Low oxygen storage modulates invertase activity to attenuate cold-induced sweetening and loss of process quality in potato (*Solanum tuberosum* L.)

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Abstract

Russet Burbank and Innovator are mid- to late-season frozen-processing cultivars with inherently different dormancy periods and susceptibilities to low temperature-induced sweetening (LTS). In contrast to Russet Burbank, which is highly prone to accumulation of reducing sugars (Glc + Fru) when stored below 8-9 °C, Innovator tubers exhibit moderate resistance to LTS and retain process quality longer at lower temperatures (4-6 °C). However, Innovator's LTS resistance is not robust and often varies across production regions. Here we show that low O₂ storage modulates LTS to reveal metabolic differences intrinsic to these cultivars. Changes in tuber respiration, process quality, reducing sugars, sucrose, starch phosphorylase and invertase activities were compared at 4 and 8 °C in 2.5 and 21 kPa O₂ over a 212-d storage period. Tuber respiration declined rapidly as O₂ level decreased from 21 to 2.5 kPa during acclimation at 8 °C. Respiration rates then fell further as the temperature was lowered from 8 to 4 °C, but this response was greatly muted for tubers at 2.5 versus 21 kPa O₂. Tubers at 21 kPa O₂ completed their cold-induced respiratory acclimation response (RAR) within 7 d compared with 13 d at 2.5 kPa O₂, and the RAR was much greater for tubers at 2.5 kPa O₂. While reducing sugars increased most rapidly in tubers over the first 30 d at 4 °C, Innovator tubers had lower invertase activity and sweetened less than Russet Burbank tubers, characterizing its LTS-resistant phenotype. Low O₂ greatly attenuated these initial LTS responses for both cultivars; however, the effect was only temporary in Innovator. LTS resumed in Innovator tubers from 93 to 212 d with reducing sugar levels increasing to equal that of Russet Burbank tubers stored at 4 °C and 21 kPa O₂. Activities of α -1,4 glucan phosphorylase (SP) were higher in Russet Burbank than Innovator and increased progressively over the storage period regardless of temperature and oxygen concentration. Innovator tubers stored at 2.5 kPa O₂ had higher SP activities from 30 to 153 d at 4 and 8 °C compared with tubers stored at 21 kPa O₂, which correlated well with increased sucrose buildup and earlier sprouting. The low O₂-mediated inhibition of LTS was largely a consequence of reduced invertase activities.

1. Introduction

Maintaining raw product quality during long-term storage of potatoes destined to frozen processing (French fries) is highly dependent on cultivar and storage temperature. Storage at less than 9 °C effectively extends storage life by reducing tuber respiration, fresh weight loss, disease pressure and sprouting (Burton 1966; Schippers 1977; Burton 1978; Wiltshire and Cobb 1996). Retention of process quality at lower temperatures, however, is compromised by the induction of low-temperature sweetening (LTS) in susceptible cultivars. LTS involves the catabolism of starch into reducing sugars (Glc + Fru) (Isherwood 1976; Marquez and Anon 1986; Sowokinos 2001; Malone et al. 2006), which then serve as substrates for the Maillard reaction during frying (Denny and Thornton 1941; Habib and Brown 1956; Mazza 1983; Fuller and Hughes 1984; Roe and Faulks 1991; Mottram et al. 2002; Stadler et al. 2002; Kumar et al. 2004). In potato, products of the Maillard reaction can give rise to undesirable off-flavors, dark pigments and acrylamide. This latter product has been identified as a ‘probable human carcinogen’ in many processed foods (IARC 1994; Tareke et al. 2000; Tareke et al. 2002; Stadler and Scholz 2004). Breeding for resistance to LTS can mitigate these problems; however, the degree of cold tolerance expressed by LTS-resistant cultivars is often modulated significantly by management, environmental conditions and stress (Zommick et al. 2014ab), and this translates into variation in retention of process quality under cold storage conditions. Understanding the metabolic bases for variation in LTS-resistant phenotypes of cultivars important to the processing industry will lead to more effective breeding for stability of the LTS-resistant trait, as well as improved management recommendations to maximize retention of process quality during storage.

Innovator and Russet Burbank are dual purpose (fresh and frozen processing) russet skin potato cultivars that vary in dormancy and resistance to LTS. Russet Burbank is a late season, long dormancy cultivar, which remains the industry standard for frozen processing in North America. By contrast, Innovator has early- to mid-season maturity, shorter dormancy and moderate resistance to LTS. While Russet Burbank tubers are highly susceptible to the accumulation of reducing sugars when stored below 8-9 °C, Innovator tubers can maintain relatively low reducing sugar concentrations and thus acceptable

process quality when stored at lower temperatures (4-6 °C). However, the degree of LTS resistance expressed by Innovator is often variable across production regions, which is no-doubt attributable to poorly understood management x environment interactions that affect tuber physiology in general and sweetening metabolism in particular. The mechanism of resistance to LTS in Innovator is not known and is critical to understanding how management and environment interact to alter the expression of this important phenotype.

Previous work has indicated that low-oxygen storage can attenuate sweetening of potatoes stored at low temperatures (Pflug et al. 1964; Butcbaker et al. 1967; Workman and Twomey 1969; Harkett 1971; Sherman and Ewing 1983; Parkin and Schwobe 1990; Schwobe and Parkin 1990; Schouten 1992; Schouten 1993; Zhou and Solomos 1998). However, the efficacy of this technique is highly dependent upon oxygen concentration, timing, and cultivar (Butcbaker et al. 1967; Workman and Twomey 1969; Sherman and Ewing 1983; Parkin and Schwobe 1990; Schouten 1992; Mawson 1998). Here we use storage in 2.5 and 21 kPa O₂ atmospheres to manipulate the LTS responses of Russet Burbank and Innovator tubers to gain a better understanding of the metabolic bases for their differential sensitivities to sweetening. While not economically feasible for commercial storage, low O₂ modulated cold sweetening to reveal cultivar-dependent differences in LTS-resistant metabolism and associated changes in process quality.

