Original Article

The making of giant pumpkins: how selective breeding changed the phloem of Cucurbita maxima from source to sink

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ABSTRACT

Despite the success of breeding programmes focused on increasing fruit size, relatively little is known about the anatomical and physiological changes required to increase reproductive allocation. To address this gap in knowledge, we compared fruit/ovary anatomy, vascular structure and phloem transport of two varieties of giant pumpkins, and their smaller fruited progenitor under controlled environmental conditions. We also modelled carbon transport into the fruit of competitively grown plants using data collected in the field. There was no evidence that changes in leaf area or photosynthetic capacity impacted fruit size. Instead, giant varieties differed in their ovary morphology and contained more phloem on a cross-sectional area basis in their petioles and pedicels than the ancestral variety. These results suggest that sink activity is important in determining fruit size and that giant pumpkins have an enhanced capacity to transport carbon. The strong connection observed between carbon fixation, phloem structure and fruit growth in field-grown plants indicates that breeding for large fruit has led to changes throughout the carbon transport system that could have important implications for how we think about phloem transport velocity and carbon allocation.

Key-words: carbon transport; fruit size; growth; crop yield; vascular; cucurbit; photoassimilate.

INTRODUCTION

Over centuries, humans have selectively bred agricultural plants to allocate more resources to fruit and seed production than their native progenitors (Gifford & Evans 1981). An outgrowth of this success has been the advent of competitive fruit growing, which in some species has led to an increase in fruit size by a factor of 100 (Hu et al. 2011). In 2014, the record for the largest fruit was set by a pumpkin that weighed 1056 kg (Guinness World Records, 2014) and, based on the rate of new world records, this size will be surpassed in the next year. Competitive fruit growing has largely escaped the notice of the scientific community because these fruits do not have agricultural value, but giant fruits provide an opportunity to ask how plant anatomy and physiology might limit carbon allocation to reproduction. The regulation of fruit growth has implications both in agricultural systems where it can impact yield, and in natural systems where it can influence plant fitness. There is also growing evidence that it has implications for species distributions along with other aspects of plant phenology (Morin et al. 2007; Savage & Cavender-Bares 2013).

Sugar is translocated in the phloem from source tissue to multiple carbon sinks throughout the plant, but the factors that determine how much carbon is allocated to any one sink remains largely unknown. Many researchers believe that the limiting factor in fruit growth is sink activity (Gifford & Evans 1981; Patrick 1988; Wardlaw 1990; Körner 2003), which is determined by a variety of processes including phloem unloading, starch synthesis, sugar accumulation, cell cycle regulation and cellular metabolism (Patrick 1997; Bihmidine et al. 2013). However, other researchers believe that carbon limits growth and that fruit size is determined by source tissue. Proponents of source limitation emphasize pruning and CO₂ addition studies that demonstrate the ability of plants to increase their yield when there is more carbon available or a higher source to sink ratio (Cure & Acoc 1986; Roitner-Schobesberger & Kaul 2013). Meanwhile, supporters of sink limitation cite the fact that classic breeding efforts typically led to changes in carbon partitioning and not carbon acquisition (Gifford & Evans 1981, but see recent work on wheat by Reynolds et al. 2009), and some modern agricultural plants exhibit lower photosynthetic capacity than their wild progenitors (Lawlor 1995). Despite the polarizing nature of this debate, it is possible that both the source and sink play a role in carbon partitioning, and their relative importance depends on developmental stage (Savage et al. 2013) and the aspect of plant productivity that is examined (Patrick & Colyvas 2014).

Another possibility is that fruit size is determined by constraints on vascular transport, specifically the ability of carbon and nutrients to move into actively growing fruit. On a cellular level, phloem conductivity is determined by sieve tube diameter and sieve plate structure (Jensen et al. 2012), but there is limited information on the relationship between phloem conductivity and transport capacity (Mullendore et al. 2010). As a result, debate about whether vascular structure constrains carbon transport has focused on measurements of phloem transport rate. The consistency of mass transport rates calculated using fruit growth has led many to suggest that there might be a limit to phloem transport...

However, studies that manipulate phloem cross-sectional area by girdling or selective pruning show that phloem transport can increase when translocation area is constricted (Milthorpe & Moorb 1969; Passioura & Ashford 1974; García-Luis et al. 2002). These manipulation experiments indicate that sieve tubes can transport sugar at higher velocities than previously believed but do not explain why phloem transport in non-manipulated plants appears stable (Peuke et al. 2001; Windt et al. 2006; Jensen et al. 2011; Savage et al. 2013).

This question has come up repeatedly in the literature and led researchers to suggest that phloem transport may be regulated to maintain a steady flow of resources and signals under normal growing conditions (Windt et al. 2006). Although there is no direct mechanism proposed for this type of regulation, many phloem models indicate that phloem transport might be constrained by phloem loading, sap viscosity, phloem pressure and xylem water potential (Hölttä et al. 2006; Jensen et al. 2011, 2013), and control on any one of these parameters could impact phloem stability.

