SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Growing Conditions
Comparison among three different cultivars of spinach (Spinacia oleracea L.: Packer, Asgrow XPH
1450 and Asgrow XPH 1510) led to the selection of Asgrow XPH 1510, due to its retention of a
vegetative state under short days and its tendency to flower rapidly under long-day conditions. Seeds of
cv. Asgrow XPH 1510 (Asgrow Seed Company, Gonzales, CA), were sown in flats of peat-vermiculite
mix under greenhouse conditions (approximately 24° C during the day and 18° at night) and treated with
regular applications of liquid fertilizer. The plants were grown in an 18 h photoperiod (long day
inductive conditions), with daylight supplemented by overhead lights. Seedlings emerged from the soil
in 7-10 days, showing the first flower buds about 18 days later. Plants were subsequently transplanted
to 12.5-cm pots and axillary branches bearing leaves (as opposed to inflorescences) were removed for
simplicity. This study marked different flowering stages by weeks. Prior to the stage designated "onset
of flowering", there are no flowers or flower buds visible. Sampling of female (pistillate) plants
continued through the seventh week, whereas that of male (staminate) plants ceased after the fifth week
because at that time they senesced. Variation in the rate of development and in size occurs, but
similarity of appearance served as a factor in selecting plants at each flowering stage.

Radioactive Assimilate Distribution
At least 2 days prior to treatment, plants were moved to growth chambers, where they were exposed to
18-hour day lengths, with 175 µE.m⁻².s⁻¹ of photosynthetically active radiation at 19° C during the day
and 17° C at night. Three to four hours after the start of the light period, individual plants were exposed
to \(^{14}\)CO₂ for 1 hour. A thick, transparent polyethylene bag (10 cm x 20 cm or 10 cm x 40 cm), which
also contained a 3mL plastic vial, was placed over a single leaf or over the entire young inflorescence
region. Unless otherwise noted, the labeled leaf was located immediately below the inflorescence
region, designated L-1. Leaves within the flower-bearing portion are indicated by positive L numbers, with the lowest leaf that subtends an axillary raceme being referred to as L1. The bags were sealed around the petioles with Time adhesive paper tape and petroleum jelly, whereas Cling florists' adhesive sealed the inflorescence regions in their longer bags in an airtight manner. A syringe was used to inject 1 µCi of NaH$^{14}$CO$_3$ in 20µL of water into the vial, followed by 200 µL of 0.6 M HCl to release the $^{14}$CO$_2$. The sealed bag contained approximately 1.5 µM of CO$_2$, including 0.0182 µM of $^{14}$CO$_2$. The injection hole was sealed with Scotch transparent adhesive tape. The plant was left to assimilate the $^{14}$CO$_2$ for 1 hour under growth chamber conditions, after which the bag was removed in a fume hood. The plant was then returned again to the growth chamber for a variable chase period (usually 3 hours).

To determine the utilization of carbon over a longer term, both staminate and pistillate plants in the third week of floral development were left for chase periods of one, two, three, or four days following labeling of the leaf immediately below the inflorescence region (L-1) for one hour.

Following the post-labeling chase period, plants were dissected. Plant organs or regions were placed in aluminum weigh boats lined with a small piece (approximately 5 cm square) of paper tissue (Kimwipes), which served to absorb any liquid released from the plant parts following cutting and freezing. The regions included: the labeled leaf, the apical bud (the unexpanded shoot in which the leaves and racemes cannot be conveniently separated, usually about 5 mm in length), leaves within the inflorescence, flowers, stem of the inflorescence, and shoot below the inflorescence (often divided into leaves and stem). After the recording of their fresh weights, the samples were covered with wire mesh and then frozen on dry ice.

Following freezing, samples were oven-dried for 2 days at 60° C. After the sample dry weights were recorded, a sample oxidizer was used to combust the plant material. Combustion of samples was carried out in one of two different instruments, one a Packard (Downers Grove, IL 60515) Sample Oxidizer, the other a Harvey (Patterson, NJ) Biological Oxidizer OX300. The Packard instrument placed the radioactive CO$_2$ in contact with a chemical absorbent solution, Carbosorb2 (Packard
Instrument Company), followed by Liquiscint (National Diagnostics) scintillation fluid for detection. A combined scintillant/carbon absorber solution trapped the CO$_2$ in the Harvey oxidizer. In some cases, if the mass of the tissue exceeded that reasonably combustible, analysis of two 0.2g subsamples of the dry, ground tissue provided the basis for extrapolation to the total dry mass. Recoveries were maintained at 90% or better, as measured by the combustion of standards. A Beckman LS1801 (Beckman Instruments, Fullerton, CA 92634) scintillation counter was used to measure the radioactivity of the samples with disintegrations per minute calculated based on a standard curve and quench corrections.