2. Materials and methods

2.1. Plant materials, storage temperature and controlled atmosphere regimes

Potato tubers (*Solanum tuberosum* L., cvs. Russet Burbank and Innovator) were obtained from a commercial grower (Burley, ID) on October 1, 2013 (harvested September 26) and stored at 10 °C (95% RH) for two weeks to facilitate wound healing. The effects of storage temperature (4 vs 8 °C), oxygen concentration (21 vs 2.5 kPa O₂), and storage duration (0, 30, 93, 153, and 212 d) on retention of process quality, low temperature sweetening and associated enzyme activities were evaluated for each cultivar. Treatments were arranged factorially as indicated in Fig. 1. Tubers of each cultivar (170-284 g per tuber) were initially blocked for size (fresh weight) into four replicates (3 tubers per replicate) and placed into

13-L sealed storage chambers (Gamma Plastic Company, San Diego, CA, USA) equipped with inlet and outlet ports (Fig. 1C). Each chamber contained 12 tubers of each cultivar (4 replicates of 3 tubers per cultivar). The tubers rested on an internal grate situated 6 cm above the floor of each chamber to allow incoming air to enter and circulate from beneath the pile (Fig. 1A and B). The chambers were connected to separate manifolds which supplied CO₂-free air (21 kPa O₂, balance N₂) or 2.5 kPa O₂ (balance N₂) at 1.5 mL s⁻¹ for the duration of storage. The 2.5 kPa O₂ atmosphere was generated using a custom-built Permea nitrogen generator (Permea Inc., St. Louis, MO, USA) as described in Blauer et al. (2013). All tubers were acclimated initially for 8 d at 8 °C (95% RH) prior to lowering the temperature to 4 °C (95% RH) for half the chambers. Chambers were removed from the manifolds and tubers sampled at the designated storage interval (Fig. 1) to assess changes in sprout development, fry process quality, tuber carbohydrates, invertase, invertase inhibitor and starch phosphorylase activities.

2.2. *Whole tuber respiration*

Tuber respiration rates were monitored to determine the effects of O₂ concentration on the initial respiratory acclimation responses (RAR) to decreasing temperature (8 to 4 °C) and as an indicator of changes in metabolic rate over 155 d of cold-sweetening. The outlet atmosphere from two of the 212-d, 21 and 2.5 kPa O₂ chambers was directed through an LI-6262 infrared CO₂ gas analyzer (LI-COR Inc., Lincoln, NB, USA) as described previously (Zommick et al. 2014b). Carbon dioxide concentrations were quantified at 2.5-h intervals through the initial 8-d acclimation period at 8 °C and continuing through 155 d of storage at 4 °C. Tuber respiration rates (CO₂ evolution) are reported as µg kg⁻¹ s⁻¹. Respiration rates are thus the average of 12 Russet Burbank and 12 Innovator tubers (24 tubers total; equivalent mass for each cultivar) at each O₂ concentration. No attempt was made to characterize potential cultivar-dependent differences in respiration rates, which have been shown to be minor relative to the effects of decreased O₂ (Blauer et al. 2013) and temperature (Zommick et al. 2014b).

2.3. *Sprouting, fry process quality, and tissue sample preparation*

Sprout fresh weight (grams per tuber) was determined individually for all 24 tubers in a treatment chamber at 30, 93, 153 and 212 d, and samples of tuber tissue were taken for analysis of glucose (Glc),

fructose (Fru), sucrose (Suc), invertase and starch phosphorylase activities (described below). For each cultivar, tubers of the three replicates (four tubers per replicate) were cut in half along the apical to basal axis slightly off center, and a complete 1.5-mm-thick slice (periderm intact) was taken from the cut surface of the smaller half with a mandoline cutter to represent the whole tuber. The single slices from each of the three tubers per replicate were combined, collectively frozen (-80 °C) and lyophilized. The lyophilized samples were ground with mortar and pestle, sieved through a 60-mesh screen (0.246 mm) and stored for further analysis after all samples had been collected and processed.

French fry process quality was also evaluated at each sampling date using methods detailed in Zommick et al. (2014a). French fry planks (9.5-mm-thick x 2.9-cm-wide x length of tuber) were cut longitudinally along the apical to basal axis from the larger half (see above) of each tuber (one plank per tuber) and the 12 planks representing a cultivar from a particular treatment chamber were collectively fried for 3.5 min at 191 °C in vegetable oil. A Photovolt reflectance meter (Model 577, Photovolt Instruments Inc., Indianapolis, IN, USA) was used to measure light reflectance from the apical and basal ends of each fry plank as an indicator of the extent of Maillard browning (fry darkening) associated with buildup in reducing sugars (RS; Glc + Fru). Reflectance data were plotted versus days in storage and translated into USDA 0 (light) to 4 (dark) color values. Photovolt reflectance unit readings ≥ 31 = USDA 0; 25-30 = USDA 1; 20-24 = USDA 2; 15-19 = USDA 3; and ≤ 14 = USDA 4 (Stark et al., 2016). USDA values >2 and differences in reflectance values ≥ 9 between apical and basal portions of fries constitute unacceptably dark and/or non-uniform fry color, respectively (Driskill et al. 2007).

2.4. Carbohydrate analysis

Tuber carbohydrates were extracted from lyophilized tissue by modifying the methods of Zommick et al. (2014a). Lyophilized tissue (250 mg) was extracted by vortexing with 3 mL 60% (v/v) methanol in triethanolamine-HCl (TEA) buffer (30 mM, pH 7.0). The homogenate was centrifuged (10,000g, 15 min) and the supernatant analyzed for Glc, Fru and Suc using enzymatic methods (Bergmeyer et al. 1974; Bernt and Bergmeyer 1974) modified for analysis on microplates (Knowles et al.