Giant pumpkins (Cucurbita maxima Duchesne) are a benchmark of competitive fruit growing because of their overall size and their increase in size in the last 100 years (Hu et al. 2011). Different than many other large agricultural fruits (Nátrónová & Nátr 1993; Chevalier et al. 2014), giant pumpkins do not exhibit higher ploidy in their vegetative material or fruit (Tatum et al. 2006; Nakata et al. 2012). All competitively grown pumpkins are derived from Hubbard squash that was brought to the United States in the late 1700s and originated in temperate regions of Argentina (Sanjur et al. 2002). Hubbard squash breeding predates world records including foliar sprays that contain hormones and nutrients, also use products that claim to increase plant or fruit growth. Some growers also use products that claim to increase plant or fruit growth including foliar sprays that contain hormones and nutrients, and soil treatments that encourage micro-organisms such as mycorrhiza (Langervin 1993).

MATERIALS AND METHODS

Plant material and growth conditions

Seed for three varieties of C. maxima (Atlantic Giant pumpkin, Mammoth Gold pumpkin and Hubbard Golden squash) were purchased from the Sustainable Seed Company (Covelo, California, USA), variety numbers 167131, 164441, 16758, respectively. One set of plants was grown in a greenhouse at the Arnold Arboretum of Harvard University in 20 gallon Smart Pots (High Caliper Growing Systems, Oklahoma City, Oklahoma, USA) in Fafard Soil Mix 52 (Sungro Horticulture, Agawam, Massachusetts, USA) and fertilized with Neptune’s Harvest Organic Fish and Seaweed Blend Fertilizer (Gloucester, Massachusetts, USA), 2-3-1, every 2 weeks. Two cohorts of three plants per variety were allowed to grow for 5 months (June to November 2013 and December to May 2014), all attaining lengths greater than 12 m. Because giant pumpkins are challenging to successfully pollinate under greenhouse conditions, the plants flowered but produced only a few fruit despite frequent hand-pollination. The greenhouse temperature was between 23 and 27 °C in the day and 18 and 22 °C at night with the humidity held between 55 and 65%. Supplemental light was used in the autumn and winter to maintain a minimum photoperiod of 12 h.

A second set of plants was grown in Sunshine Mix #1 (Sungro Horticulture) in 1 gallon pots in a walk-in Conviron growth chamber (Coviron, Winnipeg, Manitoba, Canada) with a CMP 6050 control system. The photoperiod was set at 14 h and the light-level (photosynthetically active radiation; PAR) at 160 μmol m−2 s−1. The temperature fluctuated between 23 °C during the day and 18 °C at night and the humidity was held constant at 65%.

Several growers graciously allowed us to collect samples from their field-grown Atlantic Giant plants. Some of the plants in this study produced pumpkins that ranked in the top five at the Topsfield Fair competition (Topsfield, MA, USA) in 2013. Because we were studying pumpkins cared for by different growers, there was variation in the treatment of and conditions under which plants were grown. It is common practice to encourage rooting by burying the vine and to prune the plant to reduce secondary carbon sinks. Only one giant pumpkin is produced per plant. Giant pumpkins are grown under a variety of soil conditions, and typically fertilized until they attain leaf nutrient levels comparable with those measured in previous winning plants. Some growers also use products that claim to increase plant or fruit growth including foliar sprays that contain hormones and nutrients, and soil treatments that encourage micro-organisms such as mycorrhiza (Langervin 1993).

Gas exchange, specific leaf area and growth rate

We measured gas exchange on the first three fully expanded leaves of each plant in one cohort grown in the greenhouse using a Li-Cor 6400 (Li-Cor Biosciences, Lincoln, Nebraska, USA) when the plants were two months old and flowering. Measurements were made using a clear top chamber under ambient conditions [light = 250 μmol m−2 s−1, reference CO2 = 4.3 × 10−4 mol mol−1, leaf temperature = 29 ± 1 °C (SD) and reference humidity = 33% ± 3 (SD)] between 1000 and 1200 h. When the plants were between 3 and 4 months old, we measured light response curves on two leaves per plant using the autoprogram LightCurve and a red-blue light source (Li-Cor, 6400-02B). Measurements were made at 12 light levels from 0 to 2000 μmol m−2 s−1 between 1000 and 1400 h under the following conditions: reference CO2 = 4.3 × 10−4 mol mol−1, leaf temperature = 29 ± 1 °C (SD) and reference humidity = 32% ± 8 (SD). Gas exchange was measured on three to six mature leaves of six field-grown Atlantic Giant plants that were between 2 and 3 months old on 7 September 2013 and were producing large fruit. Measurements were taken using a clear top chamber under
ambient conditions [light = 1400 μmol m⁻² s⁻¹, reference CO₂ = 3.8 × 10⁻⁴ mol m⁻³ s⁻¹, leaf temperature = 21 ± 2 °C (SD) and reference humidity = 54% ± 5 (SD)].

Total plant biomass and leaf area was measured on 9–10 plants per variety grown in the growth chamber for 2 months. Specific leaf area was measured by collecting three rectangular pieces of leaf tissue (18.75 cm²) from six leaves per plant in one greenhouse cohort and five plants in the field. We also estimated the total leaf area of seven field-grown Atlantic Giant plants based on their leaf number and average leaf size (n = 10).