Because of variation in the total amount fixed and exported, the most convenient measure of the carbon demand of a group of structures is the percent of the total export to that structure, which provides an indication of the relative importance of a given structure as a sink for the particular leaf labeled. Total export was calculated by a summation of the total recovered radioactivity from all harvested plant parts except the labeled leaf. This relative specific activity (RSA, calculated by dividing the dpm/g dry weight of an individual organ by the average dpm/g dry weight for the plant) provides a mass-independent measure of sink strength. At least 3 plants of each type were tested in each experiment and each experiment was repeated. As sampling is destructive, comparison from one week to the next necessarily involves different individuals.

**Analysis of Respiration and Photosynthesis**

A Licor 6200 (Lincoln, NE) portable infrared gas analyzer was used to determine the photosynthesis and respiration rates. The cuvette of the Licor was equipped with a laser light source (Quantum Devices) set to emit a light level comparable to that in the chambers (175µE m$^{-2}$s$^{-1}$). A 15 s period, after a minute or more of stabilization, was used to measure gas exchange in the 1 dL cuvette. Ambient CO$_2$ in the growth chamber ranged from 380-400 ppm. Photosynthesis testing took place 4-5 hours after the beginning of the light period, a time chosen to correspond with the end of the assimilation period in the experiments on carbon partitioning. Organs examined included 4 leaves, from the one just below the
inflorescence region (L-1) to the third leaf with an axillary inflorescence (L3). The calculation of photosynthetic rate depended on the leaf area enclosed in the cuvette, either marked and measured or the maximum exposed area of 12.5 cm² (determined by the spacers placed in the cuvette). Respiration of the inflorescence was measured by CO₂ evolution in darkness. 3.5 cm regions of the inflorescence, with the leaves removed, were enclosed in the Licor 6200 cuvette, with thick foam padding on both top and bottom, to seal around the stem. This was repeated following removal of the flowers and fruits. Because of the slower rate of respiratory gas exchange the measuring period was increased to 1 minute for analysis of CO₂ production.

A Licor leaf area meter determined the areas tested for photosynthesis in marked leaves, and the areas of whole leaves within and below the inflorescence. Excised leaves, or parts of leaves, were placed on a transparent plastic conveyor belt and passed through the machine, which measured their areas.

**Carbohydrate Analysis**

After a 3-hour period of recovery from the photosynthesis measurements, samples of each plant's tissue were removed for carbohydrate analysis. Tissue samples were placed in pre-weighed Eppendorf tubes containing 1.3 mL of 80% ethanol. A cork borer was used to cut 5 0.5-cm disks, avoiding the midvein, from each of the 4 leaves tested for photosynthetic rate. Depending on size, inflorescence leaf samples consisted of whole leaves or partial leaves, approximating the 2.5 cm² total area of the leaf disks. Two fruit and flower samples were taken: tissue from the apical area of the inflorescence and from the region of the inflorescence tested for gas exchange. Small segments were cut from several internodes in both the 3.5 cm of the flowering region measured for photosynthesis (the same 3.5 cm from which fruit or flowers were sampled) and in the oldest part of the stem, the area below the inflorescence, using dissecting scissors.
After several days of immersion in the 80% ethanol, the tissue samples were chopped with a small metal spatula. Microcentrifugation was used to separate the soluble sugars from the starch and other insoluble structural material. Different sized aliquots of the ethanol solution placed in 96-well plastic microtiter plates were evaporated and returned to 25 µL volumes with water. This volume was then treated with sequential enzymatic reactions based on a procedure from (Cairns, 1987) to reveal, colorimetrically, concentrations of glucose, fructose and sucrose. The fundamental reaction involves the coupled reactions of glucose-6-phosphate dehydrogenase (G6PDH) with glucose-6-phosphate (G6P) and transfer of the electrons to 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide (MTT) which, on reduction, becomes purple. This process is mediated by phenazine methosulfate (PMS). Bovine serum albumin (BSA) prevents the dye from precipitating. Glucose and fructose in the sample is converted into G6P and fructose-6-phosphate (F6P) respectively using ATP and hexokinase. The initial reaction solution included: NAD (8 mM); MTT (2 mM); PMS (6 mM); imidazole-HCl (0.2 M); MgCl2 (0.01M); BSA (4mg/mL); ATP (4mg/mL); hexokinase (0.4 U/mL); G6PDH (0.2 U/mL).

