction/response to oxidative stress
SGN-U282935	gi|225433632	Leucine-rich repeat receptor-like protein kinase	35	1.70 ± 0.05	1.16 ± 0.05	Auxin biosynthetic process/ protein amino acid phosphorylation
SGN-U269839	gi|57012986	Starch-associated protein R1	332	1.69 ± 0.09	1.15 ± 0.08	Starch catabolic process/cold acclimation/phosphorylation
SGN-U271598	gi|24429927	Late embryogenesis abundant protein	57	1.69 ± 0.07	1.17 ± 0.17	Leaf senescence/defense response
SGN-U269228	gi|1169586	Fructose-1,6-bisphosphatase	305	1.67 ± 0.17	1.10 ± 0.14	Carbohydrate biosynthetic process
SGN-U270440	gi|41176619	4-alpha-glucanotransferase	575	1.67 ± 0.13	1.07 ± 0.11	Maltose catabolic process/starch catabolic process
SGN-U273259	gi|147821809	5-AMP-activated protein kinase beta-1 subunit	77	1.67 ± 0.07	1.38 ± 0.10	Starch catabolic process
SGN-U269100	gi|10567812	Glutathione S-transferase T5	166	1.67 ± 0.02	1.00 ± 0.08	Auxin-mediated signaling pathway
SGN-U273708	gi|225424360	2-oxoglutarate-dependent dioxygenase	138	1.67 ± 0.01	1.24 ± 0.02	Ethylene biosynthetic process
SGN-U275585	gi|1813704	Aldehyde oxidase 1 homolog	48	1.42 ± 0.78	0.95 ± 0.02	Abscisic acid biosynthetic process

Recent studies show that enzymatic degradation of carotenoids by carotenoid cleavage enzymes can play an important role in controlling carotenoid levels in plants (Ohmiya et al., 2006; Beisel et al., 2010). Indeed, down-regulation of potato carotenoid cleavage dioxygenase 4, StCCD4, has been shown to increase carotenoid content (Campbell et al., 2010). The transcript level of potato *CCD1A* gene in tubers was also found to be inversely correlated with carotenoid accumulation (Zhou et al., 2011). However, data for the degradation of carotenoids by carotenoid cleavage enzymes during storage of staple food crops are rather scarce.

The *Or* gene acts as a bona fide molecular switch to trigger the differentiation of non-colored plastids into chromoplasts, an organelle with superior ability to sequester and stably store large quantities of carotenoids (Li and Van Eck, 2007; Egea et al., 2010). Chromoplasts contribute to high levels of carotenoid accumulation found in fruits and vegetables. In contrast, storage organs of many staple crops, including all cereal crops, synthesize and deposit provitamin A carotenoids primarily in amyloplast membranes, which generally have limited capacity to synthesize and stably store provitamin A carotenoids.

By examining *Or* transgenic potato tubers, we found that expression of the *Or* transgene not only increased carotenoid retention, but also stimulated continuous carotenoid accumulation during long-term storage. Detailed examination of the cellular contents of the *Or* transgenic tubers by light microscopy revealed that the amount of the orange color carotenoid lipoprotein sequestering structures increased greatly during storage and was correlated with enhanced levels of accumulated carotenoids. While there is a possibility that the orange fragments were associated within amyloplast structures, the fact that *Or* confers carotenoid accumulation within chromoplasts (Lu et al., 2006) suggests that these carotenoid sequestering structures were likely the products released from broken chromoplasts. Thus, the *Or* transgene likely exerts its effect on enhancing carotenoid stability and accumulation through promoting the formation of carotenoid sequestering substructures in chromoplasts for stable storage. Carrot produces similar carotenoid sequestering substructures within chromoplasts (Li et al., 2001) and retains carotene content with continuous chromogenesis and carotenoid accumulation during storage (Booth, 1951; Kopas-Lane and Warthesen, 1995; Imsic et al., 2010). These carotenoid sequestering structures may serve two roles in promoting stable carotenoid accumulation (Li and Van Eck, 2007). First, they sequester excess carotenoids away from the chromoplast membranes, thus stimulating continuous biosynthesis. Second, they provide a stable deposition sink that protects carotenoids from degradation. Indeed, previous works have demonstrated the importance of synthesizing the components of carotenoid sequestering structures in controlling carotenoid accumulation (Deruere et al., 1994; Rabbani et al., 1998; Simkin et al., 2007).