Vascular anatomy

Six petioles and five to six stem internodes were collected per plant in each greenhouse cohort when they were 5 months old, resulting in approximately 36 samples per variety per tissue. A similar sampling was completed for five field-grown Atlantic Giant plants in September and early October of 2013. Flower pedicels were sampled from 20 developing flowers of each variety in the greenhouse and growth chamber along with 20 flowers and fruits of Atlantic Giant plants grown in the field. In this paper, we refer to the stalk of the flower and fruit as the pedicel instead of differentiating between the pedicel and peduncle because we studied continuous changes in this structure during flower and fruit development. Cross sections were made of all samples by hand and stained with 0.1% toluidine blue prior to imaging. The cross-sectional area of the xylem and phloem was estimated in 25–50% of the petiole, stem and pedicel. All tissue in the internal and external fascicular phloem was measured except the phloem fibre cap.

Samples from one cohort of greenhouse-grown plants were used to estimate average sieve tube and vessel size. Stem samples were prepared by making hand sections and staining with aniline blue (0.01% in 0.1 M PO₄ buffer). Ten sieve plates were measured in the external fascicular phloem of 35 vascular bundles per variety after imaging the samples on a fluorescent microscope using an A4 filter (Leica, Wetzlar, Germany). Petiole and pedicel samples were dehydrated in an ethanol series, embedded in paraffin and sectioned with a rotary microtome, following protocol laid out in Jensen (1962). The lumen area of 60–100 sieve tubes and vessels was measured except the phloem fibre cap.

Phloem transport velocity

Phloem transport velocity was measured in the midvein of six leaves per variety using a dye tracer technique (Jensen et al. 2011) with modifications described by Savage et al. (2013). This technique involves bleaching a fluorescent dye, carboxyfluorescein, as it moves through the phloem with a 473 nm diode-pumped solid-state laser (BML-473-20FEB, 20 mW, Lasermate, Walnut, California, USA) and determining how long it takes for the bleached area to move in front of a photodiode sensor. We attached a customized leaf clip to a bifurcating fibre optic cable that delivers the excitation light (20 mA narrow-band light emitting diode, 470 nm) and transmits the emission light to a high-gain photodiode (SHD033, International Light Technologies, Peabody, Massachusetts, USA) from the midvein. Bleaching was done for 10–15 s between 10 and 30 mm above the leaf clip, which was positioned mid-leaf. The adaxial surface of the leaf was gently abraded with fine-grained sandpaper and dye applied in the form of 0.01 M carboxyfluorescein diacetate in a 1:10 mixture of acetone to water. After taking three measurements per leaf, the leaf was removed, its area measured and the midvein sectioned near where the sensor was placed. Sections were stained with aniline blue (0.01% in 0.1 M PO₄ buffer) and the phloem cross-sectional area of the main bundle in the midvein was measured. These measurements were made on plants grown in growth chambers and brought into the lab at least 3 h prior to making measurements. All measurements were taken between 1000 and 1700 h under 300 W LED Grow Light (E.shine, Shenzhen, Guangdong, China) at a PAR of 100 μmol m⁻² s⁻¹.

Ovary and fruit development

Ovaries were collected before, during and after flowering on plants grown in the greenhouse and growth chamber. Their mass, water content and number of locules were measured and 15 ovaries/fruits per variety were sectioned transversely and stained with 1% safranin O. Using these sections, we measured the size of 20 cells in three ovary layers: (1) outer wall, the area between the epidermis and the first ring of vascular bundles, (2) mesocarp, the area between the outer wall and ovules and (3) placental region, the area where the axis of the carpels join (Sinnott 1939). Six ovaries per variety were frozen in liquid nitrogen, freeze-dried and sent to the Earth System Center for Stable Isotopic Studies at Yale University for determination of total carbon content. Similar measurements were taken on ovaries and fruit collected from the field in September and October 2013.
To determine carbon input into giant pumpkins, we obtained growth data from 16 field-grown fruits. Fruit mass was estimated based on three measurements: the circumference (parallel to the ground), the distance over the top along the main axis of the fruit and the distance over the top perpendicular to the axis at the widest point. Estimation of mass requires the use of ‘weight tables’ that were originally developed by Langevin (1993) and updated in 2013 by Team-pumpkin (www.bigpumpkins.com) to include data from over 6500 pumpkins. For each pumpkin, we fitted a model to the relationship between mass and mass-based growth rate and estimated the size of pumpkins at the time of maximum growth. We calculated fruit carbon accumulation (g of C per day) by multiplying mass-based growth rate (g of fresh mass per day), percent dry mass (g of dry mass per g of fresh mass) and fruit carbon content (g of C per g of dry mass). Percent dry mass and fruit carbon content were determined based on logistic regressions on fruit mass using field data. Then, we estimated the total carbon influx into the fruit by assuming a constant fruit respiration rate of 2 μmol g⁻¹ h⁻¹, which is an estimate of respiration during rapid fruit expansion in another variety of C. maxima (Irving et al. 1997). Because respiration is often higher prior to expansion, our estimate of fruit carbon requirements during early fruit development is likely low, but our estimates should be more accurate during the stages of fruit growth that are the focus of these analyses. We determined volumetric transport and linear phloem transport velocity using the following equations:

Volumetric transport (cm³ day⁻¹) = carbon influx (g day⁻¹)/sap carbon content (g cm⁻³)

Linear velocity (mm s⁻¹) = volumetric transport (mm³ day⁻¹)/cross-sectional area (mm²)

For these calculations, we assumed that phloem sap concentration was between 18 and 21%, which is within the average range of previously reported values and is close to the concentration predicted for optimal transport efficiency (Jensen et al. 2013). We also determined an upper estimate of daily carbon fixation of field-grown plants by assuming they exhibit constant photosynthetic activity for 15 h (the average length of a day in July in Massachusetts, USA).