2009). The assay is based on the stoichiometric reduction of NADP (monitored at A_{340}) resulting from the enzymatic conversion of Glc and Fru to 6-phosphogluconate (via glucose-6-phosphate, hexokinase and phosphoglucose isomerase). Total Glc (free Glc plus that hydrolyzed from Suc) was determined by incubating a separate aliquot of extract with acid invertase. Suc concentration was calculated as the difference between moles total Glc and moles free Glc (Bernt and Bergmeyer 1974). Suc, Glc and Fru were quantified based on standard curves (0.05-2.4 mM).

2.5. *Invertase and starch phosphorylase activities*

Acid invertase and starch phosphorylase were extracted from 200 mg lyophilized tissue at 4 °C with 1 mL HEPES buffer (50 mM, pH 7.5) containing 15 mM $MgCl_2 \cdot 6H_2O$, 2 mM $Na_2EDTA \cdot 2H_2O$, 5 mM $NaHSO_3$, 10% (v/v) glycerol, 2% (w/v) PVPP and 2 μL protease inhibitor cocktail (2.5 mL L^{-1} leupeptin, chymostatin, pepstatin and antipain, and 20 mL L^{-1} 4-aminobenzamidine and benzamidine). The extracts were centrifuged (10,000g, 15 min, 4 °C) and protein precipitated from the supernatant with 60% (w/v) $(NH_4)_2SO_4$. The pelleted protein (10,000g, 20 min, 4 °C) was resolubilized in the original extraction buffer (without PVPP) and stored at -18 °C until further analysis. Acid invertase activity was measured colorimetrically in the presence (basal activity) and absence (total activity) of its endogenous inhibitor by a microplate modification of Brummell et al. (2011) as outlined in Zommick et al. (2013). Activities were expressed as μmol sucrose hydrolyzed $s^{-1} kg^{-1}$ protein ($U kg^{-1}$). Tuber soluble protein was quantified (Bradford, 1976) using protease-free BSA (Sigma-Aldrich, St. Louis, MO) as a standard.

Changes in activities of the plastidic (L-type; low glycogen affinity) and cytosolic (H-type; high glycogen affinity) isoforms of starch phosphorylase (α -1,4-glucan phosphorylase) over the 212-d storage period were assessed by activity gel electrophoresis (Steup 1990). The two isoforms were separated on 8% native polyacrylamide gels containing 0.65% (w/v) glycogen (100 μg protein per lane). Following electrophoresis, the gels were incubated (37 °C for 120 min) in 100 mM MES buffer (pH 6.2) containing 12 mM Glc-1-phosphate and 0.5 g L^{-1} soluble potato starch as a primer for starch synthesis. The gels were then stained with KI/I to visualize and compare starch synthesizing activities of the two isoforms.

2.6. Data analysis and presentation

Treatment effects were analyzed in an overall 4-way factorial analysis of variance (ANOVA) with sums of squares partitioned into single degree-of-freedom contrasts for main effects (cultivar, temperature, oxygen, storage duration) and their interactions (Table 1). Similarly, 3-way factorial ANOVA's analyzed the effects of cultivar, temperature and oxygen concentration on sprouting, process fry color (Photovolt reflectances of apical and basal ends of fry planks), Suc, reducing sugars and invertase activity ($\pm$ inhibitor) separately at each sampling date. Data are plotted with least significant interval bars (i.e. $\pm 0.5\text{LSD}$, $P < 0.05$; Mason et al. 2003) versus time in storage.

3. Results

3.1. Tuber respiration

Tubers were initially acclimated to 2.5 or 21 kPa O_2 atmospheres at 8 °C for 8 d (Fig. 2A) before dropping the temperature to 4 °C for the LTS-phase of study. Tubers responded to the lower O_2 with an immediate and substantial decline in respiration rate. Respiration rate (CO_2 evolution) fell from 1.39 to 0.25 $\mu\text{g kg}^{-1} \text{s}^{-1}$ during the initial 5 d of storage in 2.5 kPa O_2 at 8 °C (Fig. 2A). Respiration rates of tubers in both O_2 atmospheres remained relatively constant over the next 3 d at 8 °C, and then fell rapidly over the next 35-47 h in response to decreasing the temperature to 4 °C. On an absolute basis, the temperature-induced decrease in respiration rate was 4.3-fold greater in tubers held at 21 kPa O_2 compared with 2.5 kPa O_2 . However, the percentage decline in respiration rate during this time period was approximately 32% regardless of O_2 atmosphere. Respiration rates then increased to a maximum at ca. 7 (21 kPa O_2) and 13 d (2.5 kPa O_2) before declining to relatively low levels over the remainder of the 155-d monitoring period at 4 °C.

The cold-induced (4 °C) respiratory acclimation response (RAR) is the difference between the initial minimum and maximum rates of respiration at 4 °C. To directly compare the effects of O_2 atmosphere on tuber RAR's, respiration rates were normalized to the initial rates (=100%) at the transition to the LTS phase (i.e. zero-time at 4 °C) and plotted (Figs. 2B and C). Differences in the rates of establishment, time to completion (from trough to peak) and magnitude (percent increase from trough

to peak) of the RAR's were affected by O₂ concentration (Fig. 2C). Tubers at 21 kPa O₂ completed their RAR about 8 d earlier than tubers at 2.5 kPa O₂. The changes in respiration rate ($\mu\text{g kg}^{-1} \text{ s}^{-1}$) during acclimation (from trough to peak) were 17 and 7% for tubers at 2.5 and 21 kPa O₂, respectively. Following the RAR, the respiration rate of tubers at 21 kPa O₂ decreased through 55 d and then remained relatively constant (at ca. 55% of the zero-time rate) through the remainder of the 155-d monitoring period. By contrast, the respiration rate of tubers held at 2.5 kPa O₂ declined following completion of the RAR to ca. 65% of the zero-time rate, which was maintained from 40 to 90 d. Respiration then increased through 155 d as the low-oxygen tubers emerged from dormancy and began to sprout (Fig. 2B).