Interestingly, the *Or* transgene in potato tubers not only conferred greater accumulation of total carotenoids, but also enhanced the relative extent of β-carotene synthesis during long-term cold storage. β-carotene accounted for 15 and 35% of total carotenoid content in the *Or* tubers at 0 and 5 months of storage, respectively. The increased biosynthesis of β-carotene along with other carotenoids is likely associated with the *Or*-regulated stability of PSY protein, which provides greater enzyme activity for continuous biosynthesis during storage. PSY is the rate-limiting and the first commitment enzyme in the carotenoid biosynthetic pathway (Cazzonelli and Pogson, 2010). Previous reports have shown that overexpression of PSY in non-photosynthetic tissues dramatically increases total carotenoid content and substantially enhances biosynthesis of β-carotene in canola seeds (Shewmaker et al., 1999), potato tubers (Ducreux et al., 2005), rice endosperm (Paine et al., 2005), and *Arabidopsis* seeds and calli (Lindgren et al., 2003; Maass et al., 2009). Similarly, the enhanced stability of PSY protein in the *Or* tubers was correlated with increased total and substantial β-carotene biosynthesis during long-term cold storage. This observation suggests an important role for the *Or*-regulated stability of PSY for continuous provitamin A synthesis during storage. It should be noted that *Or* might also affect the stability of other carotenoid biosynthetic enzyme proteins for the continuous biosynthesis, as expression of phyotene desaturase and lycopene β-cyclase has been shown to greatly enhance β-carotene levels compared with expression of PSY only in transgenic potato (Diretto et al., 2007).

Provitamin A carotenoid bioavailability is affected in part by chromoplast sequestering structures in fruits and vegetables (Fleshman et al., 2011). Provitamin A carotenoids in the carotenoid–lipoprotein sequestering structures in the *Or* transgenic potato tubers were shown to be bioaccessible as determined by using the coupled *in vitro* digestion/Caco-2 cell model. The extent of micellarization during digestion and uptake by Caco-2 cells was similar to that in other fruits and vegetables (Chitchumroonchokchai et al., 2004; Ornelas-Paz et al., 2008; Fleshman et al., 2011). Examination of the relationship between provitamin A bioaccessibility and its content in tubers from the two *Or* lines showed that cell accumulation was proportional to the amounts of provitamin A carotenoids present in the tubers.

The *Or* gene induces the differentiation of chromoplasts, which creates a plastid-localized sink with enhanced capacity for sequestration and stable storage of carotenoids. While control of carotenoid sequestering structure formation increased the sink strength, the *Or* gene likely regulates, in addition to PSY stability, other cellular processes that were accelerated during tuber cold storage to further enhance β-carotene accumulation. To provide additional insight into the network associated with the *Or*-enhanced carotenoid accumulation, an iTRAQ-based proteomics strategy was employed to examine the proteins differentially expressed in the control and *Or* transgenic tubers at different periods of storage. Interestingly, we found that three major functional groups of proteins represented a relatively large number of the *Or* up-regulated proteins. They were heat shock proteins, glutathione *S*-transferases, and proteins participating in carbohydrate metabolism.