### Statistical analysis

Comparisons were made among varieties using analysis of variance (ANOVA), analysis of covariance (ANCOVA) and Tukey’s honest significant difference (HSD), and among plant traits using linear (LR) and multiple linear regression (MLR). Statistical analyses were completed using R, version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria). All statistical tests were conducted using averages of each trait at a plant level except the analyses of ovaries and sieve plates, which were each considered independent. Tests with α < 0.05 were deemed significant.

### Results

#### Photosynthesis and growth

The photosynthetic activity of the three varieties of C. maxima was not significantly different (F_{2,24} = 0.52, P = 0.6) and averaged 9.5 ± 0.4 μmol CO₂ m⁻² s⁻¹ (SE) under ambient morning light levels in the greenhouse. At the time of these measurements, plants were flowering but there were no fruits developing. The three varieties also exhibited similar light curves (Supporting Information Fig. S1). They only differed their total leaf area (F_{2,26} = 15.2, P < 0.0001) and the amount of reproductive material (i.e. flowers) produced during a 2 month period (F_{2,26} = 3.9, P = 0.03) with Hubbard squash having a higher leaf area and a lower flower production than the other two varieties (Table 2). In the field, Atlantic Giants had lower photosynthetic activity than greenhouse-grown plants under similar light conditions (1400 μmol m⁻² s⁻¹, Supporting Information Fig. S1). They also had lower specific leaf area and a greater total leaf area at the same age (Tables 1 & 3).

#### Vascular anatomy

In Atlantic Giant and Mammoth varieties, a greater proportion of the cross-sectional area of the petioles (F_{3,19} = 32.55, P < 0.0001) and the amount of reproductive material (i.e. flowers) produced during a 2 month period (F_{2,26} = 3.9, P = 0.03) with Hubbard squash having a higher leaf area and a lower flower production than the other two varieties (Table 2). In the field, Atlantic Giants had lower photosynthetic activity than greenhouse-grown plants under similar light conditions (1400 μmol m⁻² s⁻¹, Supporting Information Fig. S1). They also had lower specific leaf area and a greater total leaf area at the same age (Tables 1 & 3).

### Table 1. Leaf traits of three C. maxima varieties grown in a greenhouse

<table>
<thead>
<tr>
<th>Traits</th>
<th>n</th>
<th>Atlantic Giant</th>
<th>Mammoth</th>
<th>Hubbard squash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific leaf area (cm² g⁻¹)</td>
<td>6</td>
<td>381 ± 3</td>
<td>400 ± 20</td>
<td>403 ± 7</td>
</tr>
<tr>
<td>A_{200} (μmol m⁻² s⁻¹)</td>
<td>3</td>
<td>19.0 ± 1</td>
<td>17.3 ± 1</td>
<td>16.0 ± 1</td>
</tr>
</tbody>
</table>

Average values are listed ± one SE along with the number of plants sampled per variety (n). There were no significant differences between varieties (α = 0.05).

### Table 2. Growth and leaf area of three C. maxima varieties raised in growth chambers

<table>
<thead>
<tr>
<th>Traits</th>
<th>n</th>
<th>Atlantic Giant</th>
<th>Mammoth</th>
<th>Hubbard squash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf area (m²)</td>
<td>10</td>
<td>0.36 ± 0.06a</td>
<td>0.34 ± 0.05b</td>
<td>0.59 ± 0.05b</td>
</tr>
<tr>
<td>Aboveground GR (g day⁻¹)</td>
<td>10</td>
<td>0.322 ± 0.02</td>
<td>0.301 ± 0.01</td>
<td>0.343 ± 0.01</td>
</tr>
<tr>
<td>Belowground GR (g day⁻¹)</td>
<td>10</td>
<td>0.025 ± 0.00</td>
<td>0.021 ± 0.00</td>
<td>0.021 ± 0.00</td>
</tr>
<tr>
<td>Reproductive GR (g day⁻¹)</td>
<td>10</td>
<td>0.015 ± 0.00a</td>
<td>0.014 ± 0.00a</td>
<td>0.006 ± 0.00a</td>
</tr>
</tbody>
</table>

Average values are listed ± one SE along with the number of plants sampled per variety (n). Growth rates (GR) are on a dry mass basis. Significantly different values (Tukey HSD, α = 0.05) are bold and indicated with letters.

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Phloem in giant pumpkins

Table 3. Leaf and fruit traits of Atlantic Giants grown in the field

<table>
<thead>
<tr>
<th>Traits</th>
<th>n</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leaf area (m²)</td>
<td>7</td>
<td>100 ± 20</td>
</tr>
<tr>
<td>Specific leaf area (cm² g⁻¹)</td>
<td>5</td>
<td>170 ± 20</td>
</tr>
<tr>
<td>A1400 (μmol CO₂ m⁻² s⁻¹)</td>
<td>5</td>
<td>12 ± 1</td>
</tr>
</tbody>
</table>

Photosynthetic activity (A1400) is reported under ambient field conditions. Average values are listed ± one SE along with the number of plants sampled for each measurement (n).