3.2. Tuber dormancy and sprouting

No sprout growth was evident from either cultivar following 30 d of storage; however, the low-oxygen environment stimulated sprouting as early as 93 d in both cultivars at 8 °C (Table 2), and even at 4 °C in Innovator where growth was insufficient for fresh weight determination but sprout initiation was clearly visible (Fig. 3). As expected, tubers broke dormancy and sprouted earlier when stored at 8 °C compared with 4 °C regardless of cultivar and O₂ atmosphere (Table 2; Fig. 3). Innovator tubers emerged from dormancy earlier than Russet Burbank at both temperatures and oxygen concentrations. These data characterized the cultivar x temperature x O₂ concentration interaction ($P<0.05$) on dormancy break at 93 d of storage (Table 2). By 153 d at 8 °C, Innovator tubers had 1.7- and 6.3-fold more sprout growth than Russet Burbank tubers when stored at 2.5 and 21 kPa O₂, respectively.

3.3. Process quality (Fry color, Reducing Sugars, Sucrose)

Regardless of O₂ concentration and cultivar, storage of tubers at 4 °C resulted in darkening of fries and reduced process quality (Figs. 4 and 5, $P<0.001$) due to significant buildup in reducing sugars when compared with tubers held at 8 °C (Fig. 6AB). While these cold-induced changes were greatest during the initial 30 d of storage, the extent of LTS and associated changes in fry color over the 212-d storage period differed by cultivar and depended on temperature and O₂ concentration (cultivar x temperature x O₂ x duration, $P<0.01$; Table 1). Russet Burbank tubers were more susceptible to LTS than Innovator tubers. The reducing sugar concentrations in Russet Burbank tubers stored at 4 °C increased

70- and 48-fold over the initial 30 d of storage at 21 and 2.5 kPa O₂, respectively (Fig. 6A). By contrast, the cold-induced increase in reducing sugars of Innovator tubers was less (52-fold at 21 kPa O₂ and 28-fold at 2.5 kPa O₂), culminating in 26% (21 kPa O₂) and 42% (2.5 kPa O₂) lower concentrations when compared with Russet Burbank tubers at 30 d, and characterizing the superior LTS-resistant phenotype of Innovator tubers (Fig. 6AB). Following the initial increase, reducing sugar concentrations in Russet Burbank tubers at 4 °C remained constant from 30 to 212 d regardless of O₂ atmosphere and this was also the case for Innovator tubers stored at 21 kPa O₂. However, in contrast to Russet Burbank, the reducing sugar content of Innovator tubers stored at 2.5 kPa O₂ increased 2.4-fold from 93 to 212 d to equal that of Russet Burbank tubers stored at 21 kPa O₂ (Fig. 6B). Hence, over the first 93 d of storage, low O₂ inhibited the LTS response of Innovator more than Russet Burbank, but this effect dissipated with further time in storage (Fig. 6AB), resulting in progressive deterioration (darkening) of process (fry) color (Figs. 4 and 5). Innovator tubers, however, maintained acceptable fry color (USDA 0-1, basal end) regardless of temperature and O₂ concentration throughout the 212-d storage period (Figs. 4 and 5). Color of the basal ends of Russet Burbank fries became unacceptable (USDA 3) by 30 and 153 d for tubers stored at 21 and 2.5 kPa O₂, respectively (Figs. 4 and 5). Storage of tubers in 2.5 kPa O₂ was therefore more effective in slowing the cold-induced loss of process quality in Innovator than Russet Burbank tubers.

While fry color values in the USDA 0-2 range constitute acceptable fry quality, uniformity of color from apical to basal ends of fry planks is also an important criterion. Uniformity of fry color was considered unacceptable when the absolute difference in Photovolt reflectance (ref) between basal and apical ends was equal to or greater than nine reflectance units. Innovator tubers maintained uniform fry color (difference = 2.6-7.4 ref units) over the 212-d storage period regardless of storage temperature and O₂ concentration (Fig. 4). Conversely, except for the zero-time samples, Russet Burbank tubers held at 4 °C and 21 kPa O₂ produced non-uniform fry color (basal minus apical reflectance units ≥ 9) throughout the 212-d storage period. Low O₂ atmosphere delayed the development of non-uniform fry color in Russet Burbank tubers through the initial 30 d, beyond which color uniformity became unacceptable. Uniformity

of fry color for Russet Burbank tubers stored at 8 °C was acceptable at all sampling dates and O₂ concentrations except when stored for 153 d at 21 kPa O₂ (color difference = 9 ref units).

Changes in tuber sucrose concentrations during storage differed by cultivar and depended on temperature and O₂ concentration (cultivar x temperature x oxygen x duration, $P < 0.05$) (Table 1). The sucrose concentration of Russet Burbank tubers was equal to Innovator tubers at zero-time (Fig. 6). From 0 to 30 d at 4 °C and 2.5 kPa O₂, concentrations increased 41 and 63% in Russet Burbank and Innovator tubers, respectively. By comparison, storage at 21 kPa O₂ (4 °C) reduced this initial buildup in sucrose to 11% for Russet Burbank and 45% for Innovator tubers. These trends in initial sucrose accumulation were opposite to those for reducing sugar buildup in both cultivars at similar O₂ concentrations. From 30 to 212 d at 4 °C, sucrose concentrations in Russet Burbank tubers declined 26% at 2.5 kPa O₂ compared with 20% in tubers stored at 21 kPa O₂. A similar trend was evident for Innovator tubers stored at 21 kPa O₂ (4 °C) where sucrose concentration declined by 36% from 30 to 212 d. At 2.5 kPa O₂ and 4 °C, however, sucrose content of Innovator tubers fell 33% from 30 to 153 d and then increased by an equivalent amount through 212 d. Sucrose concentrations in tubers stored at 8 °C decreased progressively in both cultivars over the storage period (55% on average) to comparable levels at 212 d and were not affected by O₂ atmosphere.