Heat shock proteins are a group of chaperones that participate in protein folding or unfolding, protein assembly or disassembly, and translocation into organelles (Sun et al., 2002; Wang et al., 2004). The *Or* gene encodes a protein containing a DnaJ cysteine-rich zinc binding domain (Lu et al., 2006). This domain is highly specific to DnaJ-like molecular chaperones (Lu and Cyr, 1998), suggesting that the OR protein may function in association with the molecular chaperone system. Thus, it is not surprising to find that the *Or* transgene affected the expression of a number of chaperone proteins. We identified six *Or* up-regulated HSPs. Among them, five were small heat shock proteins (sHSPs), which were specific *Or*-regulated sHSPs rather than those in response to cold storage (Yang et al., 2011). sHSPs have been suggested to have unique physiological functions in plants, such as in enhancing cellular membrane stability (Ahn and Zimmerman, 2006) and in preventing protein aggregation with chaperone activity (Eyles and Gierasch, 2010). Previous studies in tomato show that expression of a number of tomato sHSPs is induced in fruits during ripening (Lawrence et al., 1997). One of the sHSPs, HSP21, was overexpressed in tomato and found to regulate chromoplast differentiation and promote carotenoid accumulation during fruit ripening (Neta-Sharir et al., 2005). This sHSP is suggested to be necessary for obtaining maximal carotenoid accumulation by promoting carotenoid biosynthetic enzyme activities (Neta-Sharir et al., 2005). Furthermore, there is emerging evidence showing the role of plastid outer envelope membrane proteins in chloroplast biogenesis (Inoue, 2011). A recent report demonstrates that a sHSP functions as a cofactor in the targeting of proteins to the plastid outer membranes for plastid differentiation and development (Kim et al., 2011). The sHSPs specifically up-regulated by *Or* may play important roles in the *Or*-regulated plastid development and carotenoid accumulation, although their exact contributions remain to be investigated.

Glutathione *S*-transferases represent a large and diverse group of enzymes whose expressions are associated with responses to stress, hormones, and developmental changes (Moons, 2005). Although glutathione *S*-transferases are among the highly expressed proteins and have been suggested to have various physiological roles, their endogenous functions remain poorly understood in plants (Dixon et al., 2010). We identified four glutathione *S*-transferases that were specifically up-regulated by *Or* rather than by cold storage. Whether and how increased amounts of these glutathione *S*-transferases are related to the *Or*-regulated carotenoid accumulation are not clear.

High levels of carotenoid accumulation in chromoplasts of fruits and roots are associated with the accumulation of sugars in many cases. Indeed, several previous studies have shown that sugars stimulate chromoplast biogenesis with increased carotenoid accumulation (Huff, 1983; Iglesias et al., 2001; Horner et al., 2007). Coincidentally, we observed general increased levels of sucrose and reducing sugars in the *Or* transgenic tubers in comparison with vector-only control (Supplemental Figure 1), which suggests a positive correlation of sugars with the *Or*-inducing carotenoid accumulation during cold storage. A recent study also reveals a direct correlation between sugar levels and isoprenoid metabolism (Flores-Perez et al., 2010). Starch in tubers is converted into sugars during cold storage of potato. We identified a number of proteins involved in starch mobilization that were specifically up-regulated by *Or*. Starch-associated protein R1 acts as a global regulator of starch catabolism and phosphorylates starch to induce degradation (Ritte et al., 2000). Two R1 proteins were identified to exhibit differential expression. In addition, thioredoxin h, a redox protein, was found to be up-regulated in *Or* tubers. Increased expression of thioredoxin h may also enhance starch degradation by increasing the activity of starch debranching enzyme pullulanase (Cho et al., 1999). Similarly, 4-alpha-glucanotransferase, an enzyme that transfers glucosyl units for starch degradation, was also identified. Apparently, degradation of starch to produce sugars is an important process for the *Or*-regulated carotenoid accumulation. Noticeably, the abundance of an AMP-activated protein kinase was increased in the *Or* tubers. AMP-activated protein kinase, a Snf1-related protein kinase (SnRK) in plants, belongs to a group of SnRKs proteins that act as global regulators of carbon metabolism in plants (Rolland et al., 2002). It constitutes a component in plant sugar sensing and sugar signaling cascades (Rolland et al., 2002). It is likely that *Or* regulates carbohydrate metabolism in providing substrates for carotenoid biosynthesis and possibly the signaling molecules for chromoplast development, resulting in increased carotenoid biosynthesis and accumulation in the *Or* tubers.