P < 0.0001) and pedicels (F₁,₇₆ = 48.4, P < 0.0001) was phloem (Figs 1 & 2a,b), and the arrangement of vascular tissue in their pedicels was more irregular than in the Hubbard squash (Fig. 2c,d). These two varieties often contained unevenly spaced vascular bundles that did not exhibit the typical bicollateral orientation (Fig. 2e). In some cases, vascular bundles developed in the centre of the pedicel (Fig. 2f), resulting in a vein of vascular tissue feeding into the fruit that was separate from the main stalk (Fig. 2g). Vascular irregularities were more pronounced in larger field-grown fruits. These plants also exhibited 30 to 40% more phloem than Atlantic Giants grown in the greenhouse (Fig. 1).

In all three varieties, the majority of the phloem cross-sectional area was composed of sieve tubes, as indicated by the density of sieve plates observed in cross section (Fig. 2h). Average sieve tube diameter was similar across varieties in the internal (F₂,₈ = 1.25, P = 0.35; F₂,₈ = 0.54, P = 0.61) and external (F₂,₈ = 4.4, P = 0.07; F₂,₈ = 0.93) fascicular phloem in the pedicles and pedicels, respectively (Fig. 3a).

There were also no significant differences in sieve plate structure in terms of pore number per plate and pore diameter in the fascicular phloem of the pedicles (F₃,₇₆ = 2.2, P = 0.13; F₃,₇₆ = 1.0, P = 0.38) and pedicels (F₃,₇₆ = 2.1, P = 0.14; F₃,₇₆ = 0.34, P = 0.71) of the three different varieties (Figs 2i & 3b). In the stem, sieve tube diameter scaled with vascular bundle size (LR, F₁,₁₀₁ = 451, P < 0.0001, R² = 0.81, Fig. 4) but there was no difference in the relationship between sieve tube diameter and bundle size among the three varieties (ANCOVA, F₉₉,₁₀₁ = 0.04, P = 0.96).

Patterns in the xylem were less consistent across organs. The pedicel was the only organ with a higher proportion of xylem on a cross-sectional area basis in Atlantic Giant and Mammoth varieties compared with the Hubbard squash (F₅,₇₆ = 21.3, P < 0.0001, Fig. 1c). In the petiole, field-grown Atlantic Giants had a similar amount of xylem compared with greenhouse-grown Mannoths, but not the other greenhouse-grown varieties (F₃,₁₉ = 5.34, P = 0.01, Fig. 1a). The greenhouse varieties did not differ in their stem xylem but had less xylem than field-grown Atlantic Giants (F₃,₁₉ = 13, P < 0.0001, Fig. 1b). There was no relationship between the cross-sectional area of the xylem and ovary diameter (MLR slope, T₅,₇₆ = 0.073, P = 0.94), and vessel diameter in the pedicles (F₃,₇₆ = 1.44, P = 0.31) and pedicels (F₃,₆ = 2.14, P = 0.19, Fig. 3c) of the three varieties was the same.

Phloem transport velocity

Linear transport velocity in the phloem midvein was faster in vascular bundles with a smaller cross-sectional area, resulting in a linear relationship between velocity and bundle area across plants (LR, F₁,₄₆ = 46.9, P < 0.0001, Fig. 5). This means that there were no varietal differences in phloem volumetric flow rate (F₂,₃₅ = 0.06, P = 0.94). Across samples phloem transport occurred at a rate of 0.0093 ± 0.0015 mm³ s⁻¹ (SD). Phloem cross-sectional area was not correlated with leaf area across varieties (LR, F₁,₁₆ = 3.47, P = 0.08, data not shown).

Ovary and fruit development

Ovaries of the three varieties of C. maxima were not significantly different in their mass at flowering when grown under controlled conditions (F₃,₇₆ = 2.45, P = 0.11, Table 4). They also had a similar carbon content (F₃,₇₆ = 1.47, P = 0.26), water content (F₃,₇₆ = 2.33, P = 0.12) and cell size in their mesocarp (F₃,₇₆ = 0.56, P = 0.6) and placental region (F₃,₇₆ = 3.35, P = 0.051, Table 4). However, Hubbard squash

Figure 1. Cross-sectional area of phloem (grey) and xylem (black) in (a) petioles, (b) stems and (c) pedicels of three varieties of C. maxima: Atlantic Giant (AG), Mammoth (M) and Hubbard squash (H) grown in a greenhouse. Data are also reported for AG that were grown by competitive pumpkin growers in the field. Significantly different values (Tukey HSD, α = 0.05) are indicated with letters and error bars are ± one SE.

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ovaries were often oblong, had more green on their epidermis (Fig. 2j–l) and had longer pedicels than the Atlantic Giant and Mammoth pumpkin varieties. They also had smaller cells in their outer ovary wall ($F_{2,27} = 14.4, P < 0.0001$), fewer locules in their fruit ($F_{2,108} = 21, P < 0.0001$) and a notably softer epidermis that could easily be bruised.