3.4. Invertase and starch phosphorylase activities

Changes in basal (inhibitor present) and total (inhibitor absent) invertase activities over the 212-d storage period were affected by cultivar, storage temperature and O₂ concentration (Table 1). During the initial 30 d of storage at 4 °C, activities increased more in tubers stored at 21 kPa O₂ than 2.5 kPa O₂ in tubers of both cultivars (Fig. 7). The basal invertase activity in Russet Burbank tubers at 4 °C and 21 kPa O₂ then remained relatively constant through 212 d, but total activity continued to increase linearly ($R^2 = 0.99$, $P < 0.01$), reflecting a significant increase in the activity of invertase inhibitor in tubers from 30 to 212 d. By contrast, over the same storage period, basal invertase activity in tubers stored at 2.5 kPa O₂ (4 °C) decreased linearly (by 57%; $R^2 = 0.90$; $P < 0.05$) while total activity remained relatively constant through 212 d, which also reflects an influence of the inhibitor in regulating invertase activity. On

average, the basal invertase activities in Russet Burbank tubers stored at 8 °C (21 kPa O₂) were lower than in tubers stored at 4 °C (21 kPa O₂) from 30 to 212 d of storage but temperature had little effect on basal activities at 2.5 kPa O₂. Total invertase activities in Russet Burbank at 8 °C were higher than basal activities, reflecting the influence of invertase inhibitor; however, in contrast to 4 °C storage, total activities remained relatively constant throughout the 212-d storage period at 8 °C for tubers stored at both O₂ levels.

The initial low temperature-induced increase in basal invertase activity of Innovator tubers held at 21 kPa O₂ was attenuated relative to Russet Burbank tubers, resulting in 44% lower activity by 30 d at 4 °C (Fig. 7). However, basal invertase activity continued to increase in Innovator tubers through 93 d (4 °C, 21 kPa O₂), reaching levels comparable to Russet Burbank (4 °C, 21 kPa O₂) through the remainder of the 212-d storage period. Like Russet Burbank, total invertase activity in Innovator tubers stored at 4 °C increased more at 21 kPa O₂ than 2.5 kPa O₂ and was significantly higher than the basal activity at most sampling times. At 8 °C, basal invertase activity in Innovator tubers was low and constant with no effect of O₂ level through 153 d of storage. The basal activity at 8 °C then increased from 153 to 212 d to become significantly higher in Innovator tubers held at 21 kPa O₂ compared with 2.5 kPa O₂. In contrast to Russet Burbank, total invertase activity in Innovator tubers stored at 8 °C and 21 kPa O₂ increased over the 212-d storage period, but remained relatively constant when stored at 2.5 kPa O₂.

Activities of the cytosolic (SPH; high glycogen affinity) and plastidic (SPL; low glycogen affinity) isoforms of α -1,4-glucan phosphorylase increased with storage time, regardless of temperature and O₂ level (Fig. 8). Russet Burbank tubers had higher activities of both isoforms than Innovator tubers at harvest and throughout storage, which correlated with higher reducing sugars (Fig. 6) and darker fry colors (Figs. 4 and 5), particularly for tubers stored at 4 °C. Effects of temperature and O₂ concentration on activities of the SPL isoform from Russet Burbank tubers were difficult to resolve due to inherently high activity; however, tubers stored at 4 °C and 21 kPa O₂ had higher activity of the cytosolic (SPH) isoform than tubers held at 8 °C and 21% O₂ (Fig. 8). In contrast to Russet Burbank, storage of Innovator tubers at 2.5 kPa O₂ induced higher activities of both isoforms from 30 to 153 d at 4 and 8 °C compared

with tubers stored at 21 kPa O₂ (Fig. 8), which correlated well with the low O₂-induced stimulation of sprouting (earlier dormancy break) observed for this cultivar (Fig. 3; Table 2). Similarly, activities of the plastidic isoform in Innovator tubers stored at 8 °C increased more rapidly and were generally higher than in tubers stored at 4 °C (Fig. 8), likely also reflecting earlier dormancy break at the higher temperature.

4. Discussion

The LTS responses of Russet Burbank and Innovator tubers, two mainstream frozen processing (French fry) cultivars with respective low and moderate resistances to LTS, were modulated by low O₂ storage for a better understanding and characterization of differences in their cold sweetening behavior. Reports of the effects of low O₂ storage on reduction of sweetening and retention of postharvest quality of LTS-susceptible potato cultivars have been mixed, largely due to the range of O₂ concentrations used. Oxygen levels too high do not mitigate LTS as much as lower concentrations (Workman and Twomey 1969; Harkett 1971). Conversely, atmospheres too deplete of O₂ induce anaerobic metabolism, which leads to the production of lactate, ethanol, off-flavors, increased decay and a complete loss of process quality (Lipton 1967; Sherman and Ewing 1983; Shouten 1992). The O₂ concentration in our studies (2.5 kPa) was chosen based on previous work suggesting maximum reduction of LTS without invoking anaerobic metabolism (Harkett 1971; Sherman and Ewing 1983; Parkin and Schwobe 1990; Khanbari and Thompson 1994).

As expected, lowering the O₂ concentration from 21 kPa to 2.5 kPa greatly reduced tuber respiration, which fell even further when the temperature was lowered from 8 to 4 °C. Respiration rates then increased as tubers acclimated to 4 °C, characterizing cold-induced respiratory acclimation responses (RAR) as defined by Zommick et al. (2014b). Zommick et al. (2014b) established a positive correlation between RAR and total sugar buildup in cold resistant and susceptible cultivars stored at 4 °C and 21 kPa O₂ over a 30-d period of sweetening. Here we demonstrate that O₂ concentration greatly affected the kinetics of the cold-induced RARs (averaged over cultivars). When assessed as percent increase in respiration from trough to peak (Zommick et al. 2014b), the RAR for tubers at 2.5 kPa O₂ was 17% compared with only 7% for tubers at 21 kPa O₂ where the buildup in total sugar concentration (RS + Suc)

was greatest. Low O₂, however, slowed development of the RAR relative to tubers stored at 21 kPa O₂, which likely reflects the limited availability of substrate (O₂) and need for energy to fuel the attenuated sweetening metabolism. These results are in contrast to Zhou and Solomos (1998) where no RAR was evident from tubers stored at 1.5 kPa O₂ during LTS at 1 °C. Absence of a RAR under these conditions may have been due to the extremely low temperature or the induction of anaerobic metabolism, which would not have invoked increased CO₂ production initially, as the first formed product of anaerobiosis in potato tubers is lactate (Barker and Saifi 1952; Barker and Kahn 1968). Indeed, storage of potato tissue at 1.5 kPa O₂ and 4 °C induced lactate formation within 65 h of storage (Knowles, unpublished results).