The loss of provitamin A carotenoids during storage of staple foods compromises their nutritional quality, which has the potential to adversely affect the health of a large number of individuals in poor rural regions who depend on such staples as their primary dietary source. Induction of chromoplast differentiation to provide an effective sink structure in storage tissues of staple crops may offer the plants a capacity to enhance carotenoid content and stably store the synthesized products during plant growth and post-harvest storage.

METHODS

Plant Materials and Treatments

Potato (*Solanum tuberosum* L. cv Désirée) vector-only control lines and transgenic lines (L29 and L93) transformed with the cauliflower *Or* gene under the control of potato granule-bound starch synthase promoter were grown in a greenhouse with a 14-h light/10-h dark photoperiod at 24°C for approximately 3 months. Mature tubers from individual plants were harvested and stored in brown paper bags at 5°C in a cold room. Following storage for 0, 1, 3, and 5 months, three tubers from each plant were peeled and cut into slices. Samples of slices were pooled, quickly frozen in liquid nitrogen, and stored at –80°C until use. Under the storage condition, no significant loss of water content in these mature tubers was observed following 5 months of cold storage.

Carotenoid Extraction and Analysis

Carotenoids from potato tubers (0.5 g fresh weight) were extracted and analyzed using a Waters HPLC system with a diode array detector (Waters, Milford, MA) following the method described by Lopez et al. (2008). Quantification of the relative concentration of individual carotenoids was achieved by comparison of the individual peak areas with a calibration curve constructed from commercial β-carotene and lutein standards (Sigma-Aldrich, St Louis, MO). All samples were analyzed in triplicate.

RNA Extraction and Quantitative RT–PCR Analysis

Total RNA from potato tubers (100 mg) was extracted following the method described by Van Eck et al. (2010). Total RNA (5 μg) was treated with DNase I (Ambion Inc., Austin, Texas) and reverse-transcribed to synthesize cDNA. The cDNA samples were used as templates in real-time PCR assays in the presence of a SYBR Green PCR master mix in an Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). The carotenoid metabolic gene specific primers used were as listed (Lopez et al., 2008). The primer sequences for the cauliflower *Or* gene were 5′-AATTCGCTGCCGGCTTTTGTAT-3′ (BoOrF) and 5′-TTGTTTCGACGGCTTCTAATGTTG-3′ (BoOrR) and for the potato *Actin* gene were 5′-AGCACCCTGTTCTGCTCACT-3′ (StActF) and 5′-GCACAGCCTGAATAGCAACA-3′ (StActR). Thermal cycling conditions consisted of a first step of denaturation at 95°C for 10 min, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 1 min at 60°C. Relative expression levels were calculated using the $\Delta\Delta C_t$ method as described by Lyi et al. (2007). Analysis of all gene expression was run in triplicate with two biological repeats.

Light Microscopic Study

Frozen potato tuber tissues were homogenized in a plastid isotonic buffer containing 0.1 M Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM NaCl, and 0.33 M sorbitol and centrifuged at 500 *g* for 2 min. The upper suspensions were dropped on microscope slides under cover slips, and examined with an Olympus BX60 microscope equipped with a Sony CCD color camera as reported previously (Li et al., 2001).

Immunoblot Analysis

Total proteins from potato tubers stored for various times were extracted with phenol and quantified with Bio-Rad protein assay (Maass et al., 2009). Proteins (80 μg) were resolved in a 12% SDS–polyacrylamide gel and analyzed by Western blot with anti-AtPSY antibody following the procedure as described (Maass et al., 2009). Signals were detected using the ECL system (GE Healthcare). Following the detection, the membranes were stripped and re-probed with anti-Actin antibody (Sigma).