After the microtiter plate reader recorded the background absorbance (at 590 nm) of the original samples, 50 µL of the above solution was added to each well. The plate was replaced in the microtiter plate reader, where absorbance was monitored until it reached a plateau (in 3-10 minutes), signaling that all the glucose had reacted. At this juncture, addition of 0.1 U of phosphoglucoisomerase (in 15 µL of solution) to each well transformed F6P into new G6P producing additional colored product. When the new plateau occurred, marking the reaction of all of the fructose-derived glucose, a 4 U supplement of invertase (in 15 µL of solution) split all of the sucrose present into glucose and fructose. The hexose-Ps then reacted with the G6PDH and MTT to form yet more of the colored product. At the third plateau, all of the soluble sugars have sequentially become glucose and caused MTT to yield the colored product. A computer program associated with the microtiter plate reader calculated the concentration of glucose in each well, by comparison with standards, then determined the fructose and sucrose originally present by subtracting the absorbance due to glucose-product from each previous stage.
Starch analysis was carried out as in (Ober et al., 1991). The pellet produced in the foregoing extraction procedure was washed with 200 µL of water, centrifuged again and decanted. 200 µL of water was added to the pellet, and the tubes heated at 90° C for 10 minutes in a heat block to gel the starch. After cooling, 800 µL of amyloglucosidase (6 U/mL) were added, and the tubes incubated 24 hours or more at 40° C. Aliquots of the samples were assayed as described for glucose above.
**Supplementary Table S1.** Descriptions and average dry weight of flowering structures for plants of the given flowering stage in weeks following the onset of flowering ±SE.

| Flowering stage (Weeks) | Pistillate (Female) |  | Staminate (Male) |
|-------------------------|---------------------|---|------------------|
|                         | Appearance          | Mass of flowers and fruits (g) | Appearance | Mass of flowers (g) |
| **Onset**               | Stigmata visible in axils 2-3 nodes below apical bud. Stem begins to elongate. | 0.017 ± 0.004 | Convex inflorescence bud no longer enclosed in young leaves. | 0.039 ± 0.008 |
| 1                       | 10 floral nodes in 10 cm. | 0.020 ± 0.002 | Anthers remain enclosed in calyxes. | 0.172 ± 0.017 |
| 2                       | Up to 30 floral nodes, in 25 cm. Tiny fruits in lowest racemes. | 0.141 ± 0.018 | Some anthesis and pollen shedding begins. | 0.127 ± 0.014 |
| 3                       | 50 or more flowering nodes. 6-7 large (about 1 cm) fruits in lower leaf axils. Fruits still tiny or unfertilized at apex. | 0.512 ± 0.035 | Copious pollen shedding - 50% anthesis or more. Leaves below inflorescence begin to show yellowing. | 0.849 ± 0.052 |
| 4                       | May or may not continue producing nodes, fruit development proceeds at apex. | 0.77 ± 0.10 | Overall yellowing in week 4, with apical bud converted to flower buds. | 0.39 ± 0.27 |
| 5                       | Fruit enlargement | 2.69 ± 0.38 | Overall senescence |
| 6                       | Fruit maturation | 2.29 ± 0.37 | |
| 7                       | Lower leaves start to senesce. Overall yellowing in week 8 | 3.06 ± 0.12 | |
Supplementary Figures

Supplementary Figure S1. Shapes and sizes of leaves from staminate and pistillate spinach plants in ascending order: leaf L1, L11, L21, L31, L41, L51, numbering from the base of the inflorescence.
Supplementary Figure S2. Photographs of staminate (A-E) and pistillate (F-J) spinach plants at various stages of development. Each is arranged clockwise from top left: A,F) Within 2 days of the onset of flowering; B,G) At the end of the first week of flowering; C,H) At the end of the second week of flowering; D,I) At the end of the third week of flowering; E,J) The tip of the inflorescence region in the fourth (E) or fifth (J) week of flowering. K, L, Close up of staminate (A) and (L) pistillate inflorescences. A: x0.4; B, F: x0.3; C: x0.25; D, E, G,H,I,J: x0.2; K, L: x0.75.