Cell division in the inner layers (mesocarp and placental region) stopped before the fruit was one kg but division in

Figure 2. Vascular anatomy of *C. maxima*. Vascular bundles in petioles of (a) Atlantic Giant and (b) Hubbard squash varieties. Bars are 250 μm. Pedicel cross sections of (c) Atlantic Giant and (d) Hubbard squash varieties. Bars are 500 μm. (e) Irregular vascular bundle in pedicel of an Atlantic Giant. Bar is 500 μm. Labels are as follows: EP and IP are external and internal fascicular phloem, respectively, F is fibres and X is xylem. (f) Central vascular bundle (black arrow) in pedicel of a flower from an Atlantic Giant stained with toluidine blue. Bar is 1 mm. (g) Central vascular bundle (black arrow) leading into the mature fruit of an Atlantic Giant. Bar is 1 cm. (h) Cross-section of Atlantic Giant stem. Callose on sieve plates is stained with aniline blue. Bar is 250 μm. (i) Sieve plate from the petiole of an Atlantic Giant. SEM micrograph. Bar is 10 μm. Ovaries of (j) Atlantic Giant, (k) Mammoth and (l) Hubbard squash varieties. Bar 1 cm.

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the outer wall appeared to last longer (Fig. 6). The exact point when cell division ended in the outer wall was unclear because we were not able to sample pumpkins that were between 10 and 100 kg. However, based on the curve fit of the data, cell division in the outer wall could have continued until the pumpkins were close to 100 kg.

Figure 3. Size of transport conduits in petioles and pedicels of three C. maxima varieties. (a) Average diameter of sieve tubes in the external, EP and internal, IP, fascicular phloem. (b) Average pore diameter in the sieve plates with the average number of pores per plate noted above each of the bars. No differentiation was made between the EP and IP for these measurements. (c) Average vessel diameter. Varieties of C. maxima are indicated by different colours and are as follows: Atlantic Giant (AG), Mammoth (M) and Hubbard squash (H). Error bars are ± one SE. None of the values are significantly different (α = 0.05).

Figure 4. Relationship between sieve tube diameter and the size of individual vascular bundles in the stems of three varieties of C. maxima. Varieties are indicated by different colours and are as follows: Atlantic Giant (AG), Mammoth (M) and Hubbard squash (H).

Model of fruit growth and phloem transport

The relationship between fruit size and phloem cross-sectional area was best fit by a quadratic model (Fig. 7a). The maximum growth rate of field-grown Atlantic Giants was approximately 20 kg d⁻¹ and the fastest growth occurred when fruits were between 143 and 370 kg. From the time of early ovary development to later stages of pumpkin growth, the carbon content of the fruit dry mass declined from 43 to 40% (LR, $F_{1,8} = 12, P < 0.0008$, Fig. 7b) and the water content increased from 91 to 98% (LR, $F_{1,38} = 88, P < 0.0001$, Fig. 7c). During this same period of time, pedicel size also increased (LR, $F_{2,23} = 584, P < 0.0001$, Fig. 7d). Assuming that respiration is 2 μmol g⁻¹ h⁻¹ (Irving et al. 1997), we estimate that Atlantic Giants transport up to 790 g of carbon per day into their fruit at the time of maximum fruit growth (Table 5). If the sap sugar concentration is between 18 and 21% (Jensen et al. 2013), this requires the movement of up to 9 L of phloem sap a day. Because there is no relationship between phloem cross-sectional area and ovary/fruit diameter, we estimated that 11.7% of the pedicel area of the field pumpkins was phloem (Fig. 7d). Considering this assumption, transport into the fruit occurs at linear velocities between 110 and 230 μm s⁻¹ at the time of maximum fruit expansion.

During the period when fruit growth was increasing, volumetric phloem transport into the fruit also increased (Fig. 8a). Because of changes in phloem cross-sectional area during this time, linear phloem transport velocity had a
slightly parabolic shape and velocity peaked prior to achievement of maximum fruit growth (Fig. 8b). We also estimated that the field-grown plants can fix up to 780 g d$^{-1}$ taking into account our measurement of total leaf area and assuming constant photosynthetic activity for 15 h (Table 5 and Fig. 9).

## DISCUSSION

Every 1–2 years, a new record is set for the size of the largest giant pumpkin. These fruit can be up to 98% water but their

### Table 4. Characteristics of ovaries of at the time of flowering

<table>
<thead>
<tr>
<th>Traits</th>
<th>Atlantic Giant</th>
<th>Mammoth</th>
<th>Hubbard squash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass (g)</td>
<td>10</td>
<td>9.0 ± 2</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Carbon content of dry mass (%)</td>
<td>6</td>
<td>45 ± 0.3</td>
<td>44 ± 0.5</td>
</tr>
<tr>
<td>Water content (%)</td>
<td>10</td>
<td>92.6 ± 0.5</td>
<td>94 ± 0.4</td>
</tr>
<tr>
<td>OW cell diameter (μm)</td>
<td>10</td>
<td>31.7 ± 1.8</td>
<td>29.3 ± 1.2</td>
</tr>
<tr>
<td>MC cell diameter (μm)</td>
<td>10</td>
<td>31.3 ± 1.5</td>
<td>33.1 ± 1.3</td>
</tr>
<tr>
<td>PR cell diameter (μm)</td>
<td>10</td>
<td>48.4 ± 3.2</td>
<td>48.9 ± 2.1</td>
</tr>
<tr>
<td>Locule number</td>
<td>43/34/31</td>
<td>4.3 ± 0.1</td>
<td>4.1 ± 0.1</td>
</tr>
</tbody>
</table>

Average values are listed ± one SE along with the number of ovaries sampled for each measurement ($n$). Significantly different values (Tukey HSD, $\alpha = 0.05$) are bold and indicated with letters. Average cell diameter is reported for the outer wall (OW), mesocarp (MC) and placental region (PR) of the fruit.