In vitro Digestion and Cell Uptake

Cubed samples of the *Or* transgenic potato tubers stored for 5 months were boiled at 100°C for 10 min. The boiled samples were homogenized and aliquots were used for simulated digestion. All standard precautions were taken during processing, simulated digestion, and cell experiments to minimize losses of carotenoids by light. Three-phase simulated digestion (oral, gastric, and small-intestinal) was performed as described by Thakkar et al. (2007). Micelle fraction was diluted with basal DMEM medium (1:4, v/v) and added to Caco-2 cells grown in a T75 flask. Cells were incubated for 4 h under standard cell culture conditions at 37°C. Monolayers were washed once with cold PBS containing albumin (2 g l^{-1}), followed by twice with cold PBS. Cell monolayers were harvested in cold PBS and centrifuged at 800 *g* at 4°C for 5 min to collect cell pellets. Carotenoids in cell pellets were immediately extracted and analyzed by HPLC as described by Chitchumroonchokchai et al. (2004).

Proteomic Analysis

Protein extraction, digestion, iTRAQ labeling, first-dimension separation, liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses, and database searching were performed essentially as detailed by Yang et al. (2011). Potato tuber proteins from the *Or* transgenic and control samples with 0 and 5 months of cold storage were extracted using the phenol extraction method as described (Yang et al., 2007). Proteins were reduced, alkylated, and then sequentially enzyme digested with Lys-C and trypsin due to the presence of high levels of protease inhibitors in potato tubers. One hundred micrograms of each digest were reconstituted in 30 μl buffer and labeled with a 4-plex iTRAQ tag according to the manufacturer's protocol (Applied Biosystem). The labeled peptides were mixed and fractionated by high pH reverse phase chromatography into 24 fractions either using a Dionex UltiMate 3000 HPLC system (Sunnyvale, CA) or on an Agilent 3100 OFFGEL Fractionator (Santa Clara, CA) using a 24-well fraction device. All of the fractions were dried and reconstituted in 20 μl of 2% acetonitrile/0.5% formic acid for nanoLC–MS/MS analysis.

All of the fractions were analyzed using an LTQ-Orbitrap Velos analysis mass spectrometer coupled with an UltiMate 3000 MDLC system (Dionex, Sunnyvale, CA). The peptides were separated on a PepMap C-18 RP nano column (3 μm, 75 μm × 15 cm) and detected by the Orbitrap through a nano ion source containing a 10-μm analyte emitter (NewObjective, Woburn, MA). Full-scan mass spectra were measured from *m/z* 400–1400 with a mass spectrometer resolution at 30 000. The top 10 most intense ions with multiple charged ions above a threshold ion count of 5000 were selected for fragmentation with normalized collision energy of 45%.

Acquired MS and MS/MS spectra were converted into MGF files using Proteome Discoverer 1.1 (Thermo) and searched using Mascot Daemon v2.3 (Matrix Science, Boston, MA) against a *Solanum tuberosum* unigene database (V4, ftp://ftp.solgenomics.net/proteins/protein_predictions_from_unigenes/single_species_assemblies/Solanum_tuberosum/) for both protein identification and iTRAQ quantitation. Only

peptides with significance scores at the 99% confidence interval were considered to be confidently identified. Proteins were identified on the basis of having at least two confidently identified peptides. To be confidently quantified, a protein had to exhibit at least two confidently identified peptides that generated a complete iTRAQ reporter ion series. The functional annotation of proteins found with a differential expression of $\geqslant \pm 1.5$-fold between 0 and 5 months of storage from two repeats was determined by Blast2go (Conesa et al., 2005).

SUPPLEMENTARY DATA

Supplementary data are available at *Molecular Plant Online*.

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