The increase in respiration observed for low-O₂ stored tubers at 4 °C versus those held at 21 kPa O₂ beyond 90 days is likely linked to the stimulation of early sprouting. Low O₂ hastens emergence from dormancy (Burton 1958; Shouten 1993). The low O₂-induced stimulation of sprouting was cultivar-dependent, with Innovator tubers sprouting earlier than Russet Burbank tubers at both storage temperatures, reflecting the inherent differences in dormancy of these two cultivars. However, the resumption of LTS (i.e. buildup in reducing sugars) in low-O₂-stored Innovator tubers at 4 °C from 93 to 212 d (see discussion below) is likely not directly attributed to the stimulation of sprouting, as reducing sugar levels remained constant despite equivalent or greater sprout development in tubers from other treatments (e.g. both cultivars at 8 °C and 2.5 and 21 kPa O₂; Innovator at 4 °C and 21 kPa O₂ at 212 d). Given that each chamber contained tubers of both cultivars, cultivar-dependent respiratory responses to low O₂ during LTS and sprouting cannot be deduced. Further work is needed to characterize the significance of low O₂ atmosphere on development of the RAR during cold sweetening of tubers expressing LTS-susceptible and resistant phenotypes. Sprout inhibitors would be useful in such studies for resolving the respiratory responses associated with low O₂-induced sprouting versus LTS.

As anticipated, Innovator tubers sweetened less and maintained superior process fry color at 4 °C and 21 kPa O₂ than Russet Burbank tubers, reflecting its LTS-resistant phenotype. While hypoxia attenuated sweetening and deterioration of fry color in both cultivars, the effect was initially greater for Innovator, resulting in lower Glc and Fru levels through 93 d at 4 °C. LTS then resumed for Innovator

tubers stored at 2.5 kPa O₂, with reducing sugar concentrations increasing linearly (2.4-fold) through 212 d to a level substantially higher than in Russet Burbank tubers held under hypoxia and equal to Russet Burbank tubers stored at 21 kPa O₂ and 4 °C. This resumption of LTS in Innovator was attended by progressive deterioration in process fry color. By contrast, 2.5 kPa O₂ restricted the LTS-induced buildup of reducing sugars in Russet Burbank tubers to a maximum of approximately 11 g kg⁻¹ dry weight, which was maintained constant from 30 to 212 d of storage. Low O₂ clearly affected the progression of LTS differently in these two cultivars, providing an opportunity to assess the extent to which invertase and starch phosphorylase were modulated by hypoxia.

The sucrose and reducing sugars that accumulate during LTS of potato are ultimately derived from starch breakdown (Isherwood 1973; Isherwood 1976). Storage temperature affects the equilibrium between synthesis and catabolism of starch and sucrose, which ultimately dictates reducing sugar levels in tubers (Davies and Viola 1992; Hill et al. 1996; Blenkinsop et al. 2004). Relative to Russet Burbank, the higher resistance of Innovator tubers to sweetening at 4 °C and 21 kPa O₂ may reflect reduced starch breakdown, enhanced starch synthesis (i.e. recycling), or both. The mechanism of low temperature-induced starch catabolism in tubers is complex and details of the initial steps are poorly understood (Bethke 2014) but the process involves the concerted action of a number of enzymes (glucan water dikinase, α and β -amylases, debranching enzyme, limit dextrinases, starch phosphorylase, and others) with sucrose being the first free sugar to accumulate before Glc and Fru (Sowokinos 1990; Davies and Viola 1992; Classen et al. 1993; Zhou and Solomos 1998). Regardless of the O₂ concentration, Glc/Fru ratio in Russet Burbank tubers stored at 4 °C averaged 1.1, which implicates inversion of sucrose as the immediate source of reducing sugars (Zrenner et al. 1996) during LTS at both O₂ levels. This was also the case for Innovator tubers stored at 2.5 kPa O₂ and 4 °C (Glc/Fru = 1.1). However, in contrast to Russet Burbank, the Glc/Fru ratio of Innovator tubers during LTS at 21 kPa O₂ was higher ($P<0.05$), averaging 1.35 from 153 to 212 d, which suggests greater contribution of Glc directly from starch (Wu and Chen 1997) and/or potentially more rapid metabolism of the Fru produced from sucrose inversion, as proposed by Wiberley-Bradford et al. (2014). Hence, in addition to invertase, low O₂ undoubtedly

selectively modulates the activities of other enzymes involved in regulating carbohydrate metabolism during LTS of resistant and susceptible varieties.

While low O₂ modulated invertase activity to limit reducing sugar buildup and improve process quality, the effect was cultivar-dependent. Basal and total invertase activities increased in tubers stored at 4 °C and 21 kPa O₂. However, this response was substantially suppressed by 2.5 kPa O₂ over the 212-d storage period in both cultivars. Our results agree with Zhou and Solomos (1998) who demonstrated reduced expression and activity of acid invertase in Russet Burbank tubers during 30 d of storage in 1.5 kPa O₂ at 1 °C. However, as noted above, the low-O₂ inhibition of LTS in Innovator was only temporary through 93 d; reducing sugars increased sharply thereafter despite very low invertase activities. Sucrose concentrations also remained relatively high in Innovator tubers at 4 °C and 2.5 kPa O₂ from 93 to 212 d. Collectively, these data reflect increased starch catabolism in Innovator tubers over this period of resumed LTS at 2.5 kPa O₂.