### Table 5. Plant carbon balance of field-grown Atlantic Giants estimated based on model of fruit growth

<table>
<thead>
<tr>
<th>Traits</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon acquisition (g of C day$^{-1}$)</td>
<td>780</td>
</tr>
<tr>
<td>Maximum growth rate (g of C day$^{-1}$)</td>
<td>570</td>
</tr>
<tr>
<td>Respiration (g of C day$^{-1}$)</td>
<td>80–210</td>
</tr>
<tr>
<td>Transport velocity (μm s$^{-1}$)</td>
<td>110–230</td>
</tr>
</tbody>
</table>

Figure 6. Cell growth in ovaries/fruit of field-grown Atlantic Giants. Ovary layers are as follows: outer wall (OW), mesocarp (MC) and placental region (PR). Cell division completes shortly before inflection point.

Figure 7. Modelling fruit development in competitively grown Atlantic Giant pumpkins. (a) Summary of mass-based growth data from field-grown pumpkins. Curves represent the best-fit quadratic equations for each fruit. (b, c) Decline in carbon content and increase in water content during pumpkin growth. Stages of fruit/flower development are marked with different colours. (d) Increase in cross-sectional area of the pedicel during fruit growth. Solid lines are logistic regressions. Dashed lines note range of sizes where fruits exhibit maximum growth.
success appears largely tied to shifts in carbon partitioning during selective breeding. The giant varieties, Atlantic Giants and Mammoths, differ from the ancestral Hubbard squash in many aspects of the carbon transport pathway from the source to the sink. Our data suggests that integration of these organs prevents any one part of the pathway from independently limiting carbon transport. Below, we consider the implications of our results to the three major parts of the carbon supply chain: the source, the transport system and the sink.

Is fruit growth carbon limited?

There is little evidence that differences in carbon acquisition can explain variation in the fruit size among varieties because all the plants exhibited a similar photosynthetic capacity on a leaf area basis when flowering, and Atlantic Giants, the variety with the largest fruit, had the smallest leaf area when grown under controlled conditions (Tables 1 and 2). Considering that plants can increase their photosynthetic capacity when there is high sink activity (Barrett & Amling 1978; Hall & Milthorpe 1978), it is possible that during the transition from flowering to fruiting, which we did not capture in this study, the three varieties diverge in their photosynthetic activity. However, recent work suggests that cucurbits and other plants that symplastically load carbon into their phloem have limited ability to up-regulate photosynthesis because their vein density determines their carbon export, and high carbon accumulation leads to down-regulation of photosynthesis (Amiard et al. 2005). This physical constraint makes it unlikely that pumpkins could increase their photosynthetic activity to a level that could explain differences in their fruit production. Moreover, all varieties exhibited similar growth rates despite the greater allocation of Mammoths and Atlantic Giants to reproduction (i.e. flower production) when grown under controlled conditions.

Our results are consistent with the observation that selective breeding for increased yield is typically associated with shifts in carbon partitioning and not higher rates of photosynthesis in eudicots (Gifford & Evans 1981, but see Reynolds et al. 2009 for discussion on wheat). Nevertheless, in field-grown Atlantic Giants, daily carbon fixation was close to the amount required to achieve maximum fruit growth rate (Table 5). This is surprising because our estimates of carbon fixation should be inflated by the assumptions that photosynthetic activity was constant during the day and that leaf area did not increase during the growing season. Taking these factors into account, it is apparent that the majority of carbon produced by Atlantic Giants is allocated to their fruit during the time of rapid expansion. This link between the source and sink could be caused by feedbacks between photosynthesis and sink activity (Geiger 1976; Watson & Casper 1984) including changes in carbon assimilation during fruit production (Barrett & Amling 1978; Hall & Milthorpe 1978) or changes in sink activity in response to sugar signaling (Smith & Stitt 2007).

Is fruit growth phloem limited?

One of the largest differences between giant pumpkins varieties and the ancestral Hubbard squash is the proportional area of their petioles and pedicels that contained phloem (Fig. 1). Because there is limited variation in sieve plate

Figure 8. Modelling phloem transport into the fruit of 16 field-grown Atlantic Giants prior to them achieving their maximum growth rate. (a) Estimate of the relationship between volumetric transport rate and phloem cross-sectional area assuming a sugar sap concentration of 18.2%. A logistic sigmoidal curve is fitted to the data. (b) The relationship between linear velocity and phloem cross-sectional area. A Weibull function is fit to the data.

Figure 9. Atlantic Giant pumpkin (Cucurbita maxima) growing in the field of George and Mary Ann Hoomis in Ipswich, MA. Photograph taken by Dustin F. Haines.

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structure, sieve tube size and phloem transport velocity (Figs 3–5), we conclude that selective breeding did not change the structure and function of phloem, only the amount of phloem. This is different to what was found in the xylem, where there was no consistent change in the structure or cross-sectional area of the tissue during selective breeding. To understand the implications of the observed anatomical changes, we need to consider whether phloem transport is regulated and the impact of phloem structure on transport capacity.