Modulation of invertase by its endogenous inhibitor is a key regulatory mechanism in LTS (Greiner et al. 1999; Brummell et al. 2011; Liu et al. 2013; McKenzie et al. 2013). Invertase inhibitor activities, derived as the difference between total and basal activities, were affected by cultivar, O₂ concentration and temperature. After the initial and well documented (Pressey and Shaw 1966; Zhu et al. 2013; Zommick et al. 2014ab) increase in invertase activity during the first 30 d of cold storage at 4 °C and 21 kPa O₂, basal activity remained relatively constant over the remaining 180 d of storage for both cultivars. Total activity however, continued to increase linearly (140%) in Russet Burbank tubers from 30 to 212 d, indicating equivalent increase in the activity of endogenous inhibitor (total minus basal activity). In contrast to Russet Burbank, the increase in invertase inhibitor in Innovator tubers was delayed, with a 96% increase occurring from 153 to 212 d at 21 kPa O₂ and 4 °C. Invertase inhibitor activities in tubers of both cultivars stored at 2.5 kPa O₂ and 4 °C remained relatively low and constant from 30 to 212 d compared with tubers stored at 21 kPa O₂. Pressey and Shaw (1966) were the first to demonstrate a role for invertase inhibitor in regulating invertase activity during LTS of susceptible cultivars (Red Pontiac and Kennebec). Storage of tubers at different levels of O₂ in the studies reported

herein revealed that the extent and time course of modulation of invertase by its endogenous inhibitor likely differ for LTS resistant versus susceptible cultivars.

Recent work demonstrated that the plastidic isoform of starch phosphorylase (SP) is not required for mobilization of starch from leaves (Zeeman et al. 2004; Lloyd et al. 2005). The high affinity of SP for small linear maltodextrins and low affinity for high molecular weight branched chain glucans indicate a downstream role (if any) in the catabolism of starch in leaves. Similarly, silenced SPL did not attenuate total sugar (Suc + RS) buildup during LTS of potato, suggesting the plastidic isoform is not required for cold-induced starch catabolism; however, the isoform was induced in non-SPL-silenced heat-stressed tubers undergoing LTS (Knowles, unpublished results), supporting work by Zeeman et al. (2004) that indicates a possible role for SPL in stress tolerance. Notwithstanding these results, SP activities increased during the first 30 d of storage at 4 °C in both Russet Burbank and Innovator tubers, a period coincident with the greatest cold-induced increases in sucrose and reducing sugars at both levels of O₂. The activities of both isoforms increased progressively over the 212-d storage period in Russet Burbank tubers regardless of temperature and O₂ concentration. This was also evident for Innovator tubers stored at 4 and 8 °C in 2.5 kPa O₂. However, at 21 kPa O₂, the increase in SPL activity occurred from 93 to 212 d at 8 °C and from 153 to 212 d at 4 °C. Increases in the activities of SP isoforms during LTS were also shown by Zommick et al. (2014a). In addition to a potential downstream role in starch catabolism, plastidic SP can participate in starch synthesis and functions at relatively low temperatures (Fettke et al. 2010; 2012). Therefore, the effects of O₂ and temperature on SPL activity may partly reflect cultivar-dependent changes in the ability to re-synthesize starch during LTS. While more detailed analyses are needed to elucidate the role of SP (if any) in the LTS-responses of susceptible and resistant cultivars, using low O₂ storage to modulate gene expression and activities of SP isoforms during LTS may be a useful approach to clarifying its function.

5. Conclusions

Comparative evaluation of the cold sweetening responses of Russet Burbank and Innovator tubers at 2.5 and 21 kPa O₂ effectively defined differences in their LTS phenotypes. Low O₂ storage decreased

tuber respiration rate, delayed the cold-induced RAR, and attenuated sweetening. The initial low O₂-induced reduction in LTS was greater for Innovator than Russet Burbank tubers; however, this effect was only temporary for Innovator tubers, with robust resumption of LTS from 3 to 7 months of storage. The mitigation of LTS by low O₂ was largely attributable to reduced invertase activity, which led to higher sucrose levels in both cultivars. However, invertase inhibitor activity, starch phosphorylase activities, and the extent of starch breakdown (as indicated by total sugar buildup) were also differentially modulated by O₂ concentration, further characterizing the intrinsic differences in LTS phenotype of these cultivars.

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Fig. 1. Schematic diagram of the factorial arrangement of storage treatments (temperature, O₂ concentration, and storage duration) for Innovator and Russet Burbank tubers. Each respiration chamber was fitted with an internal grate (A) to elevate tubers above the air inlet port. Twelve tubers of each cultivar were sealed together in each chamber (B) and oxygen atmospheres (CO₂-free air, balance N₂) were established via the inlet and outlet ports (C). Carbon dioxide was quantified in the outlet stream from each chamber at 2.5-h intervals through 155 d of storage (see Fig. 2). Chambers were removed from the system at 30, 93, 153 and 212 d of storage to assess tuber sprouting, process quality (fry color), reducing sugars, sucrose, invertase, invertase inhibitor and starch phosphorylase activities

Fig. 2. (A) Effects of low O₂ and temperature on tuber respiration (CO₂ evolution) rates during LTS. Tubers were initially acclimated to 2.5 and 21 kPa O₂ for 8 d at 8 °C prior to lowering the temperature to 4 °C (zero-time) to induce LTS. (B) Tuber respiration rates were normalized to 100% of the zero-time (switch to 4 °C) rate to directly compare changes in response to O₂ level. (C) Details of the cold-induced respiratory acclimation responses (RAR) for tubers in 2.5 and 21 kPa O₂ over the initial 17 d at 4 °C. Respiration rates are the average of Innovator and Russet Burbank tubers.

Fig. 3. Emergence from dormancy and sprouting of Russet Burbank (RB) and Innovator (Inn) tubers during a 212-d storage period at 4 and 8 °C in 21 and 2.5 kPa O₂ atmospheres. The four tubers shown for each treatment were chosen to represent the average sprout development from a 12-tuber sample at each date (see Table 2 for an analysis of sprout fresh weights).

Fig. 4. Changes in the processing quality (French fry color) of Russet Burbank (left column) and Innovator (right column) tubers as affected by storage temperature and O₂ concentration over a 212-d storage interval. Color was measured with a Photovolt reflectance meter as light reflectance from the

apical (middle row) and basal ends (bottom row) of fry planks and plotted along with the average reflectance (top row). High numbers indicate lighter fry color. USDA fry color ratings of 0 (light) to 4 (dark) are defined by the USDA axis and indicated by the color shading (values >USDA 2 constitute unacceptably dark color by industry standards). ANOVA results are presented in Table 1. Non-overlapping bars at each sampling (days in storage) indicate significant difference (LSD, $P<0.05$) between means ($n=12$). Fry samples are shown in Fig. 5.

Fig. 5. Changes in the process color and uniformity of sample French fry planks from Russet Burbank and Innovator tubers stored at 4 and 8 °C in 21 and 2.5 kPa O₂ atmospheres over time. Fry samples are oriented with the apical ends up. *Numbers indicate USDA color ratings of the basal ends of fries (see Fig. 4) (USDA values >2 are unacceptable by industry standards). Each fry plank is from a different tuber and the 4 planks shown were selected to represent the average color from a 12-tuber sample for each treatment.

Fig. 6. Changes in reducing sugar (Glc + Fru) and sucrose concentrations of Russet Burbank (A and C) and Innovator (B and D) tubers during 212 d of storage at 4 and 8 °C in 21 and 2.5 kPa O₂ atmospheres. ANOVA results are presented in Table 1. Non-overlapping bars at each sampling (days in storage) indicate significant difference (LSD, $P<0.05$) between means ($n=12$).

Fig. 7. Changes in the activities of acid invertase in the presence (basal activity) and absence (total activity) of its endogenous inhibitor during storage of Russet Burbank (top row) and Innovator (bottom row) tubers stored at 4 and 8 °C in 21 and 2.5 kPa O₂. ANOVA results are presented in Table 1. Non-overlapping bars at each sampling (days in storage) indicate significant difference (LSD, $P<0.05$) between means ($n=12$).

Fig. 8. Changes in the starch-synthesizing activities of cytosolic (SPH) and plastidic (SPL) starch phosphorylase in Russet Burbank (A) and Innovator (B) tubers during storage at 4 and 8 °C in 2.5 and 21 kPa O₂ for 212 d. The high (SPH) and low affinity (SPL) isoforms were separated on glycogen-containing native gels, which were then incubated with G-1-P and starch to stimulate starch synthesis. Each lane represents a pooled sample of four replicates representing 12 tubers.

Table 1. Sources of variation and levels of significance (*P*-values) for the effects of cultivar, storage temperature (4 vs 8 °C), oxygen atmosphere (2.5 vs 21 kPa O₂) and storage duration (0, 30, 93, 153, and 212 d) on process quality (French fry color), sugar concentrations and invertase activities in Russet Burbank and Innovator potato tubers (see Figs. 2-5).

Sources of Variation	Fry Color ¹	Reducing Sugars	Sucrose	Invertase activity	
				+Inh	-Inh
Significance levels (<i>P</i> values)					
Cultivar (C)	0.01	0.01	ns	0.05	0.05
Temperature (T)	0.01	0.01	0.01	0.01	0.01
Oxygen (O)	ns ²	0.01	0.01	0.01	0.01
Duration (D)	0.01	0.01	0.01	0.01	0.01
C x T	ns	0.01	ns	ns	ns
C x O	ns	0.05	ns	ns	ns
C x D	0.01	0.01	0.01	0.01	0.01
T x O	ns	0.01	0.01	0.01	0.01
T x D	0.01	0.01	0.01	ns	0.01
O x D	0.01	0.01	ns	0.01	0.01
C x T x O	0.05	0.01	ns	ns	ns
C x T x D	ns	ns	ns	ns	0.01
C x O x D	0.05	0.01	ns	ns	ns
T x O x D	0.01	0.01	0.05	0.05	0.05
C x T x O x D	0.01	0.01	0.05	ns	ns

¹Average of apical and basal fry color (Photovolt reflectance).

²ns, not significant.

Table 2. Sprout growth from Russet Burbank and Innovator potato tubers as affected by storage temperature and oxygen concentration.

Cultivar	Temp	O ₂	Days in Storage		
			93	153	212
	<i>°C</i>	<i>kPa</i>	<i>Sprout fresh weight (g/tuber)</i>		
Burbank	4	2.5	0c	0c	0.37b
		21	0c	0c	0.03b
	8	2.5	0.23b	3.37b	ND ¹
		21	0c	0.78c	ND
Innovator	4	2.5	0c	0.20c	2.08a
		21	0c	0c	0.75b
	8	2.5	0.63a	5.69a	ND
		21	0.09bc	4.89ab	ND
LSD _{0.05}			0.16 ²	1.66	1.07
Cultivar (C)			<0.01 ³	<0.01	<0.01
Temperature (T)			<0.01	<0.01	-
Oxygen (O)			<0.01	<0.05	<0.01
C x T			<0.01	<0.01	-
C x O			<0.05	ns	ns
T x O			<0.01	ns	-
C x T x O			<0.05	ns	-

¹Not determined

²Least significant difference, $P < 0.05$. Letters indicate mean separation within a column by LSD ($P < 0.05$).

³ P -values for indicated sources of variation.

Fig. 1. Herman et al.

Fig. 2. Herman et al.

Fig. 3. Herman et al.

Fig. 4. Herman et al.

Fig. 5. Herman et al.

Fig. 6. Herman et al.

Fig. 7. Herman et al.

Fig. 8. Herman et al.