In the leaves, we found that translocation area does not limit carbon efflux from a leaf because vascular bundles with the highest phloem cross-sectional area have the slowest phloem transport (Fig. 5). These data suggest that the amount of carbon exported from these leaves is set at a leaf level because of the rate of carbon fixation (if carbon is limited under experimental conditions) and/or phloem loading (if vein density controls carbon export, as described above). As a result, the amount of phloem in giant pumpkin petioles does not appear to impact their ability to export carbon from their leaves under the environmental conditions used in this study.

Different than in the petiole, in the pedicel, linear transport velocity exhibits a slight parabolic relationship with phloem cross-sectional area, which leads to an increase in volumetric transport during fruit expansion (Fig. 8). A similar pattern was documented by Evans et al. (1970), who noted that varieties of winter wheat with larger kernels had more phloem and greater mass transport into their reproductive organs. In both of these studies, increased carbon flux resulted from the production of new phloem and not an increase in phloem transport velocity. This pattern is consistent with the idea that phloem transport is regulated and suggests that high carbon flux requires more sieve tubes in situations where transport is not source limited, that is, in the leaves, or sink limited, that is, when growth in fruit declines (Fig. 5).

Our estimates of phloem transport velocity in pedicels are consistent with that range of values measured in this (Table 5) and other studies on cucurbits (Crafts & Lorenz 1944; Mullendore et al. 2010; Savage et al. 2013). However, if the cross-sectional area of the phloem in the pedicel is decreased by 3%, which is the difference between the Atlantic Giant and Hubbard squash varieties, we estimate that transport would need to occur at velocities as high as 310 μm s⁻¹. Although this is within the range of velocities measured in other species (Jensen et al. 2011), it is higher than those recorded for cucurbits, which typically exhibit velocities of 100–230 μm s⁻¹. It is also important to note that we likely underestimate transport velocity in these analyses because we assume that the entire cross-sectional area of the phloem is involved in sugar translocation.

As mentioned above, the interpretation of our anatomical data is dependent on whether there is a limit to phloem transport under normal growth conditions. Until we gain a better understanding of the relationship between phloem structure and function and determine whether transport is regulated directly or indirectly to maintain consistent transport rates within species, we cannot understand the full implications of this and other similar studies. What we can conclude is that there is coordination between sink activity and the development of supporting vascular tissue, and as fruit expands, there appears to be a balance between how much carbon is produced, the transport capacity of the vascular tissue and fruit growth.

**Is fruit growth sink limited?**

The fact that phloem transport velocity appears to decline prior to maximum fruit growth (Fig. 8b) suggests that phloem transport is not limited by vascular tissue but by sink activity or feedbacks between the source and sink. If this is true, varietal differences in fruit size could be partially explained by differences in fruit development. Contrary to findings in C. pepo (Sinnott 1939), there was little evidence that ovary size and cell size played a role in determining fruit growth in giant pumpkins (Table 4). Instead, the main structural difference among varieties was locule number. However, it is unlikely that this trait alone can explain fruit size because this feature was highly variable within varieties, and many competitively grown pumpkins contain only 3 or 4 locules according to growers.

Another option is that fruit size is primarily tied to temporal differences in sink activity. In C. pepo, varietal differences in fruit size are partially explained by the length of cell division and the size of cells that can divide (Sinnott 1939, 1942). Although we do not have data on the length of cell division in all three varieties, we found that field-grown Atlantic Giants exhibit similar developmental patterns to those found in C. pepo and that cell division in the outer fruit wall in C. maxima continues well after flowering (Fig. 6). Additionally, the fruit from the three varieties in this study have different times for maturation. According to our seed source, Hubbard squash matures in 100 d, while the Mammoth and Atlantic Giant varieties take 110–120 d. Competitive growers of Atlantic Giants often grow plants up to 140 d. If varieties with larger fruits have longer periods of cell division and extended growing seasons, these two factors alone could explain a great deal of the variation that exists in their fruit size.

One final structural change that occurred during breeding of giant pumpkins was the development of a tender skin, which is softer and smoother than the skin of Hubbard squash. This change is associated with macroscopic (Fig. 2j–l) and microscopic (Table 4) alterations in the outer ovary wall. The reason that this change could be important is because tissue expansion can be limited by the elasticity of outer tissue layers (Savaldi-Goldstein et al. 2007). If pumpkin size is partially controlled by the ability of the outer layers to maintain integrity during expansion, changes in these layers including the epidermis could play a critical role in explaining varietal differences. In pumpkins, the importance of the outer layer may be enhanced by the fact that the inner tissues often fall apart as fruit expands, leaving an empty cavity.

**CONCLUSIONS**

Instead of finding evidence that one factor limits fruit growth in pumpkins, we show that source and sink activity are...
coupled at the whole plant level in the field and that fruit size appears to scale with phloem transport capacity across varieties. We show that selective breeding for larger fruit has led to a suite of changes in the carbon transport system from source to sink and highlight the need for a better understanding of phloem regulation and the relationship between phloem structure and function. These issues serve as a major limitation to our current understanding of carbon transport and our interpretation of both vascular structure and measurements of phloem transport velocity.

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REFERENCES


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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Figure S1.** Light curves for three varieties of *Cucurbita maxima*. Error bars are ± one SE.